GENETIC CONSEQUENCES OF PLATE TECTONICS: CYTONUCLEAR DISCORDANCE IN PHRYNOSOMATID LIZARDS OF BAJA CALIFORNIA, MEXICO

by

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ABSTRACT

Genetic Consequences of Plate Tectonics: Cytonuclear Discordance in Phrynosomatid Lizards of Baja California, Mexico

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The peninsula of Baja California of northwestern Mexico has a complex geological history. Since the early Miocene, intense plate tectonic activity, volcanism, and sea level changes have combined to form a diverse landscape. The geological history has had profound effects on the regional biota. I evaluated the genetics of three common phrynosomatid lizards in relation to data from geology, paleontology, and stratigraphy to provide a better understanding of the historical biogeography of this region. I also addressed the evolutionary significance of temporary population fragmentation and the genetic interactions of reunited populations. Using sequence data from three mitochondrial genes (ca 2,000 bp), I reconstructed the genealogical histories of the zebra-tailed lizard (*Callisaurus draconoides*) and the black-tailed brush lizard (*Urosaurus nigricaudus*) based on samples spanning the entire peninsula and insular populations from the Gulf of California. The genealogies revealed several deep divergences (up to 11% sequence divergence in cytochrome *b*), supporting the existence of temporary seaways across the mid-peninsular, Isthmus of La Paz, and Cape regions, and extensive

inundation of the peninsula in the vicinity of Loreto. My results also suggest a significantly deeper time frame for the historical biogeography of Baja California than currently postulated. This includes evidence of a mid-peninsular seaway of late Miocene age (ca 7 million years ago). This event is also a more parsimonious explanation for the geographically congruent mitochondrial DNA (mtDNA) breaks of numerous species than the effects of climate on genetics, such as the cyclic pattern of Quaternary glaciation events. The deep divergences in mtDNA strongly contrast with variation in allozyme loci, which suggests limited population differentiation consistent with gene flow along the peninsula. This cytonuclear discordance stems from the different modes of inheritance of mtDNA and nuclear DNA (maternal versus biparental inheritance) and lack of recombination in mitochondria. Accordingly, mtDNA differentiation is a poor proxy for biparental population differentiation. Cytonuclear discordance is particularly strong in old secondary contact zones in the black-tailed brush lizard and the side-blotched lizard (*Uta stansburiana*). These contact zones show very little geographical overlap of divergent mtDNA lineages, suggesting some form of selection against mtDNA dispersal across contact zones.

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Taking on a doctoral research project and writing a multidisciplinary dissertation is a huge undertaking, pushing you not only to explore unknown scientific grounds, but also to test your own skills and strengths. No doubt, it is personally a great learning experience and achievement to complete such a task. Yet regardless of my own hard work, this dissertation would not have come about without the help, guidance, and friendship of numerous people to whom I am greatly indebted.

My first thanks go to my supervisor, Bob Murphy. Taking on a lab volunteer from Sweden with zero experience in herpetology and a passion for ornithology seems like a risky approach to recruiting doctoral students for a herpetology aficionado. Yet you allowed me to explore some potential ideas instead of feeding me a project. Knowing little of what type of doctoral projects were feasible, let alone interesting, made it difficult for me to "find a project" at first. Yet, as you knew, projects don't start fully formed but commence with an interesting question that develops as time progresses. Your approach to supervising let me initiate an interest in a topic and develop it into my own doctoral research scheme. In addition, you thoughtfully pushed me in the right direction if I ventured astray. While I have many times doubted my skills as a researcher, I believe that your "hands-off" supervision has allowed me to develop a foundation of academic independence, which no doubt will be of great importance in my future career as an evolutionary biologist. Thanks amigo!

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numerous aspects of my research. Allan also guided me to explore important research on contact zones, most notably the work of Nick Barton and colleagues, which was an eye-opener and a great source of inspiration to me. As a non-geneticist with expertise in spatial dynamics, Marie-Josée Fortin added a most valuable aspect to my thesis committee, providing insights that complemented the strong component of molecular phylogenetics, which characterized the rest of my committee. With her ever-positive approach, Marie-Josée has spurred me to keep on track. Dan Brooks and Deborah McLennan offered discerning guidance to the development of my doctoral research and my schooling in phylogenetics and historical biogeography. Doug Currie offered wonderful camaraderie and comments, treating me as a colleague despite being my superior as an established professor. A sincere thanks to Jim Rising, Gerry De Iuliis, Roger Hansell, Peter Kotanen, and Helen Rodd for inviting me to get involved in their courses as a teaching assistant. In particular, the field courses in St Andrews, New Brunswick, and Churchill, Manitoba, were unforgettable experiences. After all, evolution takes place in nature, and what better aspect of the field to drive home the message of natural selection than polar bears lurking on the arctic tundra? Additional faculty members, like my 'Swedish connection' Locke Rowe (who drives a Volvo), have also provided input and wit, for which I am grateful. A special thank go to Josie Valotta who helped with administrative issues concerning graduate studies.

Being housed at the Royal Ontario Museum (ROM) has in many ways been an interesting experience. Amy Lathrop provided an endless array of help and tips in the Laboratory of Molecular Systematics, as well as good friendship, despite taking a dislike to my "devil music" (A. Lathrop, personal communication). Ross MacCulloch

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While I am an evolutionary biologist, much of my research has been dependent on data from geology, paleontology, and related fields. My studies benefited greatly from correspondence with numerous scientists, including Luis Delgado-Argote, Bill Drake, Brian Hausback, Jack Holt, Mitch Lyle, Tobias Schwennicke, Judith Terry Smith, Joann Stock, Paul Umhoefer, Andrea Zanchi, and in particular Ana Luisa Carreño. I also want to thank the herpetologists Robert Espinoza and Tod Reeder for providing tissue samples of lizards and Gary Adest for help with my re-evaluation of allozyme data.

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TABLE OF CONTENTS

Abstract ..... **ii**

Acknowledgements ..... **iv**

Table of Contents ..... **ix**

List of Tables ..... **xiv**

List of Figures ..... **xv**

List of Appendices ..... **xvii**

Chapter 1 – Introduction **1**

The peninsula of Baja California and its peculiar biodiversity 1

Tectonic insights into the history of Baja California 4

Biogeography in a tectonic setting 7

Challenges and opportunities for Baja Californian biogeography 9

Objectives of the dissertation 11

Thesis organization 12

References 14

Chapter 2 – Deep genealogical history without population differentiation: Discordance between mtDNA and allozyme divergence in the zebra-tailed lizard (*Callisaurus draconoides*) **24**

Abstract 24

Introduction 25

Materials and methods 26

Population sampling 26

DNA extraction, amplification, and sequencing 32
Data analysis 34
Results 35
Sequence variation 35
Genealogical relationships 36
Discussion 39
General 39
Genealogical history 40
Genealogies and geological history 42
Implications of an older time frame 49
Acknowledgements 52
References 53
Chapter 3 – Deep biogeographical history and cytonuclear discordance in the black-tailed brush lizard (*Urosaurus nigricaudus*) of Baja California 65
Abstract 65
Introduction 66
Materials and methods 71
Population sampling 71
DNA extraction, amplification, and sequencing 71
Data analysis 73
Results 75
Sequence variation 75
Genealogical relationships 77

Discussion 80
Genealogical history 80
Historical biogeography 82
An emerging picture of peninsular biogeography 89
Maternal and biparental histories 90
Acknowledgements 93
References 94
Chapter 4 – Deep genealogies and the mid-peninsular seaway of Baja California 110
Abstract 110
Introduction 111
Biogeographical doubts 114
Beyond the Quaternary 115
Vicariant legacy 116
Conclusions 119
Acknowledgements 119
References 120
Chapter 5 – Simple identification of mitochondrial lineages in contact zones based on lineage-selective primers 127
Abstract 127
Introduction 128
Materials and methods 129
Specimen sampling 129
DNA extraction, amplification, and sequencing 133

Sequence data analysis 134
Design of lineage-selective primers 135
Haplotype identification 136
Results 138
Sequence variation and genealogical identity 138
Haplotype identification 139
Discussion 143
Acknowledgements 146
References 147
Chapter 6 – Characterization of an old mitochondrial DNA contact zone in the black-tailed brush lizard *Urosaurus nigricaudus* and development of lineage-selective primers for maternal ancestry monophyly analysis 151
Abstract 151
Introduction 152
Materials and methods 153
Specimen sampling 153
DNA extraction, amplification, and sequencing 156
Sequence data analysis 157
Design of lineage-selective primers 158
Haplotype identification 159
Results 161
Sequence variation and genealogical identity 161
Haplotype identification 164

Discussion 165

Acknowledgements 166

References 167

Chapter 7 – Conclusions 171

Introduction 171

The historical biogeography of Baja California: an emerging picture 171

Cytonuclear discordance: when maternal and biparental histories disagree 174

The evolution and maintenance of contact zones 175

References 179

LIST OF TABLES

Table 2.1. Number, subspecific designation (specific for outgroup taxa), collection locality, and GenBank accession number of the specimens used in this study..... 27

Table 2.2. Pairwise genetic comparisons of mtDNA and allozymes between selected specimens.....37

Table 2.3. Divergence data (uncorrected *p*-distances) for lizard species whose mtDNA genealogies have been suggested to support the historical existence of a mid-peninsular seaway and include representatives from Isla Ángel de la Guarda... 48

Table 3.1. Pairwise genetic comparisons of mtDNA and allozymes between selected specimens of *Urosaurus nigricaudus*..... 76

Table 5.1. Information for the 15 specimens sequenced in this study and used to develop lineage-selective primers.....130

Table 5.2. Nucleotide variation within and between the two mtDNA haplogroups and the diagnostic differences at the sites of the two lineage-selective primers..... 137

Table 5.3. Examples of contact zones with divergent mtDNA lineages that are amenable to identification using lineage-selective primers as described herein..... 144

Table 6.1. Information for the 13 specimens of *Urosaurus nigricaudus* evaluated in this study and used to characterize the mtDNA contact zone and develop lineage-selective primers for MAMA..... 155

Table 6.2. Nucleotide variation within and between the two mtDNA haplogroups and the diagnostic differences at the sites of the two lineage-selective primers..... 160

LIST OF FIGURES

Figure 1.1. Map of southwestern North America showing Baja California of northwestern Mexico and the associated Gulf of California..... 3

Figure 1.2. Map of Baja California and the Gulf of California showing the main tectonic features of the ocean floor (present time).....5

Figure 2.1. (a) Strict consensus of the combined data set (6 MPTs, CI = 0.617, RI = 0.825). (b) Map over northwestern Mexico and southwestern United States showing collection localities of specimens of *Callisaurus draconoides*..... 29

Figure 3.1. Map of northwestern Mexico and southwestern United States showing collection localities of specimens of *Urosaurus nigricaudus*..... 69

Figure 3.2. Strict consensus of the combined data set (8 MPTs, CI = 0.569, RI = 0.820)..... 78

Figure 4.1. Map of Baja California showing the approximate location of the hypothesized mid-peninsular seaway..... 113

Figure 5.1. Study location and specimen sampling. (a) Map of Baja California showing the mid-peninsular break in *Uta stansburiana* mtDNA and (b) a map of the region that was sampled..... 132

Figure 5.2. Genealogy of mitochondrial lineages in *Uta stansburiana*, emphasizing the mid-peninsular contact zone..... 140

Figure 5.3. Mitochondrial lineage identification based on lineage-selective PCR.....142

Figure 6.1. Sampling of specimens for mitochondrial lineage identification. (a) Map

of Baja California of northwestern Mexico showing the range of *Urosaurus nigricaudus* (shaded area) and the secondary contact zone across the Isthmus of La Paz where two highly divergent mtDNA lineages meet. (b) Sampling locations for specimens used to assess nucleotide variation within and between the two mtDNA lineages..... 154

Figure 6.2. Genealogy of mitochondrial lineages in *Urosaurus nigricaudus*, emphasizing the Isthmus of La Paz contact zone..... 162

LIST OF APPENDICES

Appendix 3.1. Number, taxonomic designation, collection locality, tissue number, and GenBank accession number of the specimens used in this study..... 108

"Scientists still do not appear to understand sufficiently that all earth sciences must contribute evidence toward unveiling the state of our planet in earlier times, and that the truth of the matter can only be reached by combining all this evidence."

Alfred Wegener, *The Origin of Continents and Oceans* (1966; translation of 1929 edition)

Chapter 1

Introduction

The peninsula of Baja California and its peculiar biodiversity

Vast areas of northwestern Mexico and the southwestern United States experience desert-like conditions with low amounts of annual rainfall and scorching sunshine. Traditionally, four major deserts have been recognized: the warm Sonoran and Chihuahuan Deserts, the cool Mojave Desert, and the cold Great Basin Desert (Fig. 1.1; Shreve 1942). These deserts encompass a peculiar array of biodiversity (Morafka *et al.* 1992; Hafner & Riddle 2005). For example, the peninsula of Baja California[1] of northwestern Mexico and its associated islands display a high number of endemic species; including plants, mammals, reptiles, and ants (Wiggins 1980; Johnson & Ward 2002; Lawlor *et al.* 2002; Murphy & Aguirre-León 2002; Riemann & Ezcurra 2007). This distinct biodiversity led Hafner (1981) to propose the recognition of a new regional

[1] The peninsula of Baja California is commonly referred to as simply 'Baja California.' This may create some confusion as the northern state on the peninsula bears the same name. Therefore, the state of Baja California will herein be referred to as 'Baja California (Norte)' to avoid confusion.

desert, the Peninsular Desert of Baja California, emphasizing its unique evolutionary history as distinct from the remainder of the Sonoran Desert.

What forces have shaped the biodiversity of Baja California? While adaptation to desert conditions may seem important, the answer is much more complex. Even though components of the North American regional deserts may be old, current desert conditions are actually very young, having been strongly affected by recent glaciation cycles (Axelrod 1979; Van Devender 1990; Van Devender 2002). Baja California contains several types of habitat other than the widespread desert, including pine-oak woodland, chaparral, and mixed conifer forest of the elevated regions of Sierra Juárez and Sierra San Pedro Mártir in the north, coastal sage vegetation along the northern Pacific coast, arid tropical scrub along the heights of the southern Sierra de la Giganta and across the Isthmus of La Paz, and pine-oak woodland, chaparral, and dry tropical forest on the slopes of Sierra la Laguna in the Cape Region (Axelrod 1979). This range of habitats offers conditions suitable to a variety of species, including xerophilic and mesophilic organisms (Murphy 1983a; Hafner & Riddle 1997). Accordingly, earlier studies emphasized the variety of habitats as an explanation for the diversity of animals and plants (Stager 1960; Truxal 1960; Wiggins 1960). Some authors also stressed the influence of the cyclic nature of Plio-Quaternary climate in shaping the regional biodiversity, by generating waves of dispersal of different taxa into Baja California (Orr 1960; Savage 1960). However, all of these studies were based on the idea of a geologically stable peninsula, a concept that was subsequently overturned.

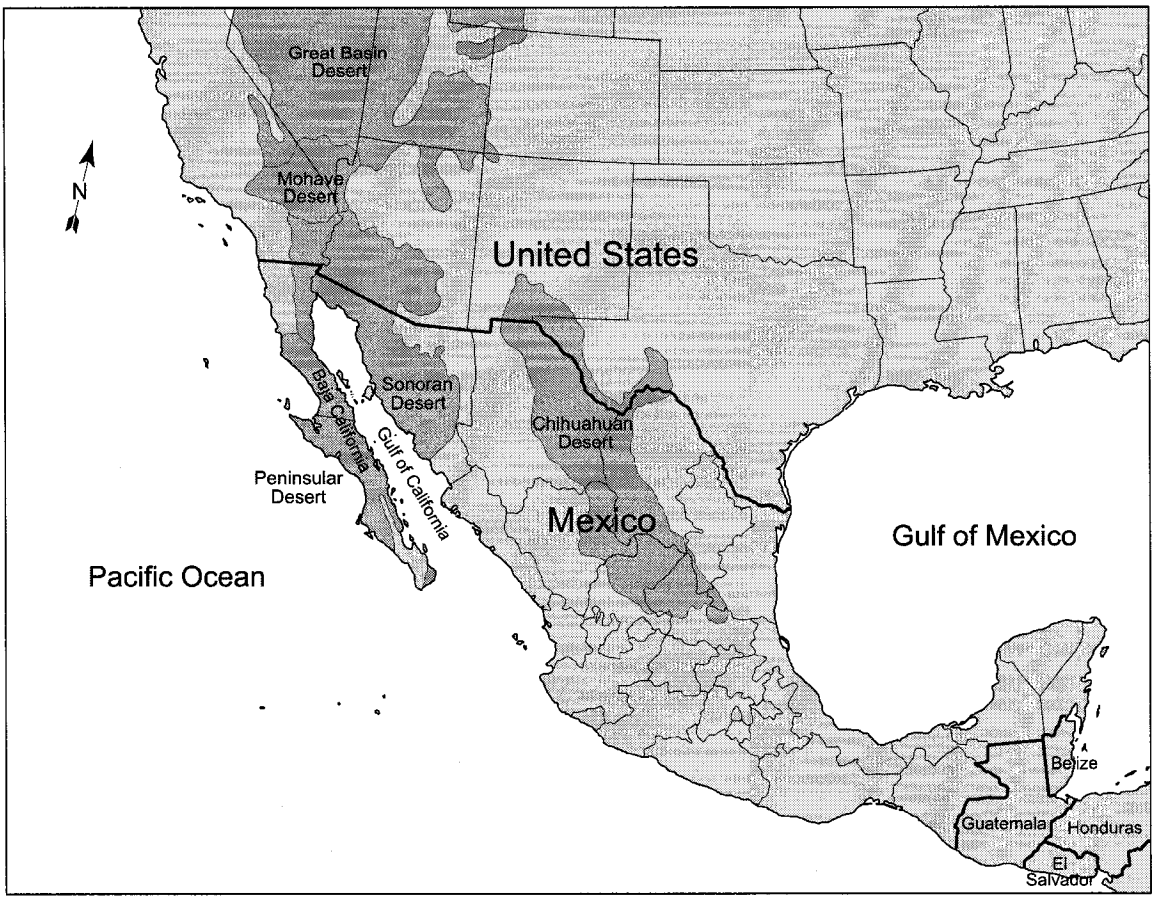


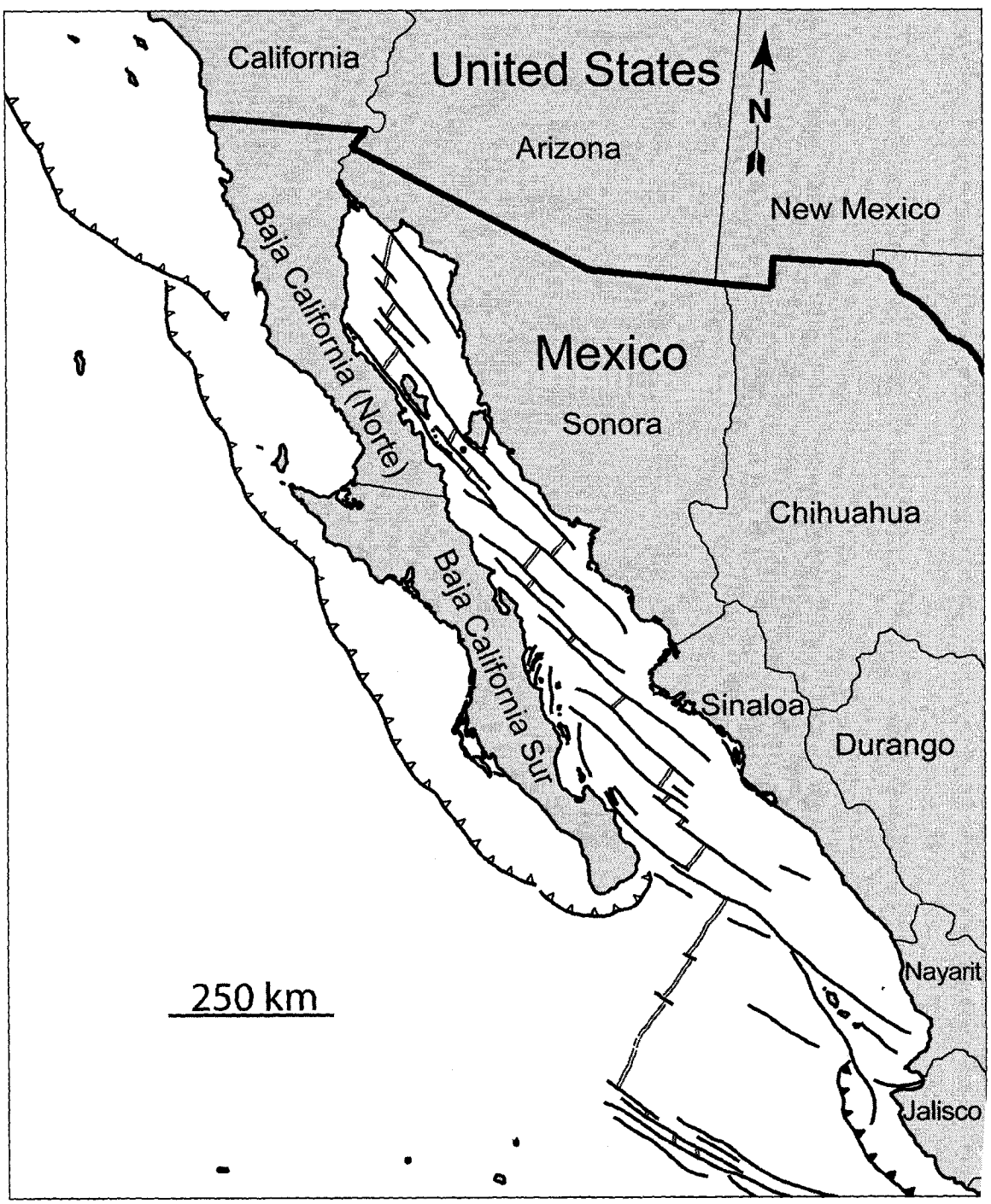
Figure 1.1. Map of southwestern North America showing Baja California of northwestern Mexico and the associated Gulf of California. Spanning approximately 1,300 km, Baja California is the fourth-longest peninsula in the world. Only the Malay, Antarctic, and Kamchatka peninsulas are longer. However, they have been more severely affected by Quaternary climate cycles than Baja California, either through the direct action of glaciation itself, or indirectly due to sea-level changes. Shaded areas depict the major regional deserts following Shreve (1942) and Hafner & Riddle (1997).

Tectonic insights into the history of Baja California

Following the development of modern 'plate tectonics' theory in the early 1960's (Hess 1962), several geological studies provided a new understanding of the tectonic history of Baja California and the Gulf of California[2] (e.g., Larson *et al.* 1968; Atwater 1970; Anderson 1971; Karig & Jensky 1972; Gastil & Jensky 1973; Moore 1973). A picture of the peninsula as a land mass broken off from the North American plate during late Tertiary times soon emerged (Fig. 1.2). During the late Mesozoic and most of Cenozoic time, the Pacific margin of Baja California was the site of subduction of the oceanic Pacific plate under the continental North American plate. At this stage the future peninsula was attached to the rest of the North American continent (Carreño & Helenes 2002). Heavy volcanism and orogenesis associated with this subduction lead to significant development of the peninsular ranges by middle Miocene (Gastil *et al.* 1979; Hausback 1984; Sawlan & Smith 1984). Subduction ceased about 12 million years ago (Mya; Lonsdale 1989; Spencer & Normark 1989; Stock & Hodges 1989), after which the tectonic development of the Gulf of California and gradual transferal of Baja California to the Pacific plate proceeded in three distinct stages (Umhoefer *et al.* 2002). During the first stage, approximately 12–6 Mya, a region of weakened continental crust roughly parallel to the coastline, but 250 km inland, underwent severe extension and developed into a rift zone, the "proto-Gulf of California" (Karig & Jensky 1972). In the second stage, about 6–3.5 Mya, the boundary between the Pacific and North American plates shifted eastward into the Gulf of California and became linked with the San Andreas

[2] The Gulf of California is also known as the Sea of Cortés.

Figure 1.2. Map of Baja California and the Gulf of California showing the main tectonic features of the ocean floor (present time). A long, fossilized subduction zone (lines with open triangles) is found west of the peninsula, where the Pacific plate submerged under the North American plate until ca 12 million years ago (Lonsdale 1989). Active submergence (line with filled triangles) still occurs along the coast of Jalisco and southward. The Gulf of California is characterized by transform fault zones (single lines), along which the peninsula is sliding northwestward. Ridges (double lines) are found at approximately perpendicular angels to the transform faults. These are accretion zones for new oceanic crust, extending the ocean bottom and widening the mouth of the Gulf of California. Tectonic characteristics follow Dauphin & Simoneit (1991) and have been simplified to show patterns pertinent to the historical biogeography of the region.


California
United States
Arizona
New Mexico
N
Baja California (Norte)
Mexico
Sonora
Chihuahua
Baja California Sur
Sinaloa
Durango
Nayarit
Jalisco
250 km

Fault system of southern California (Lonsdale 1989; Stock & Hodges 1989). Finally, at approximately 3.5 Mya, Baja California became part of the Pacific plate and the modern Gulf of California, characterized by transform faulting and generation of new oceanic crust, formed (Lonsdale 1989). According to this scenario, the peninsula was perceived as a giant piece of land broken off from mainland Mexico in a single, intact portion.

Biogeography in a tectonic setting

With a new synthesis of the geological evolution of Baja California and the Gulf of California (Gastil *et al.* 1983), it became clear that the complex history of the peninsula had greatly affected the regional biota, an influence that subsequent analyses of historical biogeography tried to incorporate (Murphy 1983a; Murphy 1983b; Grismer 1994). Unfortunately, these studies merely fitted species patterns to the most current geological model of peninsular evolution, and lacked a rigorous phylogenetic methodology. The problem was accentuated by heavy reliance on *tectonic* history for explaining biological phenomena, while largely neglecting stratigraphical data associated with the *paleogeography* of the gulf waters (e.g., Smith *et al.* 1985; Smith 1991), which pointed to older dates for vicariant events. For example, the biogeographical scenario of Grismer (1994) was based closely on the tectonic history of Stock & Hodges (1989). However, the Gulf of California as a body of water was simply in existence prior to the tectonic Gulf of California, meaning that biogeographical scenarios over-emphasizing the tectonic history were temporally too shallow.

The advent of molecular tools in the 1990's, in particular the sequencing of mitochondrial DNA (mtDNA), allowed for a finer level of hypothesis testing based on sound genealogies. The first such evaluation of a peninsular species was performed on the side-blotched lizard (*Uta stansburiana*). The study revealed a deep genealogical break midway on the peninsula (Upton & Murphy 1997). Upton and Murphy suggested that a vicariance event had generated this pattern; the mid-peninsular region had been transversed by a temporary seaway approximately 1 Mya (Upton & Murphy 1997). This was a bold interpretation, as no other genealogies with which to assess biogeographical congruence were available. However, congruent mtDNA patterns in two other lizards, *Aspidoscelis tigris* (Radtkey *et al.* 1997) and *Sauromalus* (Petren & Case 1997), followed the same year. Shortly thereafter, a population genetic analysis of the black-tailed brush lizard (*Urosaurus nigricaudus*) based on allozymes supported the historical existence of another seaway across the Isthmus of La Paz (Aguirre-León *et al.* 1999). While the idea of historical submergence of parts of the peninsula had been raised before (Nelson 1921; Johnston 1924; Beal 1948; Anderson 1950; Mina-Uhink 1957; Durham & Allison 1960), the genetic work added new impetus to the study of the historical biogeography of Baja California. Genetic tools made it possible to study the history of Baja California as a 'peninsular archipelago' (Aguirre-León *et al.* 1999) and to examine the effects of 'cryptic vicariance' on species (Riddle *et al.* 2000).

Challenges and opportunities for Baja Californian biogeography

The complex history of Baja California, as indicated by the genetic studies, presents an intricate challenge to historical biogeography as well as an opportunity to evaluate various biogeographical processes. What are the effects of geological history on biodiversity? The historical relationships among mitochondrial lineages offer one of the most promising avenues for addressing this and related questions, through the discovery of congruent patterns. Mitochondrial DNA follows a maternal, non-recombining mode of inheritance[3]. This means that mitochondrial lineages form hierarchical genealogies, which are tightly linked to female history, including dispersal patterns. Furthermore, historical events that affect different species in a similar way are expected to produce similar hierarchical relationships among mitochondrial lineages. Patterns that are congruent among genealogies are therefore indicative of a common history, making mtDNA an excellent tool for reconstructing historical biogeography. Hence, the genealogical history of common, widespread species can provide clues to the emerging picture of the history of Baja California and its biota (Brooks & McLennan 1991). Such genealogies should preferably be geographically detailed, for example by linking lineage history to geological events like island formation ('genealogical anchors' *sensu* Lindell *et al.* 2005), to provide a more comprehensive temporal resolution. A more complete

[3] The status of mtDNA as a strictly non-recombining, maternally inherited molecular is receiving increasing criticism (Bromham *et al.* 2003; Rokas *et al.* 2003; Tsaousis *et al.* 2005; Ujvari *et al.* 2007). Nonetheless, I assume that no departures from the traditional view of mtDNA transmission affect the main conclusions of this dissertation, given that these events appear to be rare.

understanding of the historical biogeography of Baja California therefore requires an amalgamated assessment of data from other fields, including geology, sedimentology, stratigraphy, paleontology, and climatology. Such an approach can also address the apparent discrepancy between genetic and geological data, which suggest different time frames for the evolution of the Gulf of California, and possibly help to discern between conflicting geological views on the age of the Gulf of California (Helenes & Carreño 1999; Oskin & Stock 2003).

With a refined understanding of the historical biogeography of Baja California, various processes in evolutionary biology can be investigated. How are species affected by temporary isolation? Are genealogical patterns geographically congruent among species? What is the relative importance of cryptic vicariance versus climate conditions in shaping genealogical diversity? Are patterns of genetic diversity the same in DNA representing different types of inheritance (maternal versus biparental)? What are the genetic interactions at secondary contact zones? This thesis investigates these and related questions. Baja California presents an opportunity to address these questions in a largely one-dimensional setting along the peninsular axis. Similar histories involving temporary seaways have been deduced for other peninsulas, most noticeably the Malay Peninsula (Woodruff 2003; de Bruyn *et al.* 2005). However, Baja California is a better place to study these evolutionary processes over a long time span, due to its significantly longer history as a peninsula.

Objectives of the dissertation

In this thesis, I study the genetic histories of three abundant, wide-spread species: the zebra-tailed lizard, *Callisaurus draconoides* (Blainville 1835); the black-tailed brush lizard, *Urosaurus nigricaudus* (Cope 1864); and the side-blotched lizard, *Uta stansburiana* (Baird & Girard 1852). These lizards are all members of the family Phrynosomatidae (*sensu* Frost & Etheridge 1989), containing ten genera and approximately 120 species (Etheridge & de Queiroz 1988; Reeder & Wiens 1996). Phrynosomatid lizards are one of the most diverse groups of reptiles in Baja California, and form a conspicuous part of the ecosystem.

The main objectives of the dissertation are threefold. First, by investigating the genealogical histories of the three lizard species in comparison with the genealogies of other species and data from geology and related fields, I aim to provide a better understanding of the historical biogeography of Baja California and the Gulf of California. Second, by comparing patterns in mtDNA and characters encoded in nuclear DNA, I aim to evaluate the genetic consequences of temporary isolation and provide insights into genetic differentiation and gene flow along the peninsula. Such a comparison is pertinent given the common practice among evolutionary biologists to use mtDNA genealogies to investigate population differentiation, a practice that is being increasingly criticized because maternal history may not be a realistic approximation of biparental population differentiation (Irwin 2002; Funk & Omland 2003). Finally, by investigating the genetics of secondary contact zones, I aim to provide a more detailed picture of the interactions between reunited populations.

Thesis organization

Following this general introduction are five chapters in publication format, written in collaboration with my supervisor Robert W. Murphy (Chapters 2 through 6), our Mexican collaborator Fausto R. Méndez-de la Cruz (Chapters 2, 3, and 6), and my fellow doctoral student Andre Ngo (Chapter 4).

Chapter 2 examines the genealogical history of the zebra-tailed lizard (*C. draconoides*) based on DNA sequence data from three genes (cytochrome *b*, ATPase 6, and ATPase 8) for samples collected throughout the entire range of the species, with a focus on the peninsula of Baja California and some insular populations of the Gulf of California. The mtDNA genealogy is compared with genetic patterns of other species and discussed in light of an extensive evaluation of data from geology, sedimentology, and related fields. As a result, a scenario for the historical biogeography of Baja California that emphasizes an older time frame is suggested. Mitochondrial DNA divergence is also compared with population differentiation based on allozymes, revealing a disparity between the two character types. This study appears as an article in Molecular Phylogenetics and Evolution (Lindell *et al.* 2005).

In Chapter 3, the genealogical history of the black-tailed brush lizard (*Urosaurus nigricaudus*) is investigated. Samples from the entire range (the peninsula of Baja California including some islands) are compared based on DNA sequence data from the same mitochondrial genes as evaluated in Chapter 2. Comparisons with other genealogies, geological history, and allozymes are performed in accordance with Chapter 2. A deep biogeographical history is supported and another case of cytonuclear

discordance is discussed. This study has been accepted for publication in Biological Journal of the Linnean Society (Lindell *et al.* in press).

Chapter 4 is a discussion paper that evaluates the occurrence of the numerous congruent genealogical breaks midway on the peninsula. Contrasting scenarios for the generation of this 'mid-peninsular break'—a history of temporary isolation due to a mid-peninsular seaway versus a discontinuity in climate-based characteristics along the peninsula—are evaluated. The assessment favors a vicariant history as the most parsimonious explanation for the clustering of mtDNA breaks. This study has been published in Journal of Biogeography (Lindell *et al.* 2006).

A simple methodology for quick and reliable identification of divergent mitochondrial lineages in contact zones is developed in Chapter 5. With reference to the genealogical history of the side-blotched lizard (*Uta stansburiana*), nucleotide diversity within and between the two mitochondrial haplogroups that meet in the mid-peninsular contact zone is explored. Lineage-selective primers are developed to evaluate the genetics of reunited populations. Results show very little overlap of mitochondrial lineages in the contact zone, despite its considerable age. This study is currently available online as a Technical Article in Molecular Ecology Notes (Lindell & Murphy in press).

Chapter 6 evaluates the genealogical history and mtDNA sequence variation in the black-tailed brush lizard (*Urosaurus nigricaudus*) to characterize an old contact zone across the Isthmus of La Paz, initially revealed by the genealogical history studied in Chapter 3. As in the mid-peninsular contact zone of *Uta stansburiana* (Chapter 5), there is very little overlap of mitochondrial lineages between the reunited populations, in

strong contrast with an assessment of population differentiation based on allozyme loci. This study will be submitted for publication in a peer-reviewed journal.

Finally, in Chapter 7, I amalgamate the findings of my work on the genetics of phrynosomatid lizards on the peninsula of Baja California, comment on unresolved issues, and suggest opportunities for future research.

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Chapter 2

Deep genealogical history without population differentiation: Discordance between mtDNA and allozyme divergence in the zebra-tailed lizard (*Callisaurus draconoides*)

Abstract

The peninsula of Baja California has a complex geological history that has strongly affected the regional biota. Genealogical histories of many species have revealed congruent patterns, which suggest that the peninsula was temporarily submerged at two locations. We sequenced a total of 1953 base pairs (bp) of the mitochondrial genome for 42 specimens of the zebra-tailed lizard (*Callisaurus draconoides*). The resulting maternal genealogy supports the former existence of a mid-peninsular seaway and a Plio-Quaternary seaway across the Isthmus of La Paz. In addition, a genealogical break is revealed in the vicinity of Loreto. This genealogical break may have resulted from prolonged submergence of the Loreto Basin during the Pliocene. The mid-peninsular seaway may have occurred as early as late Miocene, at a time significantly earlier than previously hypothesized. Comparison with other genealogies and geological evidence suggests that current models on the evolution of Baja California's fauna are temporally

shallow. The deep genealogical patterns of *C. draconoides* also disagree with the very limited population differentiation previously reported for allozyme markers, suggesting that maternal history may not be an appropriate approximation for population differentiation.

Introduction

The peninsula of Baja California and associated regions in northwestern Mexico form one of the most geologically interesting areas in the world. Since early Miocene, intense tectonic plate interactions, volcanic activity, and sea level changes combined to form a remarkably diverse landscape (Carreño & Helenes 2002). Although the biogeography of this region is extremely complex, a better understanding of the history of the region is currently taking shape. With the recent advances in phylogenetic systematics and biogeographical theory (e.g., Wojcicki & Brooks 2005), analysis of the genetics of abundant, wide-spread species can add much to the emerging picture.

The zebra-tailed lizard, *Callisaurus draconoides* (Blainville 1835), occurs in much of arid western North America, from throughout most of the Baja Californian peninsula, Sonora and Sinaloa of mainland Mexico, northward into California, Arizona, Nevada, Utah, and New Mexico. Over this vast area, 10 subspecies have been recognized, including seven in Mexico (Smith 1946; Smith & Taylor 1950). *Callisaurus draconoides* also occurs on several islands in the Gulf of California (Grismer 2002; Murphy & Aguirre-León 2002).

Despite *C. draconoides* being one of the most abundant reptiles throughout its range, including Baja California, a thorough phylogenetic evaluation using molecular methods is lacking. Intraspecific genealogical patterns of a species with a circum-Gulf distribution can help to clarify the history of the region, and demonstrate how geological history has influenced evolution of lineages and populations. Several populations of *C. draconoides* have historically been accredited species status, and the status of *Callisaurus* as a monotypic genus has recently been questioned (Wilgenbusch & de Queiroz 2000). Hence, we used mitochondrial DNA (mtDNA) sequences to investigate patterns of genealogy and genetic differentiation within *C. draconoides* from the Baja Californian peninsula and its associated islands, mainland Mexico, and southwestern United States.

Materials and methods

Population sampling

Tissue samples were obtained through our own fieldwork and donations (Table 2.1). Forty-two specimens of *C. draconoides* were chosen from a wide range of both mainland and insular populations including all of the traditionally recognized subspecies (Fig. 2.1). Single specimens of *Cophosaurus texanus* and *Holbrookia maculata* were selected as outgroup taxa. The clade consisting of *Cophosaurus* and *Holbrookia* has earlier been documented as sister-group to *Callisaurus* based on allozymes and mtDNA data (Murphy & Doyle 1998; Wilgenbusch & de Queiroz 2000).

Table 2.1. Number, subspecific designation (specific for outgroup taxa), collection locality, tissue number, and GenBank accession number of the specimens used in this study. Subspecific designation of *C. draconoides* specimens follow Smith and Taylor (1950) and Grismer (2002). Tissue numbers equal field numbers, except for italicized entries, which equal catalog numbers. Tissue number abbreviations are: ROM = Royal Ontario Museum; RWM = Robert W. Murphy (deposited at ROM); TWR = Tod W. Reeder; UNR = University of Nevada, Reno.

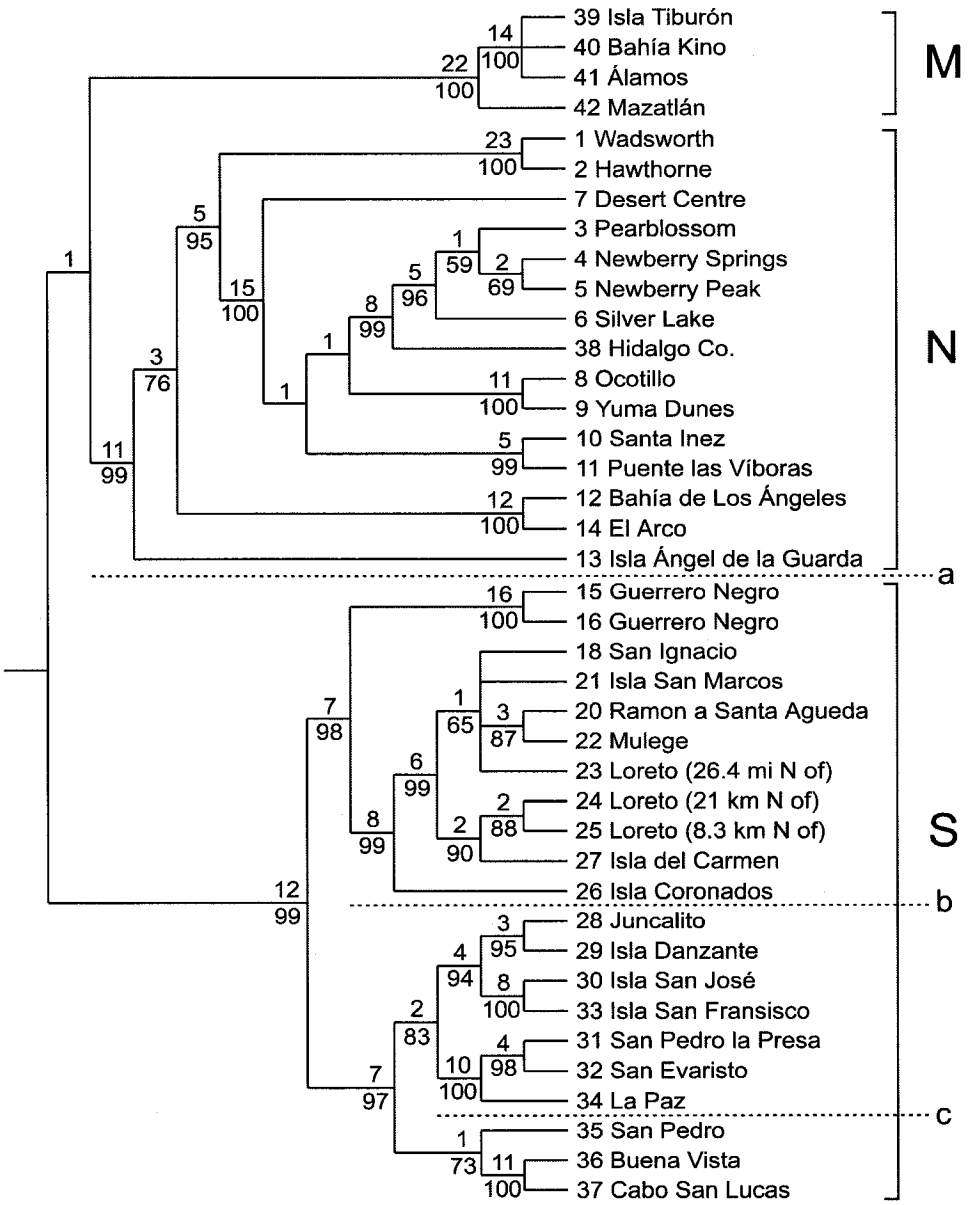
No.	Taxon	Collection locality	Tissue no.	Accession no.
Ingroup				
1	*myurus*	United States; Nevada; Washoe Co.; Wadsworth	*UNR 6136*	DQ001763, DQ001806
2	*myurus*	United States; Nevada; Mineral Co.; Hawthorne	*UNR 7313*	DQ001764, DQ001807
3	*rhodostictus*	United States; California; Los Angeles Co.; Pearblossom (4 mi N of)	*ROM 13982*	DQ001765, DQ001808
4	*rhodostictus*	United States; California; San Bernardino Co.; Newberry Springs	*ROM 14651*	DQ001766, DQ001809
5	*rhodostictus*	United States; California; San Bernardino Co.; Newberry Peak	*ROM 14745*	DQ001767, DQ001810
6	*rhodostictus*	United States; California; San Bernardino Co.; Silver Lake	ROM 03441	DQ001768, DQ001811
7	*rhodostictus*	United States; California; Riverside Co.; Desert Centre	*ROM 23225*	DQ001769, DQ001812
8	*rhodostictus*	United States; California; Imperial Co.; Ocotillo	*ROM 13781*	DQ001770, DQ001813
9	*rhodostictus*	United States; Arizona; Yuma Co.; Yuma Dunes	ROM 19874	DQ001771, DQ001814
10	*rhodostictus*	Mexico; Baja California; Santa Inez	RWM 1852	DQ001772, DQ001815
11	*rhodostictus*	Mexico; Baja California; Puente Las Víboras	ROM 37112	DQ001773, DQ001816
12	*rhodostictus*	Mexico; Baja California; Bahía de Los Ángeles	RWM 1778	DQ001774, DQ001817
13	*splendidus*	Mexico; Baja California; Isla Ángel de la Guarda	ROM 35413	DQ001775, DQ001818
14	*crinitus*	Mexico; Baja California; El Arco	RWM 1769	DQ001776, DQ001819
15	*crinitus*	Mexico; Baja California Sur; Guerrero Negro (2 km S of)	ROM 35381	DQ001777, DQ001820
16	*crinitus*	Mexico; Baja California Sur; Guerrero Negro (2 km S of)	ROM 35403	DQ001778, DQ001821
17	*crinitus*	Mexico; Baja California Sur; 83 km W of Vizcaíno	ROM 37118	DQ001779, DQ001822
18	*carmenensis*	Mexico; Baja California Sur; San Ignacio	RWM 1189	DQ001780, DQ001823
19	*carmenensis*	Mexico; Baja California Sur; San Ignacio (1 mi SW of)	RWM 1128	DQ001781, DQ001824

No.	Taxon	Collection locality	Tissue no.	Accession no.
20	*carmenensis*	Mexico; Baja California Sur; Ramon a Santa Agueda	*ROM 13576*	DQ001782, DQ001825
21	*carmenensis*	Mexico; Baja California Sur; Isla San Marcos	RWM 1369	DQ001783, DQ001826
22	*carmenensis*	Mexico; Baja California Sur; Mulege (14.2 mi W of)	RWM 1014	DQ001784, DQ001827
23	*carmenensis*	Mexico; Baja California Sur; Loreto (26.4 mi N of)	RWM 1303	DQ001785, DQ001828
24	*carmenensis*	Mexico; Baja California Sur; Loreto (21 km N of)	ROM 35376	DQ001786, DQ001829
25	*carmenensis*	Mexico; Baja California Sur; Loreto (8.3 km N of)	ROM 35375	DQ001787, DQ001830
26	*carmenensis*	Mexico; Baja California Sur; Isla Coronados	ROM 35401	DQ001788, DQ001831
27	*carmenensis*	Mexico; Baja California Sur; Isla del Carmen	ROM 35382	DQ001789, DQ001832
28	*carmenensis*	Mexico; Baja California Sur; Juncalito	RWM 1480	DQ001790, DQ001833
29	*carmenensis*	Mexico; Baja California Sur; Isla Danzante	ROM 35377	DQ001791, DQ001834
30	*carmenensis*	Mexico; Baja California Sur; Isla San José	ROM 35426	DQ001792, DQ001835
31	*carmenensis*	Mexico; Baja California Sur; San Pedro la Presa	RWM 763	DQ001793, DQ001836
32	*carmenensis*	Mexico; Baja California Sur; San Evaristo	ROM 35392	DQ001794, DQ001837
33	*carmenensis*	Mexico; Baja California Sur; Isla San Francisco	ROM 35432	DQ001795, DQ001838
34	*draconoides**	Mexico; Baja California Sur; La Paz (43 km W of)	ROM 35325	DQ001796, DQ001839
35	*draconoides*	Mexico; Baja California Sur; San Pedro (14.8 mi S of)	RWM 616	DQ001797, DQ001840
36	*draconoides*	Mexico; Baja California Sur; Buena Vista	*ROM 26796*	DQ001798, DQ001841
37	*draconoides*	Mexico; Baja California Sur; Cabo San Lucas (4 mi E of)	RWM 747	DQ001799, DQ001842
38	*ventralis*	United States; New Mexico; Hidalgo Co.	TWR 180	DQ001800, DQ001843
39	*inusitatus*	Mexico; Sonora; Isla Tiburón	ROM 38005	DQ001801, DQ001844
40	*inusitatus*	Mexico; Sonora; Bahía Kino	*ROM 15011*	DQ001802, DQ001845
41	*brevipes*	Mexico; Sonora; Álamos	*ROM 14928*	DQ001803, DQ001846
42	*bogerti*	Mexico; Sinaloa; Mazatlán	*ROM 14954*	DQ001804, DQ001847
Outgroup				
43	*Cophosaurus texanus*	Mexico; Chihuahua; 15 km N of Laboratorio del Desierto in Reserva de la Biósfera Mapimí	ROM 32048	DQ001805, DQ001848
44	*Holbrookia maculata*	Mexico; Chihuahua; 15 km N of Laboratorio del Desierto in Reserva de la Biósfera Mapimí	ROM 32049	AF302007, DQ001849

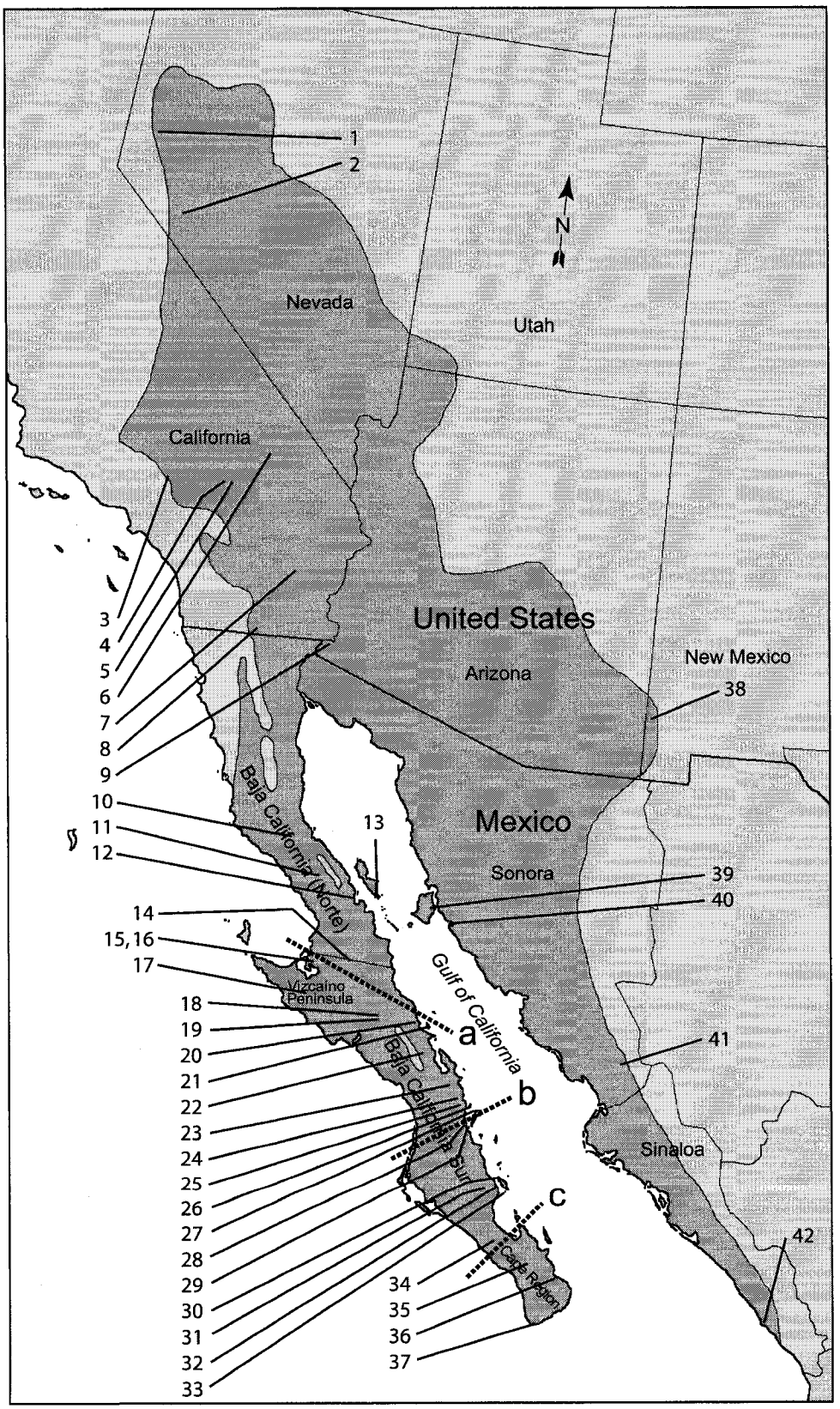
* *draconoides/carmenensis* intergradation zone

Figure 2.1. (a) Strict consensus of the combined data set (6 MPTs, CI = 0.617, RI = 0.825). Specimen numbers correspond to map localities. Nodal support is represented by Bremer decay index (DI) and bootstrap (BS) values of 50% or higher above and below the nodes, respectively. Vertical bars named M, N, and S refer to the clade of mainland Mexico, northern Baja California, and southern Baja California, respectively. (b) Map over northwestern Mexico and southwestern Unites States showing collection localities of specimens of *Callisaurus draconoides*. Specimens are clustered according to subspecies designation (Table 2.1). Horizontal dashed lines in strict consensus and on map indicate genealogical breaks discussed in text: a = mid-peninsular break; b = Loreto break; c = Isthmus of La Paz break. Range map follows Stebbins (1985) and Grismer (2002).

(a)


39 Isla Tiburón
40 Bahía Kino
41 Álamos
42 Mazatlán
M
1 Wadsworth
2 Hawthorne
7 Desert Centre
3 Pearblossom
4 Newberry Springs
5 Newberry Peak
6 Silver Lake
38 Hidalgo Co.
8 Ocotillo
9 Yuma Dunes
10 Santa Inez
11 Puente las Víboras
12 Bahía de Los Ángeles
14 El Arco
13 Isla Ángel de la Guarda
N
a
15 Guerrero Negro
16 Guerrero Negro
18 San Ignacio
21 Isla San Marcos
20 Ramon a Santa Agueda
22 Mulege
23 Loreto (26.4 mi N of)
24 Loreto (21 km N of)
25 Loreto (8.3 km N of)
27 Isla del Carmen
26 Isla Coronados
S
b
28 Juncalito
29 Isla Danzante
30 Isla San José
33 Isla San Fransisco
31 San Pedro la Presa
32 San Evaristo
34 La Paz
c
35 San Pedro
36 Buena Vista
37 Cabo San Lucas

(b)


1
2
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Nevada
Utah
California
3
4
5
6
7
8
9
United States
Arizona
New Mexico
38
10
11
12
13
Mexico
Sonora
39
40
Baja California (Norte)
14
15,16
17
Vizcaino Peninsula
Gulf of California
18
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32
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a
b
c
41
Baja California Sur
Sinaloa
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Cape Pasion
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DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from small amounts of ethanol-preserved liver or muscle tissue using a standard proteinase K extraction followed by phenol/chloroform purification (Sambrook *et al.* 1989). The remaining lysis buffer was diluted 20 times in ddH_2O and used for subsequent amplification.

Two segments of the mitochondrial genome were amplified using the polymerase chain reaction (PCR; Saiki *et al.* 1988): one cytochrome *b* region (hereafter referred to as cyt *b*; 1087 bp), which contained most (1045 bp) of the cytochrome *b* gene and a 31 bp segment of the threonine tRNA, and an *ATPase* region (hereafter referred to as *ATPase*; 866 bp) containing 30 bp of the lysine tRNA, all (168 bp) of the *ATPase 8* gene and all (682 bp) of the *ATPase 6* gene. Primers used in this study were named by their occurrence on the light or heavy DNA strand and 3' position in the *Xenopus leavis* mitochondrial genome (Roe *et al.* 1985). Two primers were used to amplify the cyt *b* region: L16355 (5'-cca tcc aac atc tca gca tga tga aa-3') and H17415 (5'-gtc ttc agt ttt tgg ttt aca aga c-3'). Two primers were used for amplification of the *ATPase* region: L9839 (5'-agc act agc ctt tta agy t-3') and L10041 (5'-gtg tgc ttg gtg tgy cat t-3'). The *ATPase* primers were designed specifically for *C. draconoides* based on primers generously supplied by O. Haddrath.

PCR reactions (25 µl) contained approximately 150 ng template DNA, 0.75 U Taq DNA Polymerase (Boehringer Mannheim), 80 µM dNTPs, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, and 10 pmol of each primer. A thermal cycler [GeneAmp PCR System 9700 (PE Applied Biosystems) or PTC 200 (MJ Research)] with the following conditions was

used: 1 step of 94°C for 2 min; 39 cycles of 94°C for 30 s, 42 to 53°C for 45 s, and 72°C for 45 s. After the final cycle, a prolonged extension step of 5 min was added. Amplified DNA was separated by electrophoresis on a 1% agarose gel and stained with ethidium bromide. DNA bands, visualized on an ultraviolet light table, were excised and eluted using a Gene Clean II kit (Bio101) and re-suspended in 10 to 25 µl ddH_2O or spun down through a filter pipette tip for 10 min with the resulting solution used for sequencing reactions. Chain-terminatory sequencing (Sanger *et al.* 1977; Hillis *et al.* 1996b) of amplified DNA was performed using the Thermo Sequenase ^{33}P-labeled terminator cycle sequencing kit (Amersham). Sequencing was performed in both directions with the same primers used in PCR amplification. In addition, L16676 (5'-tga gga caa ata tcc ttc tga gg-3'; Fu 1999) was used to sequence an internal section of the amplified cyt *b* fragment. This primer and L16355 are from regions that earlier have been established as conserved among a variety of vertebrates (Kocher *et al.* 1989). Sequencing products were separated by electrophoresis on 6% polyacrylamide-7M urea gels for 2–7 hr at 65 W. The gels were then dried onto filter papers and visualized on autoradiograph films (Kodak) after 24–96 hr exposure.

A large proportion of sequences were obtained using an ABI 377 Automated Sequencer, instead of the sequencing method outlined above, by utilizing the ABI Prism BigDye Terminator v 3.0 Cycle Sequencing Kit (Applied Biosystems) following the manufacturer's protocols. Amplified sequences derived for automated sequencing were cleaned using the QIAquick PCR Purification Kit (Qiagen), and sequencing products were cleaned using Centri-Sep Spin Columns (Princeton Separations).

Finally, the cyt *b* sequence of the *Holbrookia maculata* specimen was obtained from an earlier study (GenBank accession number AF302006; Trépanier & Murphy 2001).

Data analysis

Parsimony (Siddall & Kluge 1997; Kluge 2002) was used in a refutationist framework (Popper 1963). The sequence data were entered into BioEdit 5.0.9 (Hall 1999). The near absence of insertions and/or deletions (indels) in the cyt *b* region and total absence in the *ATPase* region made it possible to align these sequences unambiguously by eye. The two data sets were exported into MacClade 3.0.4 (Maddison & Maddison 1992) and then exported as nexus files into PAUP* 4.0b10 (Swofford 2000) for phylogenetic analysis. The data sets were also combined using MacClade in order to reconcile them on a single set of solutions. Such a total-evidence approach using character congruence has been shown to have several advantages over taxonomic congruence (Kluge 1989, 1998). Values of pairwise proportional sequence divergence (*p*-distances) were obtained using PAUP*. Individuals with identical cyt *b* or *ATPase* sequences were merged into a single OTU (operational taxonomic unit) for all phylogenetic analyses. The alignment of sequences used for analyses has been submitted to TreeBASE (study and matrix accession numbers are S1278 and M2234, respectively).

In our analyses, all characters were evaluated as unordered because there is no *a priori* reason to assume order of evolutionary change between the four nucleotide bases

of DNA (Swofford *et al.* 1996). In order to maximize the explanatory power of all lines of evidence, following reasons outlined by Frost *et al.* (2001), all analyses were conducted using equal weighting of transversions and transitions. The phylogenetic analyses using PAUP* were conducted using a heuristic search, with random addition of sequences, 100 replicates, retaining minimal trees only, using tree bisection-reconnection branch swapping saving multiple trees, and collapsing zero-length branches.

Nodal support was assessed with Bremer decay analysis (Bremer 1988, 1994) using AutoDecay 4.0.2 (Eriksson 1998) as implemented with PAUP*, and with bootstrap analysis (Felsenstein 1985) in PAUP* using 1000 replicates. We recognize that various problems have been identified for these and other common nodal support measures (e.g., Lee 2000; DeBry 2001). In both nodal support analyses, the heuristic search settings were identical to the phylogenetic analysis described above.

Results

Sequence variation

Nucleotide sequence length varied between 1081 and 1087 bp in cyt *b* due to indels that were almost exclusively located in the non-coding region between the cytochrome *b* and threonine tRNA genes. Gaps were inserted to accommodate the alignment of conserved regions, resulting in a total of 1087 aligned positions. There were 332 variable characters (30.5% of total sites), among which 226 (20.8%) were potentially phylogenetically

informative. Pairwise *p*-distances between the aligned sequences from cyt *b* revealed a large range in sequence divergence from 0.09% to 8.85% among ingroup specimens.

From *ATPase*, 866 homologous sites were examined. No insertions or deletions were observed. In total, 293 characters were variable (33.8% of total sites), with 180 (20.8%) being potentially phylogenetically informative. Pairwise *p*-distances between the aligned sequences from *ATPase* also revealed a large range in sequence divergence (0.13–8.34%), following the removal of two redundant sequences (17 and 19) that were identical to other sequences (16 and 18, respectively).

Pairwise distances based on the combined sequences (1953 bp; 40 specimens) ranged from 0.16% to 8.27% sequence divergence (Table 2.2).

Genealogical relationships

The cladistic analysis of cyt *b* yielded 24 most parsimonious trees (MPTs; length = 702 steps, CI = 0.571, RI = 0.820). The analysis of *ATPase* resulted in 4 MPTs (length = 530 steps, CI = 0.687, RI = 0.848). Individual analyses of the two gene segments were fully congruent with regard to phylogenetic relationships among clades.

Cladistic analysis of the combined data set resulted in 6 MPTs (length = 1238 steps, CI = 0.617, RI = 0.825). In the strict consensus tree, the specimens of *C. draconoides* formed three major clades, each associated with Bremer decay (DI) values of 11 or higher and bootstrap (BS) values of 99% or 100% (Fig. 2.1). One clade

Table 2.2. Pairwise genetic comparisons of mtDNA and allozymes between selected specimens. Percent sequence divergences (uncorrected *p*-distances) for combined mtDNA gene segments are given above the diagonal. Genetic distances (Nei's *D*; Nei, 1978) based on allozymes and reported by Adest (1987) are given below the diagonal for populations corresponding to localities of the present study. Specimens were selected based on relevance to the genealogy (see Fig. 2.1) and geographic location to facilitate comparison with allozyme data of Adest (1987).

	6	8	11	12	13	14	15	18	25	28	34	37	38	40	41	42
1 Wadsworth	3.36	4.09	3.58	4.27	4.65	4.25	7.15	6.83	6.91	6.74	7.31	7.43	3.75	7.34	7.12	7.82
6 Silver Lake	—	2.31	1.89	4.82	4.96	5.37	7.44	6.98	7.23	6.84	7.26	7.47	1.40	7.22	7.06	7.56
8 Ocotillo	.00	—	1.74	4.91	5.60	5.49	7.49	6.88	7.19	7.21	7.45	7.82	2.84	7.75	7.68	7.97
11 Puente Las Víboras			—	4.68	5.24	5.17	7.60	7.29	7.52	7.04	7.42	7.67	2.58	7.36	7.32	7.83
12 Bahía de Los Ángeles				—	4.99	3.25	7.70	7.35	7.45	7.31	7.55	7.93	5.21	7.59	7.43	7.46
13 Isla Ángel de la Guarda					—	5.43	7.61	7.25	7.36	7.22	7.73	8.03	5.64	7.44	7.48	7.52
14 El Arco						—	7.24	7.25	7.35	7.01	7.38	7.38	5.67	7.37	7.06	7.36
15 Guerrero Negro	.02	.02					—	2.53	2.65	3.31	3.47	3.61	7.75	7.11	7.32	7.39
18 San Ignacio								—	0.52	3.24	3.42	3.70	7.48	6.90	7.02	7.45
25 Loreto (8.3 km N of)	.03	.08					.07		—	2.98	3.21	3.58	7.73	7.03	7.27	7.76
28 Juncalito										—	1.41	1.90	7.39	6.91	7.03	7.66
34 La Paz	.03	.03					.00		.08		—	2.00	7.66	7.03	7.30	8.03
37 Cabo San Lucas												—	7.84	7.08	7.25	7.55
38 Hidalgo Co.													—	7.48	7.39	7.92
40 Bahía Kino														—	1.23	3.72
41 Álamos	.00	.00					.01		.05		.02				—	3.72
42 Mazatlán	.07	.06					.07		.16		.08				.06	—

Note: for comparison with Adest's allozyme data we selected specimens 6, 8, 15, 25, 34, 41, and 42 to correspond to Adest's populations from Nevada, Guadelup Cañon, Guerrero Negro, Loreto, La Paz, Álamos, and Mazatlán, respectively.

contained specimens from southern Baja California, including specimens from Cabo San Lucas northward to San Ignacio and Guerrero Negro. A second clade consisted of specimens from northern Baja California including those from El Arco northward as well as all specimens from the United States. This clade also included the specimen from Isla Ángel de la Guarda. The third clade consisted of the three mainland Mexican samples from Sonora and Sinaloa. This mainland group also included the sample from Isla Tiburón. The major clades of northern Baja California and mainland Mexico formed a weak sister-group relationship (DI = 1, BS < 50).

Further subdivisions occurred within both major clades from Baja California. Within the southern clade, samples from Cabo San Lucas north to Juncalito formed the sister-group to samples from 8.3 km north of Loreto northward to San Ignacio and the Vizcaíno Desert. The southernmost subclade also split between the three samples from the Cape Region and specimens from 43 km west of La Paz north to Juncalito. The northern subclade was divided between specimens from 8.3 km north of Loreto north to San Ignacio and those from the Vizcaíno Desert.

Within the northern major clade, Isla Ángel de la Guarda was the sister-group to the remaining specimens, among which a division occurred between (1) samples from El Arco to Bahía de Los Ángeles and (2) the samples from Puente Las Víboras to northward on the peninsula together with all the specimens from the United States.

Overall, deeper genealogical splits were generally associated with higher divergence estimates. The preferred tree shows a deep genealogical split midway on the peninsula associated with 7.25% sequence divergence between El Arco and San Ignacio (Fig. 2.1; Table 2.2). Younger genealogical splits occur across the peninsula around

Loreto, just north of Bahía de Los Ángeles, and over the Isthmus of La Paz, as well as across the Vizcaíno Desert.

Discussion

General

Our cladistic analyses resulted in several MPTs with single specimens representing each OTU. Two assumptions are inherent in the discussion of these MPTs. First, we assume that the characters in our data set are correlated due to genealogical association that results from the mitochondrial genome being descended as one single unit without experiencing recombination. The absence of recombination means that hierarchical, genealogical patterns will form among, as well as within, species. Second, we assume that the hierarchical relationships among mtDNA haplotypes follow maternal lineages only. Although paternal inheritance has been described as common in other taxa, most notably the mussel *Mytilus* (Zouros *et al.* 1994), it has been reported for three vertebrate species only (Gyllensten *et al.* 1991; Schwartz & Vissing 2002; Bromham *et al.* 2003; Kvist *et al.* 2003), none of which are reptiles. Hence, we assume that the geographical patterns of the genealogies are linked tightly to female history, including dispersal patterns.

Genealogical history

Taxonomy should be logically consistent with the recovered history of evolution (Wiley 1981; Frost & Hillis 1990). The deep genealogical history revealed in this study raises the question as to whether or not *Callisaurus* actually consists of several species.

The deepest genealogical split occurs between the southern Baja Californian clade and the clade consisting of samples from northern Baja California and mainland Mexico (Fig. 2.1). This split is associated with a high genetic distance of approximately 7.25% (Table 2.2). This level of genetic divergence is similar to that reported by Wilgenbusch and de Queiroz (9.7%; 2000), although their estimate is based on a base pair substitution model. Wilgenbusch and de Queiroz also questioned the status of *Callisaurus* as a monotypic genus due to the moderately high genetic distance values. The deep genealogical splits may be associated with disruption in gene flow and population differentiation.

Callisaurus draconoides is the first organism in the region for which a mtDNA genealogy encompassing a range of widespread locations from around the Gulf of California can be compared with a study of nuclear-encoded markers on a similar geographic scale. Adest (1987) reported low allozymic genetic divergences among seven populations representing six subspecies of *C. draconoides* from widely separate areas. The average genetic distance (Nei 1978) between populations based on 20 allozyme loci was $D = 0.05$. This low level of divergence is consistent with what has been reported for intraspecific differentiation in other Baja Californian reptiles (Murphy 1983). In addition, samples from Guerrero Negro (n = 6) and Guadalupe Cañon (n = 4) of Baja California

(Norte), between which the deepest genealogical split of the present study is located, showed a low genetic divergence of $D = 0.02$ (Table 2.2). The genealogical split between the clade of mainland Mexican samples and its sister-group, suggested by Wilgenbusch and de Queiroz (2000) to be the deepest split within *C. draconoides*, approximates 7.48% sequence divergence. Adest (1987) reported a total lack of allozyme differentiation in this area when comparing samples from Álamos in Sonora (n = 7) and Nevada (n = 9; Table 2.2).

The high allozyme similarity among populations, despite deep genealogical histories, indicates ongoing gene flow among populations in which the deepest genealogical splits occur. Evidence from morphological studies supports this conclusion. Both Linsdale (1932) and Bogert and Dorson (1942) argued that only one species of *Callisaurus*, *C. draconoides*, inhabits the Baja Californian peninsula. Linsdale (1932, p. 359) stated that "The range of characters separating any two of the subspecies is not large but in each case there is good evidence of intergradation where the ranges meet." They were, however, unsure about the status of the sand-dwelling form *crinitus* of the Vizcaíno Desert, which has previously been recognized as a species (Cope 1896; Norris 1958). *Callisaurus d. crinitus* is genealogically closely associated with the deep mid-peninsular split (see specimens 15 and 16 in Fig. 2.1). However, *C. d. crinitus* has been reported to intergrade morphologically with both *C. d. rhodostictus*, from the adjacent region north of the mid-peninsular break, and *C. d. carmenensis*, its other neighboring subspecies to the southeast (Mosauer 1936; Grismer 2002). It appears that specimens from the Vizcaíno Desert are not genetically isolated.

All available data from allozymes and morphology suggest that gene flow is unabated for contiguous populations of *C. draconoides*, a pattern that is true for most other species of vertebrates on the peninsula (e.g., Lawlor *et al.* 2002; Murphy & Aguirre-León 2002). Hence, in spite of the deep genealogical patterns revealed by mtDNA, and following a phylogenetic species concept, evidence suggests that only one species of *Callisaurus* inhabits the Baja Californian peninsula, southwestern United States, and mainland Mexico.

Genealogies and geological history

Even though no contiguous population of *Callisaurus* merits species status, the genealogy may add valuable information to our understanding of how the Baja Californian peninsula evolved geologically and the historical biogeography of its fauna. Recent molecular phylogenetic studies on phrynosomatid lizards have suggested the historical occurrence of trans-peninsular seaways across Baja California at the level of San Ignacio, midway on the peninsula, and the Isthmus of La Paz (Upton & Murphy 1997; Aguirre-León *et al.* 1999). Congruent genealogies of numerous other groups, including other lizards, snakes, mammals, birds and arthropods, also support the existence of these seaways (e.g., Zink *et al.* 1997; Riddle *et al.* 2000; Gantenbein *et al.* 2001; Lawlor *et al.* 2002; Murphy & Aguirre-León 2002).

The discovery of a deep genealogical split within *C. draconoides* supports the existence of a mid-peninsular seaway. Populations of *C. draconoides* appear to have been

historically isolated across this seaway, generating the genealogical split in mtDNA. Later, when the ocean receded, the populations were re-united and started to interbreed again, generating enough gene flow in nuclear DNA to explain the results of Adest (1987).

Murphy and Aguirre-León (2002) emphasized the complex and still largely unknown paleogeographical history of the Baja Californian peninsula in their discussion of a "peninsular archipelago." An evaluation of younger splits in the genealogy of *C. draconoides* combined with recent evidence from geology and paleostratigraphy reveals patterns that may help elucidate this unknown history.

In our genealogy, the haplotype from Isla Ángel de la Guarda diverges early in the northern Baja Californian clade (Fig. 2.1). It is 4.99% divergent from the haplotype from Bahía de Los Ángeles (Table 2.2). This is the only population of *C. draconoides* occurring on a deep-water island. Hence, it has not been affected by temporary land-bridge connections to the peninsula during glacial periods. Geological data suggest that Isla Ángel de la Guarda was formed around 2–3 million years ago (Mya) by a leaky transform fault (Lonsdale 1989; Delgado-Argote *et al.* 2000; Stock 2000; Carreño & Helenes 2002). The poor over-water dispersal capability of *C. draconoides* (Murphy & Aguirre-León 2002) implies that the population most likely is of the same age.

The genealogy of *C. draconoides* also suggests the occurrence of a relatively deep genetic break between maternal lineages in the vicinity of Loreto (Fig. 2.1.). Geographically close specimens display 2.98% sequence divergence (Table 2.2). No vicariance event, such as a trans-peninsular seaway, has been suggested for this region, although the deepest split in the genealogy of *Aspidoscelis* (formerly *Cnemidophorus*)

hyperythra occurring in this region (Radtkey *et al.* 1997) led Murphy and Aguirre-León (2002) to speculate on the existence of yet another peninsular island.

A clear pattern resulting from the geographical congruence among multiple genealogies, as associated with the trans-peninsular seaways of the mid-peninsular region and the Isthmus of La Paz, has, so far, not emerged in the Loreto area. However, recent evidence from the black-tailed brush lizard (*Urosaurus nigricaudus*, Lindell *et al.* in preparation) and the side-blotched lizard (*Uta stansburiana*, Hollingsworth 1999; Lindell *et al.* in preparation) also show genealogical splits in close vicinity of the *C. draconoides* break near Loreto (Lindell *et al.* in preparation). Sequence divergence across this break is similar among the three species (2.5–4.2%; homologous cyt *b* region compared).

Available geological data do not support these concordant genealogical breaks to be associated with a trans-peninsular seaway. West of Loreto lies the Sierra de la Giganta, a formidable mountain ridge that had attained most of its heights by middle Miocene (Hausback 1984; Umhoefer *et al.* 2001). The abrupt altitudinal change from the heights of the Sierra de la Giganta to the narrow low-lying areas along the eastern coast of the peninsula is part of the Gulf Escarpment, which formed during late Miocene (Stock & Hodges 1989; Umhoefer *et al.* 2002). Although no geological evidence suggests that the Gulf Escarpment and the Baja Californian peninsula were severed at the level of present day Loreto, extensive geological activity occurred in the low-lying area east of the Gulf Escarpment during Pliocene and Pleistocene (Umhoefer *et al.* 1994; Dorsey *et al.* 1997; Umhoefer *et al.* 2002). Detailed studies on the stratigraphy of the Loreto basin have suggested substantial deposition of marine sediments of Pliocene origin (McLean 1988, 1989; Piazza & Robba 1994; Umhoefer *et al.* 1994; Dorsey *et al.* 1997). There is

no evidence for sedimentation extending westward across the Sierra de la Giganta (A. Zanchi, personal communication). Umhoefer and colleagues estimated that deposition began at 3.4 Mya and lasted until 2.0 Mya (Umhoefer *et al.* 1994), while McLean estimated the dates to 3.3 and 1.9 Mya, respectively (McLean 1988).

Even if the peninsula was not completely severed by a seaway in this area, submergence of the Loreto Basin towards the base of the Gulf Escarpment during Pliocene may have isolated coastal populations and restricted latitudinal gene flow to populations west of the mountains. While such an incomplete isolation event likely did not affect all species similarly, we expect that congruent genealogical discontinuities will be discovered in other species. Further genealogical studies based on detailed sampling of this area are required to address the significance of this "Loreto break." Nevertheless, in the maternal genealogy of *C. draconoides*, the Loreto break is older than the split across the Isthmus of La Paz, implying a younger Plio-Quaternary submergence of the Isthmus region (Hausback 1984).

These genealogical patterns, inferred to be associated with known geological events, have implications for the relative dating of other genealogical splits in *C. draconoides*. If the Loreto break and isolation of Isla Ángel de La Guarda happened approximately 2–3 Mya, then the maternal genealogy of *C. draconoides* implies that the mid-peninsular seaway must have occurred significantly earlier than previously hypothesized. Therefore, the current age estimate for a mid-peninsular vicariance event of 1 Mya (Upton & Murphy 1997; Aguirre-León *et al.* 1999; Hollingsworth 1999; Riddle *et al.* 2000) appears too young, a possibility recognized by Murphy and Aguirre-León (2002). Three points supporting an older age estimate are outlined below.

Firstly, the initial proposal of a historical mid-peninsular seaway was based on the genealogy of *U. stansburiana* (Upton & Murphy 1997). In this genealogy, the Isla Ángel de la Guarda specimen splits off prior to the mid-peninsular break. Based on an estimate from tectonic evidence suggesting that Isla Ángel de la Guarda broke off from the peninsula approximately 1 Mya (Moore 1973), Upton and Murphy dated the formation of the mid-peninsular seaway at approximately 1 Mya. Therefore, the estimate for the formation of Isla Ángel de la Guarda at 2–3 Mya implies an older mid-peninsular seaway than suggested, given the genealogy of *U. stansburiana* alone.

Secondly, a number of other species show a mid-peninsular break, suggested to correlate with the genealogical break in *U. stansburiana* (reviewed in Lawlor *et al.* 2002; Murphy & Aguirre-León 2002). Of these species, only a few genealogies include specimens from Isla Ángel de la Guarda. Among the reptilian representatives, *Sauromalus* (Petren & Case 1997, 2002) and *Aspidoscelis tigris* (Radtkey *et al.* 1997) display a genealogical pattern where the Isla Ángel de la Guarda clade splits off after the mid-peninsular divergence. Hence, in agreement with the genealogy of *C. draconoides*, they support an age for the mid-peninsular seaway as older than 2–3 Mya (the current time estimate for isolation of Isla Ángel de la Guarda). The older scenario suggested by the genealogy of *C. draconoides* is further supported by the genealogy of *Pituophis*, which also shows a deep mid-peninsular break. Interestingly, the estimated divergence time of 4.7–13.2 Mya for *Pituophis* was originally suggested to have been caused by a physiogeographical event older than the mid-peninsular seaway (Rodríguez-Robles & De Jesús-Escobar 2000).

Finally, geographically nearby specimens (of the four lizard taxa) on either side of the mid-peninsular break show high genetic divergence values (Table 2.3), suggesting an old isolation event. An estimate for the mid-peninsular seaway having happened 1 Mya requires an mtDNA divergence rate of 9.0% per million years in *U. stansburiana*. Such a rate is improbable, being 6.8 to 19.1 times greater than estimates based on mtDNA from five other reptilian taxa (Zamudio & Greene 1997), despite the use of partially different gene regions.

When did the mid-peninsular seaway form? Genealogical patterns can only give us the relative dating of the event, and estimating divergence times based on molecular data is associated with uncontrollable and large sources of error (Hillis *et al.* 1996a; Ayala 1999). However, there is evidence for an earlier date for the mid-peninsular seaway, based on available sedimentological and paleontological data. Large areas adjacent to the main mountain range in the mid-peninsular region were submerged during middle Miocene to early Pliocene times, as documented by a substantial paleontological record of marine mollusks (Smith 1984, 1991). Marine sediments, currently exposed close to Santa Rosalía, also suggest deepening of Gulf waters in late Miocene to early Pliocene (Wilson 1948; Ortlieb & Colletta 1984). Based on dates from marine sediments and a series of basalt vents, Helenes and Carreño (1999) and Carreño and Helenes (2002) suggested a late Miocene seaway across the mid-peninsula. The geochronology of the Santa Rosalía sediments suggests a likely age of onset of marine deposition to 7.1 ± 0.05 Mya (Holt *et al.* 2000), lending further support to a late Miocene date for the seaway.

Helenes and Carreño (1999) and Carreño and Helenes (2002) proposed San Ignacio as the location for the seaway, as this location requires the shortest distance to

Table 2.3. Divergence data (uncorrected *p*-distances) for lizard species whose mtDNA genealogies have been suggested to support the historical existence of a mid-peninsular seaway and include representatives from Isla Ángel de la Guarda. Estimates represent divergences between geographically close specimens across the genealogical break.

Species	Mid-peninsular divergence	Sequences compared	Reference
Callisaurus draconoides	8.2%	cyt *b* region (1087 bp)	Present study
	6.0%	*ATPase* region (866 bp)	
	7.2%	combined (1953 bp)	
Aspidoscelis tigris	7.0%	cytochrome *b* (887 bp)	Radtkey *et al.* 1997
Sauromalus australis/obesus	4.5%	cytochrome *b* (902 bp)	Petren and Case, 1997
Uta stansburiana	9.9%	cytochrome *b* (555 bp)	Upton and Murphy, 1997
	7.5%	*ATPase 6* (335 bp)	
	9.0%	combined (890 bp)	

join the marine sediments from west to east across the peninsula. San Ignacio is situated less than 200 m above sea level. Although the area closer to the Gulf Escarpment east of San Ignacio reaches an altitude of almost 500 m, evidence suggests it was raised substantially during Pleistocene alone, as sedimentary terraces from nearby Santa Rosalía were elevated at least 340 m (Wilson 1948; Ortlieb 1979, 1980, 1991). The uplift is related to Plio-Quaternary volcanic activity north of Santa Rosalía associated with the Reforma and Tres Virgenes volcanoes (Schmidt 1975; Demant 1984; Ortlieb & Colletta 1984). This magnitude of elevation is exceptional and has not been observed in other areas along the peninsular Gulf coast (Ortlieb 1980). In addition, Carreño (1981, 1982) inferred 200–500 m as depositional depths for the sediments related to the hypothesized late Miocene seaway.

The proposed seaway may be the isolation event that generated the numerous concordant genealogical patterns observed. A late Miocene seaway formed around 7 Mya would correspond to divergence rates for the four lizard taxa similar to those reported by Zamudio and Greene (1997).

Implications of an older time frame

Clearly, further evidence from both geology and genealogical history is needed to elucidate the history of the peninsular archipelago. Genealogical patterns may illuminate plausible paleogeographical scenarios not yet suggested by geological models. However, an older time frame for genealogical histories, as suggested by *C. draconoides*, should be

evaluated together with geological evidence of a similar, if not yet older, time frame. Available evidence suggests that the mouth of the Gulf of California opened as early as 12.5 Mya (Hausback 1984; Lonsdale 1989), or even earlier, at 16–14 Mya (Smith 1991). Miocene marine sediments have been also reported from throughout the proto-Gulf (e.g., Smith 1991; Smith *et al.* 1994; Gastil *et al.* 1999; Delgado-Argote *et al.* 2000). These sediments pre-date the hypothesized late Miocene mid-peninsular seaway. Oskin and Stock (2003) recently re-evaluated the sedimentological setting on Isla Tiburón, suggesting that marine waters may have reached the area in late Miocene. A late Miocene extension of the Gulf waters into this area severing the distribution of *C. draconoides*, at a time similar to a mid-peninsular seaway, could explain the inability of the current data set to resolve the basal trichotomy in the genealogy of *C. draconoides*.

The deep history revealed in this study suggests that current models on the evolution of peninsular faunas are temporally shallow in relation to geological evidence, in particular those models based on a specific geological scenario. For example, Grismer (1994) based his hypothesis of the evolution of the Baja Californian herpetofauna on a fixed geological model restricting the opening of the Gulf to approximately 5.5 Mya. This led him to postulate that *C. draconoides* likely was a recent immigrant into Baja California, entering the peninsula after the expansion of mid-peninsular deserts and union of the southern peninsular regions. Our genealogy completely disagrees with this hypothesis, implying a much older time span for the presence of *C. draconoides* in the region. Similarly, while Riddle *et al.* (2000) neatly laid out evidence for cryptic vicariance in the regional fauna, they also restricted their hypothesis to the time frame of Grismer, in addition to relying on the earlier 1 Mya estimate for the mid-peninsular

seaway in their biogeographical reconstruction. Nevertheless, their conclusion about a significantly earlier scenario for the evolution of Baja California's desert biota now appears even more justified. It no longer appears reasonable to hypothesize range shifts associated with Quaternary climatic oscillations (Tanner & Banta 1977; Schmidly *et al.* 1994) or adaptation to local selection regimes (Grismer 1994) as a primary mechanism responsible for contemporary species distributions and genetic discontinuities along the Baja Californian peninsula.

Any attempt at formulating a refined hypothesis of the biogeographical history of Baja California must be based on analyses that synthesize biological and geological data (e.g., Murphy & Aguirre-León 2002). The complex history of genealogies of the same region, with associated variation and discrepancies, must be seriously considered. For example, the possibility of recurring seaways having created similar genealogical patterns in the mid-peninsular region is intriguing and could explain some of the discrepancies among genealogical topologies. Genealogical congruence already supports multiple inundations of the Isthmus of La Paz (Murphy & Aguirre-León 2002).

In this regard, genealogical splits inferred to be related to known geological events, such as the Loreto break and formation of deepwater islands such as Isla Ángel de la Guarda, may be used as "genealogical anchors" when elucidating the biogeographical history of the peninsular archipelago. The hierarchical structures of such temporally detailed genealogies will lend themselves nicely to an analysis of the historical biogeography in which hypothetico-deductive methodology can make testable hypotheses of historical area relationships (PACT; Wojcicki & Brooks 2005). Conversely, historical biogeography analyses based on genealogies conforming to a general picture (e.g. an

older seaway), but lacking the temporal restraints provided by a detailed evaluation, may erroneously suggest total congruence between genealogies affected by different vicariance events at the same location. Genealogical anchors may thus ensure a similar temporal framework of the species used in biogeographical analyses.

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Chapter 3

Deep biogeographical history and cytonuclear discordance in the black-tailed brush lizard (*Urosaurus nigricaudus*) of Baja California

Abstract

Molecular tools help us deduce historical events such as vicariance, dispersal, gene flow, and speciation. However, our inferences are inevitably linked to the nature of the characters that we use to infer history. For example, the difference in inheritance patterns of mitochondrial DNA (mtDNA) and nuclear DNA (non-recombining maternal versus recombining biparental inheritance) may lead us to propose different intraspecific histories. The peninsula of Baja California of northwestern Mexico, a region affected by a complex geological history involving temporary seaways, permits evaluation of differences between these character types. We sequenced 1,966 bp of mtDNA to reconstruct the genealogical history of *Urosaurus nigricaudus* (black-tailed brush lizard) from samples spanning the entire peninsula. The genealogy revealed several deep divergences, congruent with temporary vicariance events in the mid-peninsular, Loreto, and Cape regions, as well as a major split across the Isthmus of La Paz possibly resulting

from a late Miocene seaway. The results support an emerging picture of the historical biogeography of Baja California, which suggests that key vicariance events are older than commonly perceived. The maternal genealogy of *U. nigricaudus* sharply contrasts with variation in allozymes that suggests very little differentiation across mitochondrial breaks, consistent with a pattern of ongoing gene flow. We interpret this cytonuclear discordance in relation to the historical biogeography of the region.

Introduction

Our ability to use molecular tools to infer historical events such as vicariance, dispersal, gene flow, and speciation is dependent upon our choice of characters with which to reconstruct history. Different types of DNA may suggest different scenarios, as evident at the interspecific as well as the intraspecific level (Brower *et al.* 1996; Nichols 2001; Funk & Omland 2003). Central to this discussion is the distinction between mitochondrial DNA (mtDNA) and nuclear DNA based characters (Moore 1995), and the extent to which intraspecific genealogies correspond to population history (Edwards & Beerli 2000; Hudson & Turelli 2003; Lindell *et al.* 2005). This debate is important, as more and more species are subjected to range-wide molecular analyses with the intent of addressing species history. MtDNA has largely been the tool of choice, mainly due to its hierarchical nature of maternal inheritance and relatively rapid rate of evolution, coupled with a good understanding of its genomic features (Barton & Jones 1983; Harrison 1989). However, while these very features have been hailed as a proxy for understanding population

divergence, even to the degree of blindly extrapolating population history from the geographic patterns of mtDNA genealogies, reliance on mtDNA alone is receiving increasing criticism (Rubinoff & Holland 2005). Further studies illuminating the distinction between mtDNA and nuclear DNA based characters are needed.

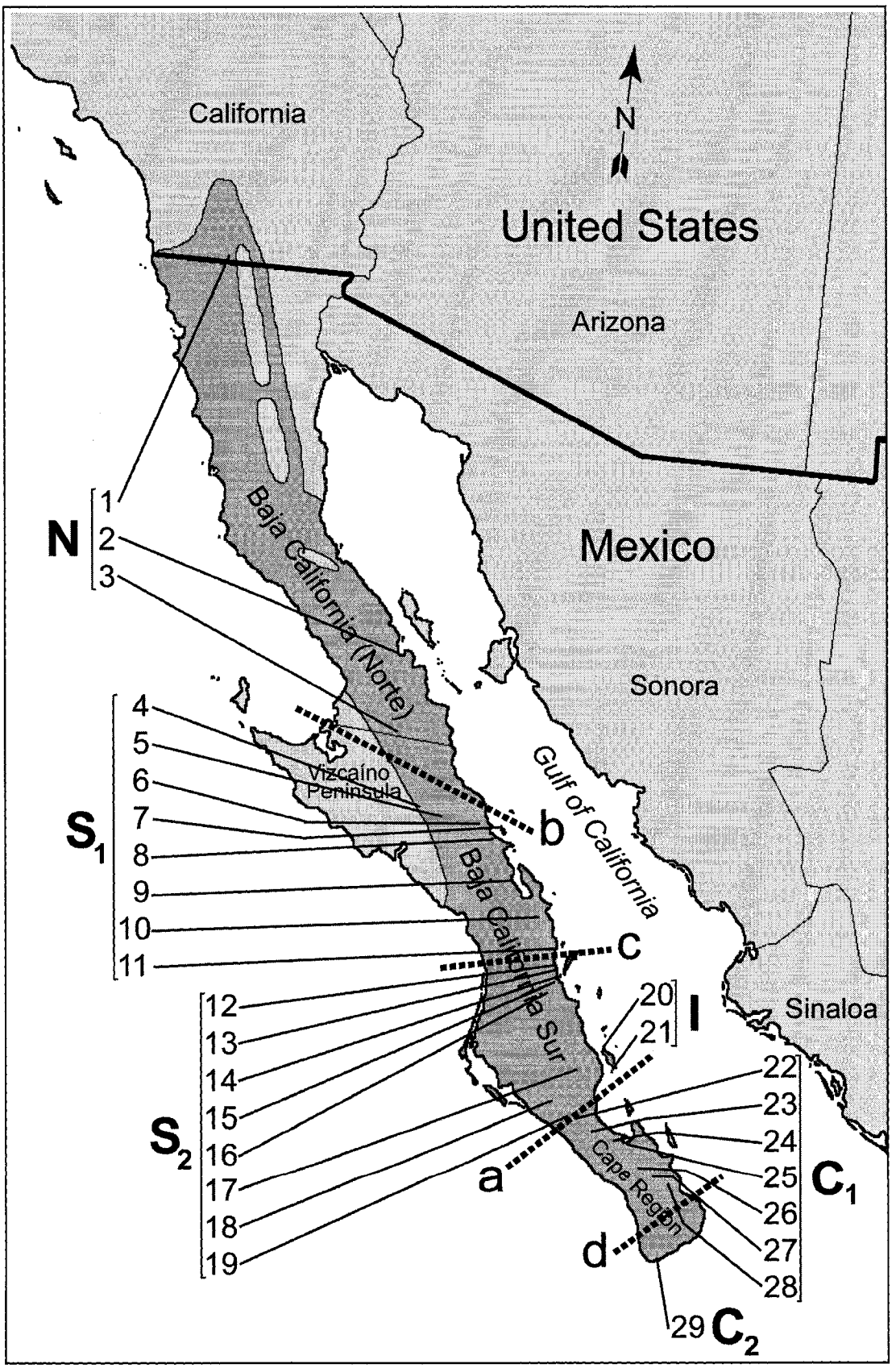
The peninsula of Baja California of northwestern Mexico has a very complex geological history (Carreño & Helenes 2002), making it an interesting region for studying genetic patterns resulting from a vicariant history. [The state of Baja California on the northern half of the peninsula will herein be referred to as 'Baja California (Norte).'] This peninsula presents a virtually one-dimensional transect along which patterns in loci representing maternal and biparental histories can be evaluated. Recent molecular studies based on mtDNA have revealed deep genealogical divergences in numerous species along the peninsula (Upton & Murphy 1997; Riddle *et al.* 2000b; Murphy & Aguirre-León 2002; Lindell *et al.* 2005). Congruent patterns among these genealogies suggest that historical seaways transversed the peninsula in at least two locations, temporarily separating populations. Secondary contact zones later formed when the populations reunited, as evident from the genetic breaks along the peninsula where highly divergent mtDNA lineages meet (Lindell *et al.* 2006).

In a recent molecular study on the zebra-tailed lizard (*Callisaurus draconoides*; Lindell *et al.* 2005), deep genealogical history based on mtDNA showed a clear discordance with low overall population differentiation in allozymes (Adest 1987). Such cytonuclear discordance may have various explanations, such as sex-differential dispersal or Haldane's rule. It remains unknown if cytonuclear discordance is common along the peninsula of Baja California, as *C. draconoides* is the only organism for which studies

based on allozymes and mtDNA of comparable size are available. However, current evidence suggests that for most organisms gene flow is strong along the entire peninsula (Murphy & Aguirre-León 2002).

The black-tailed brush lizard, *Urosaurus nigricaudus* (Cope 1864), is a common phrynosomatid lizard in Baja California, ranging continuously from the southern tip of the Cape Region, northward into southern California (Fig. 3.1). It is commonly found in trees [e.g. desert ironwood (*Olneya tesota*)] and large shrubs, but sometimes also among large boulders. The only areas on the peninsula where it does not occur are the Vizcaíno Peninsula and parts of the northeastern corner. A number of insular populations also exist in the Gulf of California (Grismer 2002; Murphy & Aguirre-León 2002). An allozyme-based study addressing population differentiation has already been conducted for *Urosaurus* in Baja California (Aguirre-León *et al.* 1999). In this study we used mtDNA sequences to investigate the genealogy of *U. nigricaudus* from the peninsula of Baja California and some adjacent islands. The history of maternal lineages was contrasted with the pattern of genetic variation in biparentally inherited allozymes. The mtDNA genealogy was also evaluated in the context of an older time-frame for the geological evolution of the peninsula and its associated biota (Lindell *et al.* 2005; Lindell *et al.* 2006).

Figure 3.1. Map of northwestern Mexico and southwestern United States showing collection localities of specimens of *Urosaurus nigricaudus*. Specimens are clustered according to genealogical relationships (see Fig. 3.2 and Results). Dashed lines across the peninsula indicate genealogical breaks discussed in text: a = Isthmus of La Paz break; b = mid-peninsular break; c = Loreto break; d = Cabo break. Species range (shaded area) follows Stebbins (1985) and Grismer (2002).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S2
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I
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C1
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C2
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a
b
c
d

Materials and methods

Population sampling

Tissue samples were obtained through our own fieldwork and donations. Twenty-nine specimens of *U. nigricaudus* were chosen from a wide range of peninsular and insular populations (Appendix 3.1; Fig. 3.1). Several samples were very close geographically (i.e. same population), yet all were treated as individual OTUs (operational taxonomic units). Seven samples of *U. graciosus*, *U. lahtelai*, and *U. ornatus* were used as a closely related outgroup (Mittleman 1942; Wiens 1993; Aguirre-León *et al.* 1999). In addition, single specimens of *Petrosaurus mearnsi*, *P. thalassinus*, and *P. repens* were chosen as distant outgroup taxa (Reeder 1995; Reeder & Wiens 1996).

DNA extraction, amplification, and sequencing

Laboratory procedures and data analysis follow those described by Lindell *et al.* (2005) for *C. draconoides*. Total genomic DNA was extracted from small amounts of ethanol-preserved liver or muscle tissue using a standard proteinase K extraction followed by phenol/chloroform purification (Sambrook *et al.* 1989). The remaining lysis buffer was diluted 20 times in ddH_2O and used for subsequent amplification.

Two segments of the mitochondrial genome were amplified using the polymerase chain reaction (PCR; Saiki *et al.* 1988): one cytochrome *b* region (hereafter referred to as

cyt *b*; 1094 bp), which contained most (1045 bp) of the cytochrome *b* gene and a 31 bp segment of the threonine tRNA, and an *ATPase* region (hereafter referred to as *ATPase*; 872 bp) containing 31 bp of the lysine tRNA, all (168 bp) of the *ATPase 8* gene, and all (682 bp) of the *ATPase 6* gene. Primers used in this study were named by their occurrence on the light or heavy DNA strand and 3' position in the *Xenopus leavis* mitochondrial genome (Roe *et al.* 1985). Two primers were used to amplify cyt *b*: L16355 (5'-cca tcc aac atc tca gca tga tga aa-3') and H17415 (5'-gtc ttc agt ttt tgg ttt aca aga c-3'). Two primers were used for amplification of *ATPase*: L9839 (5'-agc act agc ctt tta agy t-3') and H10710 (5'-gtg tgc ttg gtg tgy cat t-3'). The *ATPase* primers were designed specifically for *C. draconoides* (Lindell *et al.* 2005).

PCRs (25 μl) contained approximately 150 ng template DNA, 0.75 U *Taq* DNA Polymerase (Boehringer–Mannheim), 320 μM dNTPs, 10 mM Tris–HCl, 1.5 mM $MgCl_2$, and 10 pmol of each primer. A thermal cycler [GeneAmp PCR System 9700 (PE Applied Biosystems) or PTC 200 (MJ Research)] with the following conditions was used: 1 step of 94°C for 2 min; 39 cycles of 94°C for 30 s, 44-52°C for 45 s, and 72°C for 45 s. After the final cycle, a prolonged extension step of 5 min was added. Amplified DNA was separated by electrophoresis on a 1% agarose gel and stained with ethidium bromide. DNA bands, visualized on an ultraviolet light table, were excised and eluted using a Gene Clean II kit (Bio101) and re-suspended in 10–25 μl ddH_2O or spun down through a filter pipette tip for 10 min with the resulting solution used for sequencing reactions. Chain-terminatory sequencing (Sanger *et al.* 1977) of amplified DNA was performed using the Thermo Sequenase ^{33}P-labeled terminator cycle sequencing kit (Amersham). Sequencing was performed in both directions with the same primers used in PCR amplification. In

addition, L16676 (5'-tga gga caa ata tcc ttc tga gg-3'; Fu 1999) was used to sequence an internal section of the amplified cyt *b* fragment. This primer and L16355 are from regions that earlier have been established as conserved among a variety of vertebrates (Kocher *et al.* 1989). Sequencing products were separated by electrophoresis on 6% polyacrylamide–7M urea gels for 2–7 hr at 65 W. The gels were then dried onto filter papers and visualized on autoradiograph films (Kodak) after 24–96 h exposure.

A large proportion of sequences were obtained using an ABI 377 Automated Sequencer, instead of the sequencing method outlined above, by utilizing the ABI Prism BigDye Terminator v 3.0 Cycle Sequencing Kit (Applied Biosystems) following the manufacturer's protocols. Amplified sequences derived for automated sequencing were cleaned using the QIAquick PCR Purification Kit (Qiagen), and sequencing products were cleaned using Centri-Sep Spin Columns (Princeton Separations).

Sequence data have been submitted to GenBank (Accession Nos. EF653280–EF653357).

Data analysis

Sequence data were entered into BioEdit 5.0.9 (Hall 1999). The near absence of insertions and/or deletions (indels) in cyt *b* and total absence in *ATPase* made it possible to align these sequences unambiguously by eye, with the exception of the non-coding region between the cytochrome *b* and threonine tRNA genes. The two data sets were exported into MacClade 3.0.4 (Maddison & Maddison 2002) and then exported as nexus

files into PAUP* 4.0b10 (Swofford 2000) for phylogenetic analysis. The data sets were also combined using MacClade in order to reconcile them on a single set of solutions, following a total evidence approach (Kluge 1989). Values of pairwise proportional sequence divergence (*p*-distances) were obtained using PAUP*. The alignment of sequences used for analyses has been submitted to TreeBASE (study and matrix Accession Nos. are S1815 and M3321, respectively).

Genealogical history was evaluated using maximum parsimony. In our analyses, all characters were evaluated as unordered because there is no *a priori* reason to assume order of evolutionary change between the four nucleotide bases of DNA (Swofford *et al.* 1996). To maximize the explanatory power of all lines of evidence, following reasons outlined by Frost *et al.* (2001), all analyses were conducted using equal weighting of transversions and transitions. The phylogenetic analyses using PAUP* were conducted using a heuristic search, with random addition of sequences, one million replicates, retaining minimal trees only, using tree bisection-reconnection branch swapping saving multiple trees, and collapsing zero-length branches.

Nodal support was assessed with Bremer decay analysis (Bremer 1988, 1994) using AutoDecay 4.0.2 (Eriksson 1998) as implemented with PAUP*, and with bootstrap analysis (Felsenstein 1985) in PAUP* using 10000 replicates. In both nodal support analyses, the heuristic search settings were identical to the phylogenetic analysis described above, except that the number of replicates was reduced to 10000 and 100 for Bremer decay and bootstrap analysis, respectively.

Results

Sequence variation

Nucleotide sequence length varied in cyt *b* due to indels that were chiefly located in the non-coding region between the cytochrome *b* and threonine tRNA genes, but also found within the threonine tRNA gene. Gaps were inserted to accommodate the alignment of conserved regions, resulting in a total of 1094 aligned positions. Nevertheless, 16 characters (positions 1048–1063) were excluded from further analysis due to uncertain alignment, limiting the data set to 1078 characters. Of these, 391 positions were variable (36.3% of total positions), among which 329 (30.5%) were potentially phylogenetically informative. Pairwise *p*-distances between the aligned sequences from cyt *b* revealed a large range in sequence divergence from 0.10% to 12.01% among ingroup specimens.

From *ATPase*, 872 homologous sites were examined. In total, 365 characters were variable (41.9% of total sites), with 296 (34.0%) being potentially phylogenetically informative. Pairwise *p*-distances between the aligned sequences from *ATPase* also revealed a large range in sequence divergence (0.12% to 12.15%).

Pairwise distances based on the combined sequences (1966 bp; Table 3.1) ranged from 0.27% to 11.44% sequence divergence.

Table 3.1. Pairwise genetic comparisons of mtDNA and allozymes between selected specimens of *Urosaurus nigricaudus*. Percent sequence divergences (uncorrected *p*-distances) for combined mtDNA gene segments are given above the diagonal. Genetic distances (Nei's *D*; Nei 1978) based on allozymes and reported by Aguirre-León *et al.* (1999) are given below the diagonal for populations corresponding to localities of the present study. Specimens were selected based on relevance to the genealogy (see Fig. 3.2) and geographic location (Fig. 3.1) to facilitate comparison with allozyme data of Aguirre-León *et al.* (1999)*. Locality comparisons with both allozyme and mtDNA sequence data are in boldface; comparisons across genealogical breaks discussed in the text appear on shaded backgrounds.

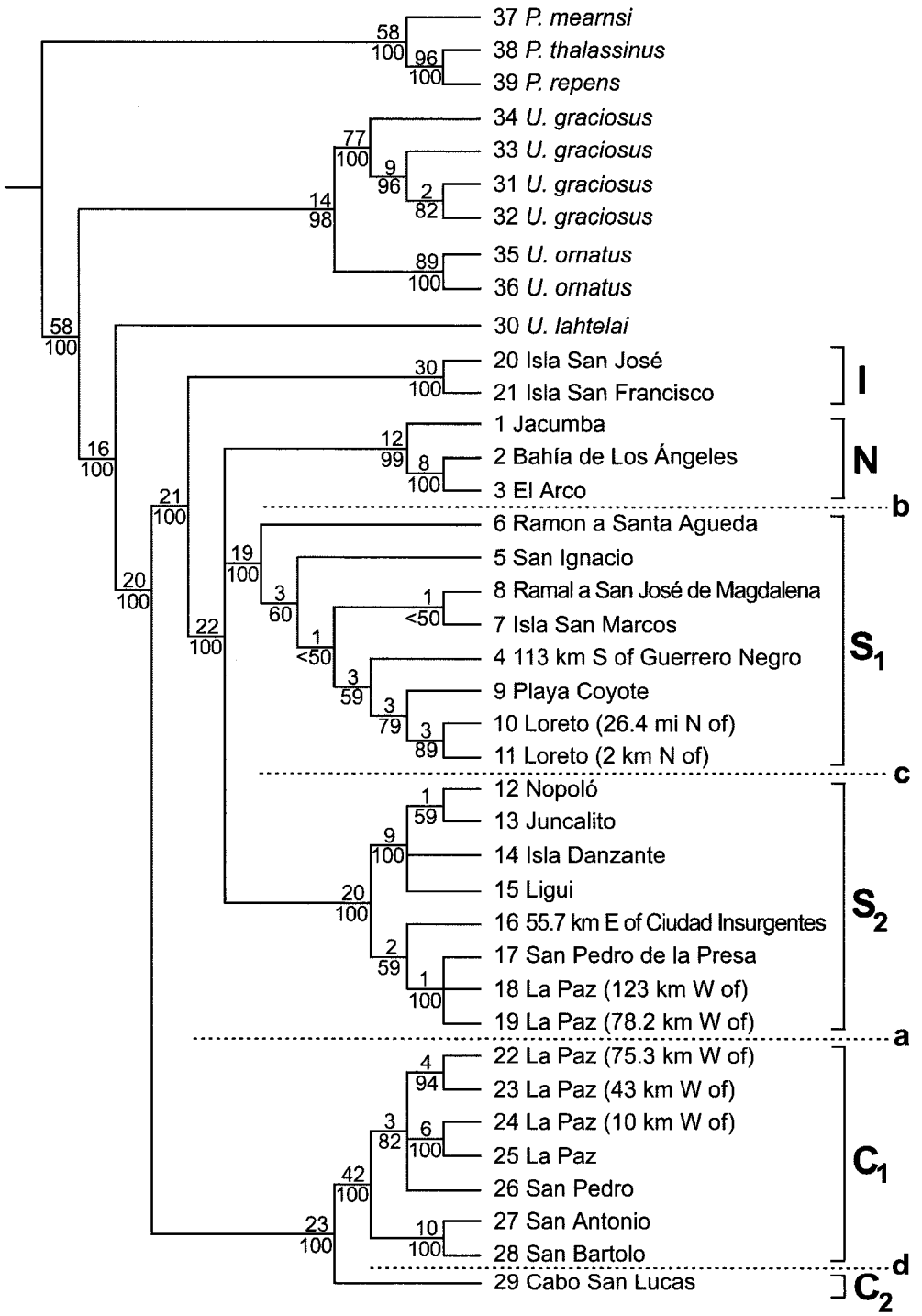
Specimen (clade)	1	**3**	4	**5**	**10**	11	12	**13**	**17**	19	20	21	22	**25**	28	**29**
1 Jacumba (N)	—	2.64	4.37	4.47	4.25	4.44	4.18	4.18	4.68	4.57	5.49	6.05	10.70	11.33	11.35	10.66
3 El Arco (N)		—	4.54	**4.64**	**4.37**	4.66	4.77	**4.66**	**5.07**	4.74	6.08	6.43	10.61	**10.99**	11.05	**10.61**
4 113 km S of Guerrero Negro (S_1)			—	1.05	1.14	1.12	4.39	4.26	4.77	4.36	6.26	6.32	10.39	10.78	10.89	10.08
5 San Ignacio (S_1)		**0.017**		—	**1.15**	1.22	4.20	**4.31**	**4.60**	4.38	5.88	5.93	10.31	**10.66**	10.67	**10.02**
10 Loreto (26.4 km N of; S_1)		**0.075**		**0.085**	—	0.42	4.06	**4.04**	**4.64**	4.35	6.22	6.30	10.07	**10.49**	10.59	**9.90**
11 Loreto (2 km N of; S_1)					—	—	4.12	4.10	4.66	4.29	6.35	6.39	10.20	10.77	10.75	10.13
12 Nopoló (S_2)						—	—	0.48	2.55	2.44	6.56	6.93	10.97	11.18	11.25	10.83
13 Juncalito (S_2)		**0.070**		**0.082**	**0.001**			—	**2.37**	2.37	6.59	6.95	11.03	**11.23**	11.34	**10.67**
17 San Pedro de la Presa (S_2)		**0.188**		**0.191**	**0.109**			**0.108**	—	1.20	6.72	7.15	10.33	**10.51**	10.61	**10.15**
19 La Paz (78.2 km W of; S_2)										—	6.74	6.95	11.02	11.16	11.44	11.10
20 Isla San José (I)											—	0.97	10.46	10.57	10.76	10.20
21 Isla San Francisco (I)												—	10.93	11.13	11.38	10.82
22 La Paz (75.3 km W of; C_1)													—	1.36	2.53	7.28
25 La Paz (C_1)		**0.193**		**0.197**	**0.117**			**0.117**	**0.001**					—	1.96	**6.65**
28 San Bartolo (C_1)															—	7.41
29 Cabo San Lucas (C_2)		**0.189**		**0.203**	**0.107**			**0.106**	**0.003**					**0.006**		—

*For comparison with the allozyme data of Aguirre-León *et al.* we selected specimens 3, 5, 10, 13, 17, 25, and 29 to correspond to the populations of Aguirre-León *et al.* named El Arco, San Ignacio, San José Comondú, Juncalito, San Pedro la Presa, La Paz, and Cabo San Lucas, respectively.

Genealogical relationships

The cladistic analysis of cyt *b* yielded 644 most parsimonious trees (MPTs; length = 1003 steps, CI = 0.540, RI = 0.805). The analysis of *ATPase* resulted in four MPTs (length = 815 steps, CI = 0.606, RI = 0.840). Individual analyses of the two gene segments were fully congruent with regard to genealogical relationships among distinct clades; only minor differences occurred among terminal branches of closely related specimens. Cladistic analysis of the combined data set resulted in eight MPTs (length = 1821 steps, CI = 0.569, RI = 0.820). In the strict consensus tree, the specimens of *U. nigricaudus* formed two major clades, each associated with Bremer decay (DI) values of at least 21 steps and bootstrap (BS) values of 100% (Fig. 3.2). A Cape Region clade consisted of eight specimens including the sample from 75.3 km west of La Paz. In this clade the specimen from Cabo San Lucas (Cabo subclade; C_2) was the sister lineage to the other seven specimens, which formed a well supported main Cape Region subclade (C_1). The sister group to the Cape Region clade consisted of all other specimens, including the remainder of the peninsula and all insular samples. In this clade the two specimens of the islands of Isla San José and Isla San Francisco (I) formed a sister group to the remaining specimens. They in turn formed three distinct subclades supported by DI values of 12 or higher and BS values of 99% or 100%: a northern subclade (N) composed of three samples from Jacumba of southern California southward to El Arco midway on the peninsula; a mid-southern subclade (S_1) consisting of samples of the nearby region from 113 km south of Guerrero Negro southward to 2 km north of Loreto; and a deep southern subclade (S_2) including samples from Nopoló (just south of Loreto) southward to 78.2 km

Figure 3.2. Strict consensus of the combined data set (8 MPTs, CI = 0.569, RI = 0.820). Specimen numbers correspond to map localities and have been arranged in a north to south direction where possible. Nodal support is represented by Bremer decay index (DI) and bootstrap (BS) values above and below the nodes, respectively. Vertical bars named C_1, C_2, I, N, S_1, and S_2 refer to the main Cape Region, Cabo, Isla San José and Isla San Francisco, northern Baja California (including southern California), mid-southern, and deep southern (excluding the Cape Region) clade, respectively. Horizontal dashed lines indicate genealogical breaks discussed in text: a = Isthmus of La Paz break; b = mid-peninsular break, c = Loreto break; d = Cabo break. The relationship among the N, S_1, and S_2 clades was weak and has been collapsed for simplification and to emphasize the genetic discontinuities along the peninsula (see Results)


37 P. mearnsi
38 P. thalassinus
39 P. repens
34 U. graciosus
33 U. graciosus
31 U. graciosus
32 U. graciosus
35 U. ornatus
36 U. ornatus
30 U. lahtelai
20 Isla San José
21 Isla San Francisco
I
1 Jacumba
2 Bahía de Los Ángeles
3 El Arco
N
b
6 Ramon a Santa Agueda
5 San Ignacio
8 Ramal a San José de Magdalena
7 Isla San Marcos
4 113 km S of Guerrero Negro
9 Playa Coyote
10 Loreto (26.4 mi N of)
11 Loreto (2 km N of)
S1
c
12 Nopoló
13 Juncalito
14 Isla Danzante
15 Ligui
16 55.7 km E of Ciudad Insurgentes
17 San Pedro de la Presa
18 La Paz (123 km W of)
19 La Paz (78.2 km W of)
S2
a
22 La Paz (75.3 km W of)
23 La Paz (43 km W of)
24 La Paz (10 km W of)
25 La Paz
26 San Pedro
27 San Antonio
28 San Bartolo
C1
d
29 Cabo San Lucas
C2

west of La Paz. Of these three groups, the northern subclade grouped with the deep southern subclade, suggesting an unanticipated genealogical connection between geographically distant samples along the peninsula. The node uniting these two subclades was however relatively weakly supported (DI = 3, BS = 60) and was collapsed in the strict consensus tree, which emphasizes the genetic discontinuities along the peninsula (Fig. 3.2).

Genealogical breaks between the nearby groups along the peninsula were associated with deep divergences (Table 3.1). The genealogical breaks of the Isthmus of La Paz, the mid-peninsula, Loreto, and on the Cape Region (a, b, c, and d, respectively, in Fig. 3.1) showed a sequence divergence of 11.02%, 4.54%, 4.12%, and 7.41%, respectively (represented by the divergence between the most closely located specimens on either side of a break). The divergence between the two insular specimens of Isla San José and Isla San Francisco and the specimen from San Pedro de la Presa on the adjacent peninsula was also substantial, averaging 6.93%.

Discussion

Genealogical history

The geographical patterns of mtDNA genealogies are tightly linked to female history, including dispersal patterns (cf. Lindell *et al.* 2005). While the status of mtDNA as a non-recombining, strictly maternally inherited molecule is receiving increasing criticism

(Bromham *et al.* 2003; Rokas *et al.* 2003; Tsaousis *et al.* 2005), we assume that no departure from the traditional view of mtDNA transmission affects the main conclusions of our discussion.

The deep genealogy of *U. nigricaudus* implies a long evolutionary history. The break across the Isthmus of La Paz (11.02%; Fig. 3.1) stands out, representing a magnitude of divergence most often associated with interspecific or higher differentiation. This deep genealogical split in mtDNA may be associated with disruption in gene flow and hence population differentiation, a common feature of secondary contact zones showing deep mitochondrial divergence (e.g., Szymura & Barton 1991; Szymura *et al.* 2000; Phillips *et al.* 2004). In fact, the populations on either side of the Isthmus of La Paz have historically been recognized as different species; *U. nigricaudus* (*sensu* Cope 1864) of the Cape Region and *U. microscutatus* (*sensu* Van Denburgh 1894) inhabiting the remainder of the peninsula. Later authors agreed with this distinction based on analyses of morphological characters (Mittleman 1942; Rau & Loomis 1977; Wiens 1993). However, data also indicate a clinal pattern in morphology across the Isthmus of La Paz, suggesting mixing of gene pools northward to San José de Comondú (Wiens 1993), approximately 250 km north of the Isthmus of La Paz.

A more recent study by Aguirre-León *et al.* (1999) investigated differentiation among populations of *Urosaurus* in Baja California based on the analysis of 25 allozyme loci. Their data showed high population differentiation ($F_{ST} = 0.436$), consistent with a history of population fragmentation along the peninsula (Aguirre-León *et al.* 1999). A reanalysis of their data with BIOSYS-2 (Swofford *et al.* 1997), including only the eleven populations of *U. nigricaudus* [hence excluding the sample of *Uta stansburiana* on Isla

Cedros (tissue number RWM 1861) that was incorrectly included as *U. microscutatus*, and the two samples of *U. lahtelai* (tissue number RWM 1246 and voucher catalogue number ROM 14378)], also yielded a pattern of overall population differentiation (F_{ST} = 0.264). However, allozyme differentiation was extremely low across the deep genealogical breaks revealed by the history of maternal lineages (Isthmus of La Paz, mid-peninsular, Loreto, and Cabo breaks; Fig. 3.1, Table 3.1), suggesting no or negligible population differentiation across the mtDNA breaks. The phylogenetic analysis of allozyme alleles (Murphy 1993) also indicated allelic dispersion, both across the Isthmus of La Paz and the mid-peninsula (Aguirre-León *et al.* 1999). In summary, compelling evidence from the studies representing biparental inheritance imply ongoing gene flow and lack of population differentiation across areas of large genealogical discontinuities. Indeed, a pattern of ongoing gene flow appears true for most other species of vertebrates on the peninsula (Lawlor *et al.* 2002; Murphy & Aguirre-León 2002; Lindell *et al.* 2005). Hence, despite the unusually deep mtDNA genealogy of *U. nigricaudus*, the current taxonomy (*sensu* Aguirre-León *et al.* 1999) is supported.

Historical biogeography

> "The history of the Gulf of California will be better understood when the data from sedimentology, paleontology, structural geology, geochemistry, and geophysics are integrated." Smith (1991: 662)

Studies in geology and related fields such as sedimentology and paleontology have significantly increased our understanding of the history of Baja California, including its biota. However, genetics, in particular the genealogical history of mtDNA lineages, can assist our understanding of the historical biogeography of Baja California. In fact, genetics may provide clues to the history of the peninsula not discernable from other data. Unfortunately, biogeographical syntheses have largely mapped genetic evidence onto an existing geological model (e.g., Murphy 1983b; Grismer 1994; Riddle *et al.* 2000b). Similarly, interpretation of genetic data has commonly relied on the vicariant history hypothesized in the influential study on *Uta stansburiana* by Upton & Murphy (1997), which suggested that the mid-peninsular region of Baja California was temporarily submerged 1 Mya. Many studies have therefore focused their discussion on patterns of Quaternary and, to a lesser degree, Pliocene age.

The strong emphasis on Plio-Quaternary history in genealogy-based studies is somewhat surprising as geological, sedimentary, and palaeontological data all point to a significantly older age of the Gulf of California, which also encompasses Miocene events. During the middle Miocene (14–16 Mya) the sea invaded the Gulf of California region through the Cabo Trough, which at that time was positioned next to the Mexican mainland (Smith 1991). Marine sedimentary rocks have been found on Isla Tiburón in the northern half of the present gulf, for which radiometric and palaeontological data suggest a middle Miocene (12–13 Mya) age (Smith *et al.* 1985; Smith 1991). In fact, "faunal distributions and radiometric data show that an early gulf similar in extent to the present one existed as early as 13 Mya..." (Smith 1991: 638). Can the temporal discrepancy

between genealogical and other data be resolved, or is the evidence of older events in the biogeography of Baja California lost in genealogical data?

Deep genealogies revealed in common, widespread species are important in trying to resolve the full span of the historical biogeography of Baja California and the associated Gulf of California. Due to the complex historical area relationships among portions of the peninsula, including its adjacent islands, the history of the peninsula must be evaluated by the relative timing of genealogical splits. DNA divergence data alone are too inaccurate to resolve the complicated history (Ayala 1999; Lindell *et al.* 2006). The genealogical data must also be interpreted alongside current information from other fields of research. Specifically, genealogical splits inferred to be related to known geological events, such as seaways or the formation of deepwater islands ('genealogical anchors'; Lindell *et al.* 2005), may allow sufficient temporal detail for biogeographical hypotheses. Following this approach, Lindell *et al.* (2005) evaluated the genealogy of *C. draconoides* in conjunction with those of other species and recent geological and palaeontological data. They suggested that the historical biogeography of Baja California, and hence the evolution of the peninsular biota, results from events that are older than generally perceived. The unusually divergent mtDNA genealogy of *U. nigricaudus* may add to this approach, as it might elucidate events related to the early evolution of the Gulf of California. Here, we provide an interpretation of the genealogy of *U. nigricaudus* in relation to other genealogies and current research from geology, sedimentology, and paleontology.

The genealogy of *U. nigricaudus* mirrors those of *C. draconoides* and additional species discussed by Lindell *et al.* (2005), suggesting that biogeographical patterns

involve a significant signal of Miocene events. In *U. nigricaudus*, the deepest break is across the Isthmus of La Paz (a in Fig. 3.1). The relative timing of this break implies an older split than the commonly documented mid-peninsular divergence. Such a pattern has previously been observed by Riddle *et al.* (2000b) in the genealogy of *Chaetodipus arenarius*, which revealed a very deep genetic discontinuity in mtDNA (12.8% divergence in cytochrome oxidase III) across the Isthmus of La Paz [see Murphy (1983a) for a similar scenario based on allozyme data]. Congruent genealogical patterns have also been found in scorpions (*Centruroides exilicauda*; Gantenbein *et al.* 2001) and leaf-toed geckos (*Phyllodactylus*; Murphy *et al.* in preparation), suggesting a common vicariant history.

Support for an old seaway across the Isthmus of La Paz from other fields remains inconclusive. While the Isthmus of La Paz is a low-lying area between the mountainous Cape Region to the south and the ridges of Sierra de la Giganta to the north, there is no evidence for continuous marine sediments across the peninsula at this location. In a detailed geological examination of Baja California Sur, Hausback (1984) described extensive sedimentary deposits across the Isthmus of La Paz. While most of the sedimentary material is of terrestrial (i.e. non-marine) origin (Hausback 1984, personal communication; Smith 1991), evidence for marine sediments exists. On the western side of the Isthmus of La Paz, near El Cien, older rocks are uncomformably overlain by shallow marine deposits of late Miocene to Pliocene origin (Heim 1922; Mina-Uhink 1957; Smith 1992; Helenes & Carreño 1999: 594). Geological data also have indicated a fault zone (the La Paz fault) at the very southern reaches of the Isthmus of La Paz (Beal 1948; Hausback 1984). The Cape Region appears to have moved north-eastward by

approximately 50 km along this fault (Hausback 1984; Lonsdale 1989), implying that the Isthmus of La Paz was markedly narrower up to at least late Miocene times. These data have been interpreted as the existence of a shallow marine seaway during Miocene that separated the geographic ranges of multiple species into an isolated Cape Region and northern populations (Schwennicke *et al.* 1996; Carreño & Helenes 2002: 31). The implied date is older than a late Miocene mid-peninsular seaway (Lindell *et al.* 2006). Nonetheless, further evidence, including additional mtDNA genealogies, is needed to address the existence of a Miocene seaway across the Isthmus of La Paz.

In *U. nigricaudus*, the lineages found on Isla San José and Isla San Francisco are also older than the mid-peninsular break, suggesting an old split from the mainland. This pattern is somewhat surprising as the deepest water depth between the islands and the peninsula currently is 60 m (Carreño & Helenes 2002; Murphy & Aguirre-León 2002). Hence, barring a deeper canal depth in the past (which is possible if sedimentation has been extensive), the islands repeatedly experienced landbridge connections to the peninsula during Pleistocene glacial periods when eustatic sea levels were lowered by 120 m or more compared to the present (Rohling *et al.* 1998). However, the old lineages may have survived since the initial separation of the islands from the peninsula. Geological data suggests that Isla San José started to separate from the peninsula during late Miocene by fault lines opening up the San José Canal from the north, and was fully separated from the peninsula in early Pliocene (A. L. Carreño, personal communication; Puy-Alquiza 1992; Drake & Umhoefer 2003). The islands may have been isolated for a long period before Pleistocene sea level changes periodically reconnected it to the peninsula. Alternatively, this haplotype group originated on the peninsula before the

isolation of the islands. This implies that at least two old lineages are coexisting on the peninsula adjacent to Isla San José. However, this scenario seems unlikely as extensive sampling (n = 133) of the Isthmus of La Paz break and nearby regions suggests that two lineages meet in a very narrow contact zone, neither of them being of the island haplotype group (Lindell & Murphy in preparation). The available evidence therefore supports a scenario by which the insular lineages resulted from an old split of Isla San José and Isla San Francisco from the mainland. Nevertheless, further sampling of these islands may reveal the coexistence of this old haplotype group with lineages from the adjacent peninsula, following landbridge dispersal events during glacial periods.

The mid-peninsular break (b in Fig. 3.1) has been central to the discussion of a cryptic vicariant history of Baja California. Indeed, the first published genealogy for a peninsular species (*Uta stansburiana*; Upton & Murphy 1997) revealed a deep split midway on the peninsula. Upton and Murphy argued that a vicariant history involving temporary submergence of the mid-peninsula had produced this pattern. They cited geological sources (Moore 1973) and hypothesized a temporary mid-peninsular seaway of Pleistocene age. Subsequent mtDNA genealogies from numerous taxa, including lizards (Petren & Case 1997; Radtkey *et al.* 1997; Lindell *et al.* 2005), snakes (Rodríguez-Robles & De Jesús-Escobar 2000), mammals (Riddle *et al.* 2000a; Riddle *et al.* 2000b; Whorley *et al.* 2004), birds (Zink *et al.* 1997; Zink *et al.* 2001), and arthropods (Crews & Hedin 2006), has corroborated the mid-peninsular seaway hypothesis. In fact, species that lack this genealogical break are few (Wong *et al.* 1998; Zink *et al.* 2000; Jaeger *et al.* 2005). Following a reinterpretation of genealogical data in conjunction with new findings in geology, Lindell *et al.* (2005) suggested that the mid-peninsular

vicariance event may have occurred 7 Mya during late Miocene. Marine submergence was extensive in the mid-peninsular region at this time and geochronology of sediments in the Santa Rosalía region point to an onset of marine deposition at 7.1 ± 0.05 Mya (Helenes & Carreño 1999; Holt *et al.* 2000; Carreño & Helenes 2002). Authors of subsequent studies have reiterated this conclusion when evaluating genealogical patterns (Crews & Hedin 2006). The genealogy of *U. nigricaudus* supports this older scenario.

The genealogy of *U. nigricaudus* is also consistent with a 'Loreto break' (c in Fig. 3.1), as found in *C. draconoides* (Lindell *et al.* 2005), *Aspidoscelis hyperythra* (Radtkey *et al.* 1997), and *Uta stansburiana* (Hollingsworth 1999; Lindell *et al.* in preparation). Detailed studies of the Loreto basin have revealed substantial deposition of marine sediments of Pliocene origin (McLean 1988, 1989; Piazza & Robba 1994; Umhoefer *et al.* 1994; Dorsey *et al.* 1997). The area was submerged approximately 3.4–2.0 Mya (McLean 1988; Umhoefer *et al.* 1994). No geological evidence points towards a transpeninsular submergence at the level of Loreto. Nevertheless, given the dramatic topography of the adjacent Gulf Escarpment, which forms the eastern side of the Sierra de la Giganta, the congruent genealogical breaks are likely the result of restricted latitudinal dispersal during this Pliocene inundation event (Lindell *et al.* 2005).

The genealogical break in *U. nigricaudus* further south on the Cape Region (d in Fig. 3.1) appears also to follow a vicariant history. Sedimentary evidence points to an extensive submergence of the San José del Cabo Trough near the tip of the Cape Region (McCloy 1984a, 1984b; Martínez-Gutiérrez & Sethi 1997). Depositional character of these sediments suggests that marine inundation was extensive during late Miocene and early Pliocene, as implied by deposition of deep-water (ca 200 m) sediments between 6.5

and 3.2 Mya (McCloy 1984b; Carreño 1992). The exact extent of this submergence is difficult to determine, but it may have completely isolated the very south-eastern portion of the Cape Region or at least come close to doing so (Martínez-Gutiérrez & Sethi 1997; Carreño & Helenes 2002). *Urosaurus nigricaudus* was likely affected by this event, and dispersal of a divergent maternal lineage to Cabo San Lucas (specimen 29 in Fig. 3.1) following complete or near isolation is possible. If this scenario is true, *U. nigricaudus* must also have continuously inhabited the Cape Region following this vicariant event, as any local extirpation due to range shifts would have erased the genealogical break. This suggests that climate fluctuations during the Quaternary (Shackleton *et al.* 1984) did not substantially shift the range of *U. nigricaudus*, at least not in this area. Similar patterns exist in *C. arenarius* (Riddle *et al.* 2000b) and *Phyllodactylus* (Murphy *et al.* in preparation). Additional studies are needed from the southern portion of the Cape Region to substantiate the existence of this 'Cabo break.'

An emerging picture of peninsular biogeography

The combined analysis of deep mtDNA genealogies and evidence from geology, stratigraphy, and paleontology provides an emerging picture of the historical biogeography of Baja California as much deeper than commonly believed. The deeper biogeographical history suggested by this and other mtDNA genealogies (Lindell *et al.* 2005) also fits a time-frame for the overall history of the peninsula and the Gulf of California; the mouth of the gulf opened sufficiently early and was followed by the

development of an extensive gulf prior to the scenario described herein and by Lindell *et al.* (2005). Additional genealogies of organisms with a long history in the area will help further clarify the older components of the historical biogeography of Baja California, possibly including the opening of the gulf itself (14–16 Mya; Smith 1991).

With a more detailed picture of the historical biogeography of Baja California emerging, it will be possible to investigate the possibility of recurring seaways, which may have inundated the Isthmus of La Paz at drastically different times, during Miocene and Quaternary. We will also have a better foundation for understanding the effects of a vicariant history on the regional fauna. For example, genealogical data already show that rates of evolution differ among taxa affected by the same vicariance events. Similarly, it is becoming increasingly clear that the maternal histories deduced from deeply divergent mtDNA genealogies are not echoed by congruent patterns in biparental histories, despite the vicariant history of the region.

Maternal and biparental histories

Baja California offers a template for understanding the disparate histories of maternal and biparental inheritance as shaped by temporary vicariance. Species with a long and fragmented history commonly show divergence both in mtDNA and allozymes (e.g., Szymura *et al.* 2000; Alexandrino *et al.* 2005). In *U. nigricaudus*, the overall population differentiation suggested by allozyme variation (Aguirre-León *et al.* 1999) seems to agree with the deeply divergent history of maternal lineages. However, allozyme differentiation

is comparatively limited across the deep mitochondrial breaks found along the peninsula (cf. Jockusch & Wake 2002). Instead, biparental differentiation is more apparent in other regions along the peninsula (Table 3.1). This cytonuclear discordance is most obvious for the highly divergent (11.02%) mitochondrial break across the Isthmus of La Paz, for which virtually no population differentiation exists in allozymes [Nei's $D = 0.001$ between La Paz and San Pedro la Presa of Aguirre-León *et al.* (1999)]. Similar cytonuclear discordance has recently been found in *C. draconoides* (Lindell *et al.* 2005), indicating ongoing gene flow along the peninsula.

What may account for the cytonuclear discordance seen in *U. nigricaudus*? In general, temporary vicariance events may have been long enough to produce initial divergence in mtDNA, but too short for differentiation in allozyme loci. Such a difference follows the significantly lower effective population size of mtDNA and shorter coalescence time compared to nuclear DNA (Moore 1995). Sufficient isolating barriers (Coyne & Orr 2004) to drive speciation did not develop during isolation, so that, upon secondary contact, the populations were reunited and gene flow resumed. If this is the case, then an approximation of mtDNA differentiation with biparental population divergence is clearly erroneous.

Following reunion of the populations, several factors may have affected the sharing of genes and hence shaped the cytonuclear discordance. Sex-differential dispersal may allow for nuclear gene flow in the absence of mitochondrial introgression. Male-mediated gene flow has been implicated in other studies (Helbig *et al.* 2001; Jockusch & Wake 2002). Unfortunately, little is known about the sex-specific dispersal patterns in *U. nigricaudus*. The reduced viability or fertility of the heterogametic sex, known as

Haldane's rule (Haldane 1922; Orr 1997), can also lead to cytonuclear discordance (Sætre *et al.* 2001). However, indirect evidence suggests that males, not females, are heterogametic in *U. nigricaudus* (Sites *et al.* 1992). Female behaviour could perhaps restrict the dispersal of opposing mitochondrial lineages if females are more prone to accept closely related females. Finally, the coevolution among mitochondrial and nuclear gene products for function in the electron transport system of mitochondrial membranes (Blier *et al.* 2001; Rand *et al.* 2004) may affect contact zones; mitochondrial introgression may be limited into a population containing mal-adaptive nuclear genes. Such linkage disequilibrium could be largely restricted to the nuclear genes with which mitochondrial genes must work, while still allowing for gene flow of non-linked parts of the nuclear genome (Bengtsson 1985; Martinsen *et al.* 2001).

The cytonuclear discordance is most striking across the Isthmus of La Paz, with no allozyme differentiation across a deeply divergent and narrow mtDNA break (Lindell & Murphy in preparation). While limited population differentiation, such as that over the Isthmus of La Paz, may be due to low overall levels of allozyme variation, this is not the case in *U. nigricaudus*. Therefore, this type of contact zone is perhaps best characterized as a "maternal boundary," emphasizing the distinct histories between mitochondrial and biparental modes of inheritance. We are currently performing a more detailed study to further address the genetic interactions at this mitochondrial secondary contact zone and the possible causes of cytonuclear discordance.

While mtDNA is an excellent tool for which historical biogeography can be addressed, mtDNA-based phylogeography appears an inappropriate tool from which to infer biparental population differentiation. Likewise, the use of mtDNA data alone in

delimiting species (DNA barcoding; Herbert *et al.* 2003; Baker & Bradley 2006) appears unsuitable for a fauna that has been deeply affected by cryptic vicariance events, such as that of Baja California (Riddle *et al.* 2000b). Hence, as increasingly recognized, any study addressing population differentiation and gene flow should, at least partially, be based on nuclear-encoded characters. Additional data based on nuclear DNA, including faster evolving microsatellite DNA loci, will provide a more detailed picture of population history, gene flow, and cytonuclear discordance in *U. nigricaudus* (in progress).

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Appendix 3.1. Number, taxonomic designation, collection locality, tissue number, and GenBank accession number of the specimens used in this study. Tissue numbers equal field numbers, except for italicized entries, which equal voucher catalogue numbers. Tissue number abbreviations are: LACM = Los Angeles County Museum; ROM = Royal Ontario Museum; RWM = Robert W. Murphy; TCWC = Texas Cooperative Wildlife Collections; USC = University of Southern California. Tissues are kept at the Royal Ontario Museum. Specimen vouchers corresponding to tissue numbers marked with an asterisk are kept in the herpetological collection of the Instituto de Biología, Universidad Nacional Autónoma de México (using the identical number).

No.	Taxon	Collection locality	Tissue No.	Accession No.
Ingroup				
1	*Urosaurus nigricaudus*	United States; California; San Diego Co.; Jacumba (3 mi E of)	*ROM 14020*	EF653280, EF653319
2	*U. nigricaudus*	Mexico; Baja California (Norte); Bahía de Los Ángeles	RWM 1786	EF653281, EF653320
3	*U. nigricaudus*	Mexico; Baja California (Norte); El Arco	RWM 1747	EF653282, EF653321
4	*U. nigricaudus*	Mexico; Baja California Sur; 113 km S of Guerrero Negro (along Hwy 1)	ROM 35373*	EF653283, EF653322
5	*U. nigricaudus*	Mexico; Baja California Sur; San Ignacio (1 mi SW of)	*LACM 128173*	EF653284, EF653323
6	*U. nigricaudus*	Mexico; Baja California Sur; Ramon a Santa Agueda (3 mi E of)	*ROM 43486*	EF653285, EF653324
7	*U. nigricaudus*	Mexico; Baja California Sur; Isla San Marcos	RWM 1345	EF653286, EF653325
8	*U. nigricaudus*	Mexico; Baja California Sur; Ramal a San José de Magdalena (junction on Hwy 1 and road to San José de Magdalena)	ROM 35371*	EF653287, EF653326
9	*U. nigricaudus*	Mexico; Baja California Sur; Playa Coyote (4 km S of)	*ROM 43497*	EF653288, EF653327
10	*U. nigricaudus*	Mexico; Baja California Sur; Loreto (26.4 mi N of, along Hwy 1)	RWM 1299	EF653289, EF653328
11	*U. nigricaudus*	Mexico; Baja California Sur; Loreto (2 km N of, along Hwy 1)	ROM 37166*	EF653290, EF653329
12	*U. nigricaudus*	Mexico; Baja California Sur; Nopoló	ROM 37154*	EF653291, EF653330
13	*U. nigricaudus*	Mexico; Baja California Sur; Juncalito	ROM 37265*	EF653292, EF653331
14	*U. nigricaudus*	Mexico; Baja California Sur; Isla Danzante	RWM 1670	EF653293, EF653332
15	*U. nigricaudus*	Mexico; Baja California Sur; Ligui (3 km S of, along Hwy 1)	ROM 37156*	EF653294, EF653333
16	*U. nigricaudus*	Mexico; Baja California Sur; 55.7 km E of Ciudad Insurgentes (along Hwy1)	ROM 35390*	EF653295, EF653334

No.	Taxon	Collection locality	Tissue No.	Accession No.
17	*U. nigricaudus*	Mexico; Baja California Sur; San Pedro de la Presa	*ROM 45239*	EF653296, EF653335
18	*U. nigricaudus*	Mexico; Baja California Sur; La Paz (123 km W of, along Hwy 1)	ROM 35344*	EF653297, EF653336
19	*U. nigricaudus*	Mexico; Baja California Sur; La Paz (78.2 km W of, along Hwy 1)	ROM 37208*	EF653298, EF653337
20	*U. nigricaudus*	Mexico; Baja California Sur; Isla San José	ROM 35436*	EF653299, EF653338
21	*U. nigricaudus*	Mexico; Baja California Sur; Isla San Francisco	ROM 35428*	EF653300, EF653339
22	*U. nigricaudus*	Mexico; Baja California Sur; La Paz (75.3 km W of, along Hwy 1)	ROM 35338*	EF653301, EF653340
23	*U. nigricaudus*	Mexico; Baja California Sur; La Paz (43 km W of, along Hwy 1)	ROM 35332*	EF653302, EF653341
24	*U. nigricaudus*	Mexico; Baja California Sur; La Paz (10 km W of, along Hwy 1)	ROM 35321*	EF653303, EF653342
25	*U. nigricaudus*	Mexico; Baja California Sur; La Paz	*ROM 23100*	EF653304, EF653343
26	*U. nigricaudus*	Mexico; Baja California Sur; San Pedro (10 mi S of, along Hwy 1)	*LACM 128210*	EF653305, EF653344
27	*U. nigricaudus*	Mexico; Baja California Sur; San Antonio (7.3mi S of, along Hwy 1)	*LACM 128211*	EF653306, EF653345
28	*U. nigricaudus*	Mexico; Baja California Sur; San Bartolo	*ROM 26192*	EF653307, EF653346
29	*U. nigricaudus*	Mexico; Baja California Sur; Cabo San Lucas (4 mi E of)	*LACM 128232*	EF653308, EF653347
Outgroup				
30	*U. lahtelai*	Mexico; Baja California; Cataviña (6.8 mi N of, along Hwy 1)	*ROM 14065*	EF653309, EF653348
31	*U. graciosus*	United States; California; Riverside Co.; Desert Centre	*ROM 43496*	EF653310, EF653349
32	*U. graciosus*	United States; California; Imperial Co.; Ocotillo	*ROM 13844*	EF653311, EF653350
33	*U. graciosus*	Mexico; Baja California; San Felipe	RWM 1165	EF653312, EF653351
34	*U. graciosus*	Mexico; Sonora; Puerto Peñasco (17 km NE of)	*ROM 13941*	EF653313, EF653352
35	*U. ornatus*	United States; Arizona; Cochise Co.; Chiracahua Mountains (5 km WSW of Dobson Peak)	*LACM 127649*	EF653314, EF653353
36	*U. ornatus*	United States; New Mexico; Hidalgo Co.; Rodeo	*TCWC 58664*	EF653315, EF653354
37	*Petrosaurus mearnsi*	Mexico; Baja California; Cataviña (6.8 mi N of, along Hwy 1)	USC 4467	EF653316, EF653355
38	*P. thalassinus*	Mexico; Baja California Sur; San Ignacio	USC 4540	EF653317, EF653356
39	*P. repens*	Mexico; Baja California Sur; La Paz (ca 30 km N of, along road towards San Juan de la Costa)	ROM 34067*	EF653318, EF653357

Chapter 4

Deep genealogies and the mid-peninsular seaway of Baja California

Abstract

Geological forces and long-term climate changes can have profound effects on species. Such effects may be manifested in the pattern and magnitude of genealogical diversity, as revealed by mitochondrial DNA (mtDNA) lineages. The relative importance of the different forces on a regional biota must be evaluated along with a good understanding of geological and climatological history. The peninsula of Baja California of north-western Mexico is one area where both geology and climate have affected the historical biogeography of the regional biota. Molecular studies based on the genealogical relationships among mtDNA lineages have made great contribution towards elucidating the historical biogeography of Baja California. Perhaps most noticeably, numerous concordant breaks in mtDNA genealogies halfway on the peninsula suggest a vicariant history in which the mid-peninsula was temporarily submerged. This vicariant explanation has recently been criticized, as no conclusive geological evidence for a continuous submergence of the mid-peninsula exists. As an alternative, a scenario based

on climatological factors has been suggested. Herein, we discuss the validity of the hypothesized mid-peninsular vicariance event and the climate-based alternative in explaining the concordant genealogical breaks. We argue that despite the significant changes in climate brought about by the glacial cycles throughout the Quaternary, a vicariant history involving a mid-peninsular seaway remains the most parsimonious explanation to the observed patterns in mtDNA genealogies.

Introduction

The effects of geological history versus long-term climate changes on genealogical diversity remain a highly debated topic (e.g., Hewitt 2004a, 2004b). The relative importance of the different forces may be evaluated only in a geologically highly active region where species cross various climate regimes. The peninsula of Baja California in north-western Mexico, a narrow tract of land approximately 1300 km long, lends itself to testing the alternative hypotheses. Its geological evolution follows a complex history of tectonism, orogenesis and paleostratigraphy (Hausback 1984; Lonsdale 1989; Helenes & Carreño 1999; Carreño & Helenes 2002). Additionally, changes to the palaeogeography of the peninsula are greatly augmented by wide-scale fluctuations in sea level associated with the glacial cycles during the Quaternary (Ortlieb 1979, 1991; Lambeck & Chappell 2001). Undoubtedly, the regional biota is strongly affected by the complicated history of Baja California.

Molecular studies, particularly those investigating the genealogical relationships among mitochondrial DNA (mtDNA) lineages, have made great contributions towards elucidating the historical biogeography of Baja California (Riddle *et al.* 2000; Murphy & Aguirre-León 2002). Based on the genealogy of mtDNA lineages in *Uta stansburiana* (side-blotched lizard), Upton & Murphy (1997) suggested that the mid-peninsular region was submerged approximately 1 million years ago (Ma), resulting in a temporary connection between the Gulf of California and the Pacific Ocean (Fig. 4.1). Importantly, geographically concordant divergences have been found in the mid-peninsular region for numerous additional mtDNA genealogies including other lizards, snakes, mammals, birds, and spiders (e.g., Zink *et al.* 1997; Riddle *et al.* 2000; Rodríguez-Robles & De Jesús-Escobar 2000; Lawlor *et al.* 2002; Murphy & Aguirre-León 2002; Crews & Hedin 2006). These data sets support the idea that a mid-peninsular seaway temporarily severed Baja California (Upton & Murphy 1997). Similar support is available from studies based on allozyme variation (Murphy & Aguirre-León 2002).

Can alternative hypotheses explain the deep genealogical break observed in much of the biota of Baja California? The main objective of this study is to evaluate the validity of the hypothesized mid-peninsular vicariance event and suggested alternatives in light of available evidence.

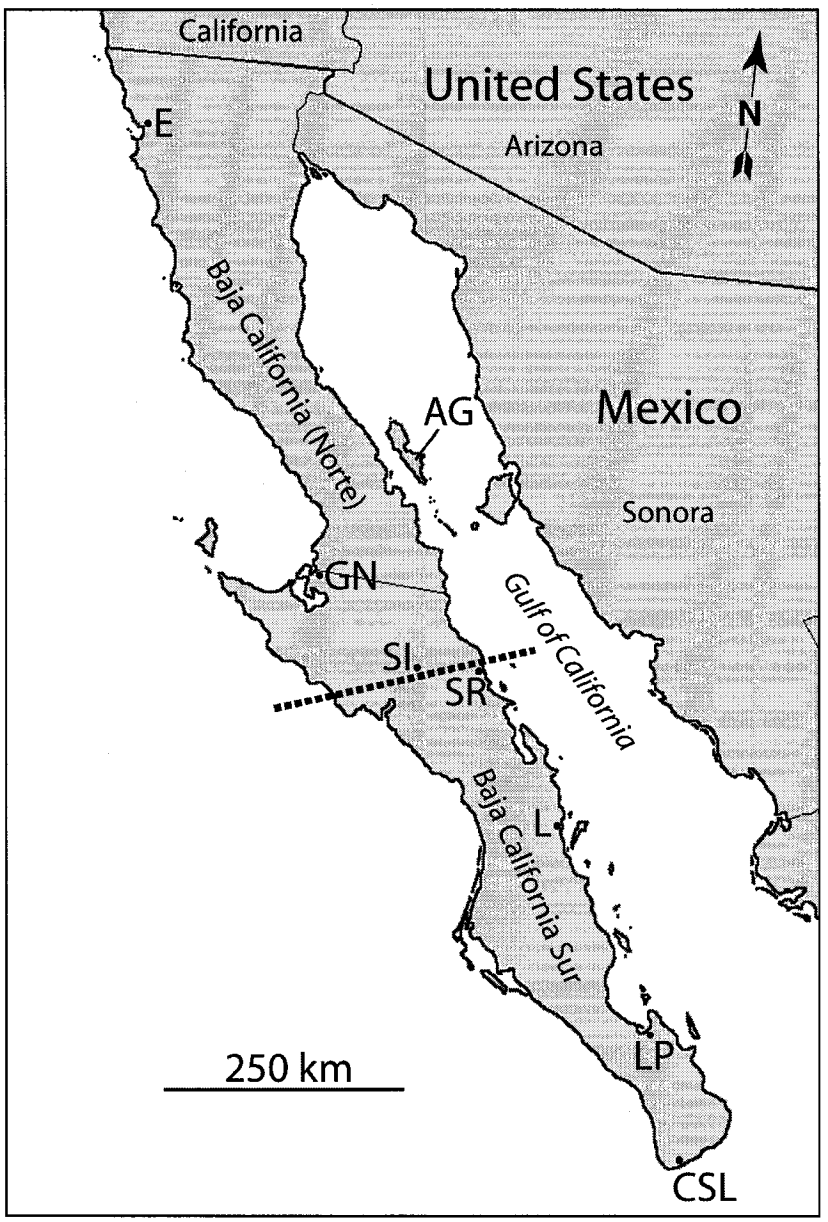

Figure 4.1. Map of Baja California showing the approximate location of the hypothesized mid-peninsular seaway. The current locations of individual genealogical breaks in mtDNA, which support a mid-peninsular vicariant history, vary (see text). For example, the genealogical break in *Uta stansburiana*, used to initially suggest the mid-peninsular seaway, occurs south of Santa Rosalía (Upton & Murphy 1997; Hollingsworth 1999). AG = Isla Ángel de la Guarda, CSL = Cabo San Lucas, E = Ensenada, GN = Guerrero Negro, L = Loreto, LP = La Paz, SI = San Ignacio, SR = Santa Rosalía.

Biogeographical doubts

While a vicariance event affecting all species similarly is the most parsimonious explanation for the congruent genealogical breaks, the existence of a mid-peninsular seaway has been challenged. Indeed, marine sediments that completely transverse the mid-peninsular region have, to date, not been found (Hafner & Riddle 2005). Grismer (2002), reflecting on differences among individual genealogies used to support the mid-peninsular seaway (in particular, geographical location and percent sequence divergence), deemed the molecular evidence for a 1 Mya mid-peninsular seaway equivocal. Rather, he suggested that other forces might have generated the geographic congruence among the mtDNA lineages. Specifically, he advocated that abrupt changes in phytogeography, geology, and weather patterns in central Baja California might have contributed to the observed genetic divergences (Grismer 2002).

Species have been affected by Quaternary glacial cycles resulting in successive range contractions and expansions (FAUNMAP Working Group 1996; Comes & Kadereit 1998; Hewitt 2000). Such climate oscillations may have affected species distributions on the peninsula as well as intraspecific genetic patterns (Hafner & Riddle 1997). Mid-peninsular genetic divergences may have formed due to temporary range fragmentation during glacial periods followed by formation of secondary contact zones during interglacials when populations reunited (cf. Schneider *et al.* 1998; Hugall *et al.* 2002).

Genealogical splits may also form within a continuously distributed species simply due to the hierarchal, female-restricted, and non-recombining inheritance of

mtDNA, given sufficient time (Hoelzer 2001; Irwin 2002). Vicariance, therefore, is not required to produce deep divergences along a narrow peninsula like Baja California.

Beyond the Quaternary

Recent work on the mtDNA genealogy of *Callisaurus draconoides* (zebra-tailed lizard) (Lindell *et al.* 2005) revealed a very deep divergence mid-way on the peninsula that was consistent with a mid-peninsular vicariance event. When evaluated with new geological evidence (Carreño & Helenes 2002, and references therein) and genealogies of reptilian taxa of similar sampling distribution [notably including specimens from the deep-water island Isla Ángel de la Guarda; *Aspidoscelis* (Radtkey *et al.* 1997), *Sauromalus* (Petren & Case 1997), and *Uta* (Upton & Murphy 1997)], a significantly older time frame for the events affecting the historical biogeography of Baja California emerges. This older scenario includes a mid-peninsular vicariance event of late Miocene or early Pliocene age (cf. Rodríguez-Robles & De Jesús-Escobar 2000). Geological and palaeontological evidence supports a mid-peninsular submergence at this time (Helenes & Carreño 1999; Carreño & Helenes 2002; Ledesma-Vázquez 2002). An extensive palaeontological record of marine mollusks demonstrates that vast areas of the peninsula were submerged during late Miocene and early Pliocene, with Pacific waters extending to the vicinity of San Ignacio (Smith 1984, 1991). Marine incursions also have been recorded on the coast of the Gulf of California near Santa Rosalía, where the onset of marine sedimentation has been estimated at approximately 7 Mya (Ortlieb 1991; Holt *et al.* 2000). The earlier date

suggested for the mid-peninsular seaway is also supported by high sequence divergences observed in additional taxa (Lindell *et al.* 2005), most recently spiders (Crews & Hedin 2006).

What do we make of the absence of continuous marine sediments between the Gulf of California and the Pacific Ocean? Currently, available geological evidence would support a very narrow (approximately 30 km) landbridge between the northern and southern halves of the peninsula close to where San Ignacio now is situated (Hafner & Riddle 2005). Such a connection would not allow any dispersal between Gulf and Pacific waters of marine species. However, multiple data sets from marine species show disjunct distributions, which only can be explained by a temporary connection across the mid-peninsular region (Upton & Murphy 1997; Bernardi *et al.* 2003; Hafner & Riddle 2005; Riginos 2005). This suggests that continuous marine deposits between the Gulf of California and the Pacific Ocean either may have been obscured by geological activity after the mid-peninsular seaway and subsequently disappeared (Hafner & Riddle 2005), or have simply not yet been discovered.

Vicariant legacy

Genealogical patterns associated with geological events, such as the formation of the deep-water island Isla Ángel de la Guarda, require a date for the mid-peninsular divergence that is earlier than the estimated age of 1 Mya (e.g., Upton & Murphy 1997; Riddle *et al.* 2000). This earlier date is beyond the scope of climate oscillations related to

glacial cycles, which began approximately 2.5 Mya (Shackleton *et al.* 1984). Accordingly, climate-driven habitat fragmentation cannot adequately explain the mid-peninsular genetic breaks observed for most taxa. Although a climatic change involving seasonality of rain patterns currently occurs in the mid-peninsular region (Hastings & Turner 1965), the formation of genetic breaks due to a long-lasting abrupt change in habitat in the mid-peninsular area (*sensu* Grismer 2002) also appears unlikely, simply because regional weather and vegetational regimes have repeatedly shifted following glacial cycles, not only altitudinally, but also latitudinally (Van Devender 1990). Indeed, latitudinal range changes of as much as 500 km have been reported for plants along the peninsula (Rhode 2002).

The older scenario for the mid-peninsular seaway of Baja California may better explain the spread in geographic location among breaks of individual genealogies. MtDNA lineages isolated on opposite sides of a seaway form a secondary contact zone once the barrier disappears. The original location of such secondary contact zones would be very similar across species affected by the isolation event. However, the location of a contact zone is not stagnant (Barton & Hewitt 1981, 1989) and well-documented examples exist of moving contact zones (e.g., Dasmahapatra *et al.* 2002; Secondi *et al.* 2003). Hence, a geographic spread among secondary contact zones established millions of years ago following a common vicariance event should be expected. Geographically separated breaks can also be produced by the process documented by Hoelzer (2001) and Irwin (2002). However, such a process would produce a much more random distribution of genetic breaks along the peninsula, rather than the apparent congregation of contact zones seen in the mid-peninsula. A more pertinent issue is the nature of the individual

contact zones, given the older suggested date: Why do the secondary contact zones in mtDNA along Baja California remain so narrow despite the millions of generations that have passed after they were established?

So, what about the molecular clock? Among taxa, substantial variation occurs in the percent sequence divergence (Murphy & Aguirre-León 2002; Lindell *et al.* 2005). Unfortunately, despite alleged advances in molecular clock theory, divergence estimates from sequence data for the mid-peninsular seaway vary to the extent of being useless (Ayala 1999; Edwards & Beerli 2000). They are too inaccurate to help us pinpoint the timing of events. The complex history of Baja California illustrates the necessity of evaluating geographically detailed genealogies with known dated geological events (Murphy & Crabtree 1985; Upton & Murphy 1997). Such "genealogical anchors" include the formation of deep-water islands (Lindell *et al.* 2005). This approach is more likely to provide the temporal detail necessary to compare genealogies in a historical biogeography analysis. It is of particular importance for regions of complex biogeographical history, such as that harboured in the "peninsular archipelago" of Baja California (Murphy & Aguirre-León 2002), where the possibility of temporally nested events (the fact that seemingly concordant events may, in fact, represent distinct histories) exists (Hafner & Riddle 2005).

Conclusions

The historical biogeography of Baja California has been strongly affected by geological and paleogeographical forces. These forces have left a genetic imprint, particularly evident in the mid-peninsular region where deeply divergent genealogies show a striking level of congruence. It is possible that forces other than an ancient seaway have significantly affected individual genealogies among those harbouring deep genetic splits in the mid-peninsular region. The alternative explanations have no support, and are countered by strong evidence. Consequently, a vicariance event affecting all species similarly remains the most parsimonious explanation for the congruent genealogical breaks around the mid-peninsular region. Future analyses based on geographically and temporally detailed genealogies will help elucidate the historical biogeography of the peninsular archipelago of Baja California and may suggest the occurrence of additional vicariance events, including the possibility of recurring mid-peninsular seaways.

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Chapter 5

Simple identification of mitochondrial lineages in contact zones based on lineage-selective primers

Abstract

A variety of research projects focus on genetic variation among and within maternal lineages as encompassed by mitochondrial DNA (mtDNA). While mtDNA often differs substantially between species, large differences may also be found within species. The evaluation of such divergent lineages, for example in intraspecific contact zones (hybrid zones), commonly involves sequencing numerous individuals. Large-scale sequencing is both expensive and labour-intensive. Based on sequences from 15 individuals, we devised a simple and quick PCR-assay for identification of divergent mtDNA lineages in a secondary contact zone of the side-blotched lizard (*Uta stansburiana*). The application uses lineage-selective primers to amplify a lineage diagnostic product, and is based on each group of mtDNA haplotypes being a monophyletic assemblage of haplotypes sharing the same maternal ancestry, deeply divergent from the other group. The assay was tested on a larger sample (n=147) of specimens from the contact zone, confirming its usefulness in quick and reliable identification of mtDNA lineages. This approach can be

modified for other species, provided diagnostic lineage variation is available, and may also be performed in simple laboratory settings while conducting fieldwork.

Introduction

Recent years have seen a surge of research projects addressing genetic variation among and within maternal lineages. Molecular research on mitochondrial DNA (mtDNA) lineages commonly involves sequencing numerous individuals. Large-scale sequencing is both expensive and labour-intensive.

Mitochondrial lineages tend to differ substantially between species, but large differences are also commonly found within species (Phillips *et al.* 2004). In cases where some sequence variation is already known, distinct lineages are often found that are of key interest, for example in contact zones (hybrid zones). A simple method for identifying such divergent lineages can greatly reduce the need for extensive sequencing (Sætre & Moum 2000), and quickly direct further sampling efforts, especially if results are attainable in simple laboratory conditions while conducting fieldwork (Barker 1994).

Numerous secondary contact zones exist along the peninsula of Baja California of north-western Mexico, reflecting a complex geological history involving temporary vicariance events since Miocene (Murphy & Aguirre-León 2002; Lindell *et al.* 2006). Genetic tools for detailed analyses of these contact zones will provide a better understanding of the peninsular fauna and the evolution and maintenance of old intraspecific contact zones.

In the side-blotched lizard (*Uta stansburiana*), a break between genealogical lineages is located just south of Santa Rosalía, about mid-way on the peninsula (Upton & Murphy 1997; Hollingsworth 1999). Here, two highly divergent mtDNA lineages form a secondary contact zone, showing a 9.0% sequence divergence based on cytochrome *b* (cyt *b*) and ATPase 6 sequence data (Upton & Murphy 1997). To ease our analysis of this contact zone, we aimed to develop a PCR assay for quick, simple, and reliable identification of the two divergent mtDNA lineages based on diagnostic nucleotide differences. The approach reduced both cost and labour and enabled us to quickly estimate the position and width of the contact zone.

Materials and methods

Specimen sampling

Fifteen specimens of *U. stansburiana* were chosen from the contact zone between San Lucas and San Bruno, south of Santa Rosalía (Table 5.1, Fig. 5.1). The specimens represented a range of locations along a roughly straight transect (Highway 1). We aimed at sampling both divergent haplotype lineages by sampling lizards up to approximately 22 km apart across the contact zone as tentatively described by Hollingsworth (1999).

Table 5.1. Information for the 15 specimens sequenced in this study and used to develop lineage-selective primers. Specimens are listed according to their geographic position from north to south along the sampling transect (approximately 22 km long). Lineage denotes mtDNA haplotype lineage: N and S correspond to the northern and southern haplotype lineage, respectively. Tissue numbers correspond to Royal Ontario Museum (ROM) Field Numbers. Specimen vouchers are held at the Laboratorio de Herpetología, Instituto de Biología, Universidad Nacional Autónoma de México, in Mexico City. Distances listed under Collection locality were measured along Highway 1 and are approximate. Sequence accession numbers are listed under GenBank. Specimens S95, S96, and S97 had identical sequences, and S161 was identical to S164.

Specimen	Lineage	Tissue number	Collection locality	GenBank
S89	N	ROM 37140	10 km N of San Lucas microwave station junction	DQ001850
S90	N	ROM 37141	(same as S89)	DQ001851
S91	N	ROM 37142	(same as S89)	DQ001852
S92	N	ROM 37143	(same as S89)	DQ001853
S93	N	ROM 37144	(same as S89)	DQ001854
S94	S	ROM 37048	At San Lucas microwave station junction [junction of Highway 1 and small road to San Lucas microwave (microondas) station, ca 0.4 miles S of San Lucas; same as "San Lucas" of Upton & Murphy (1997)]	DQ001855
S95	N	ROM 37049	(same as S94)	DQ001856
S96	N	ROM 37050	(same as S94)	DQ001857
S97	N	ROM 37053	(same as S94)	DQ001858
S159	N	ROM 37149	1.7 km S of junction of San Lucas microwave station junction	DQ001859
S73	S	ROM 37124	2.9 km S of junction of San Lucas microwave station junction	DQ001860
S161	S	ROM 37151	4.2 km S of San Lucas microwave station junction	DQ001861
S72	S	ROM 37122	8.5 km S of San Lucas microwave station junction	DQ001862
S162	S	ROM 37152	9.3 km S of San Lucas microwave station junction	DQ001863

Specimen	Lineage	Tissue number	Collection locality	GenBank
S164	S	ROM 37123	11.9 km S of San Lucas microwave station junction	DQ001864

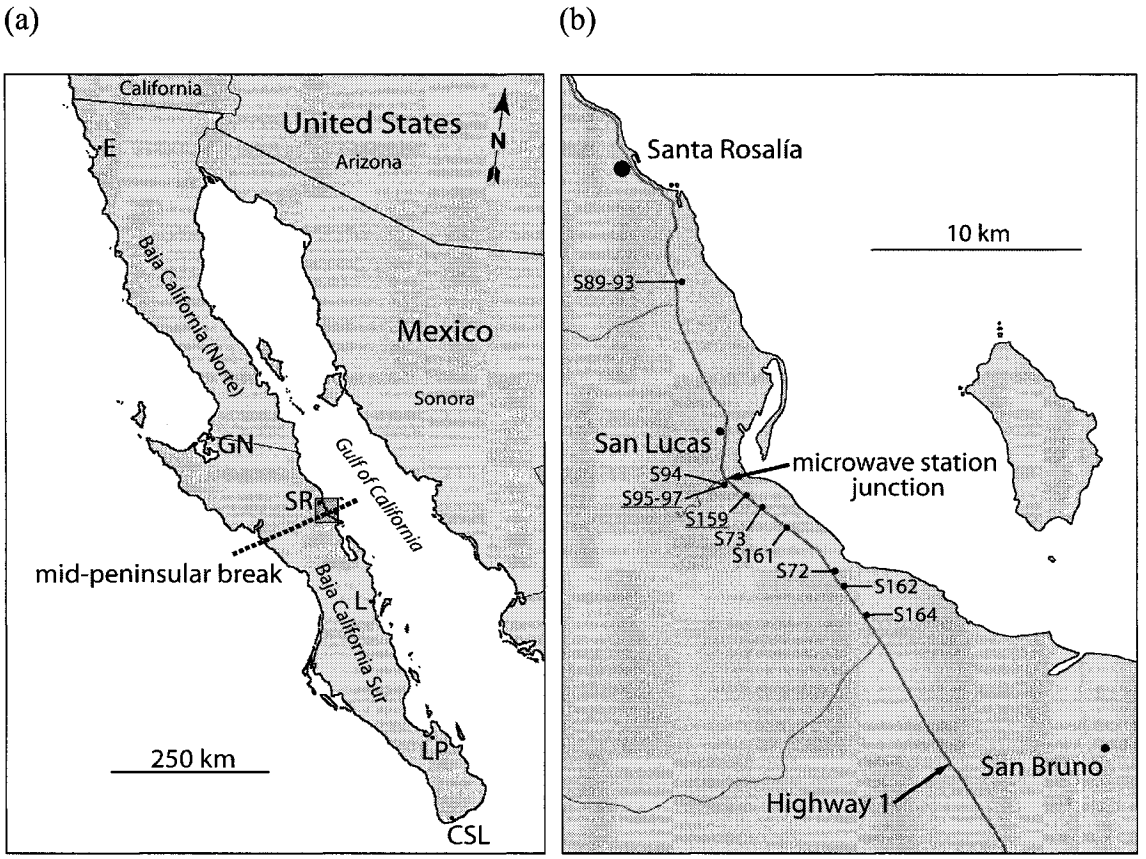


Figure 5.1. Study location and specimen sampling. (a) Map of Baja California showing the mid-peninsular break in *Uta stansburiana* mtDNA and (b) a map of the region that was sampled. The break is a secondary contact zone between two maternal lineages showing on average 10.4% sequence divergence in cytochrome *b*. Sampling region is indicated on the map of Baja California by a shaded square. Sampling was conducted along a transect (Highway 1) across the contact zone between San Lucas and San Bruno, two villages located south of Santa Rosalía. Specimens with the northern mitochondrial lineage have been underlined. CSL = Cabo San Lucas, E = Ensenada, GN = Guerrero Negro, L = Loreto, LP = La Paz, SR = Santa Rosalía.

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from small amounts of ethanol-preserved liver or muscle tissue by standard phenol extraction (Sambrook *et al.* 1989) . One segment (1081 bp) of the mitochondrial genome was amplified using the primers L16355 (5'-CCATCCAACATCTCAGCATGATGAAA-3') and H17415 (5'-GTCTTCAGTTTTTGGTTTACAAGAC-3'). This region contained most (1045 bp) of the cyt b gene plus a 28 bp segment of threonine tRNA.

PCR reactions (25 μl) contained 2 μl of template DNA (approximately 150 ng), 0.8 μl of 10 mM dNTPs, 1 μl of each 10 μM primer solution, 1x PCR buffer, 1.5 mM $MgCl_2$, and 0.75 U Taq DNA polymerase (Boehringer Mannheim). A thermal cycler [PTC 200 (MJ Research)] with the following conditions was used: 1 step of 94°C for 2 min; 39 cycles of 94°C for 30 s, 48°C for 45 s, and 72°C for 45 s. After the final cycle, a prolonged extension step of 5 min was added. Two microliters of amplified DNA were separated by electrophoresis on a 1% agarose gel and stained with ethidium bromide. DNA bands were visualized on an ultraviolet light table to verify product quality. The remaining product of each reaction was cleaned using the QIAquick PCR Purification Kit (Qiagen).

Amplified DNA was sequenced using an ABI PRISM 377 DNA Sequencer (Applied Biosystems). Sequencing was performed in both directions with the primers initially used in PCR amplification, or with the conserved internal primers L16742 (5'-GGAACAACCCTAGTTGAATGAAT-3') and H16852 (5'-GTTGTTTGAACCTGTTTCGTG-3') specifically designed for *U. stansburiana*. The

ABI PRISM BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems) was used following the manufacturer's protocols. Sequencing products were cleaned using Centri-Sep Columns (Princeton Separations).

Sequence data analysis

Sequence data were entered into BioEdit 5.0.9 (Hall 1999) and combined with the cyt *b* sequences (555 bp) of Upton & Murphy (1997). The near absence of insertions and/or deletions (indels) in the sequences made it possible to align these sequences by eye. Two indels occurred in the non-coding region between cyt *b* and threonine tRNA. One indel was observed in threonine tRNA. No indel was found in cyt *b*. The combined data set was exported into MacClade 4.05 (Maddison & Maddison 2002) and then exported as a nexus file into PAUP* 4.0b10 (Swofford 2000). Values of pairwise proportional sequence divergences (*p*-distances) were obtained using PAUP*. Individuals with identical sequences for the 555 bp segment of Upton & Murphy (1997) were merged into a single operational taxonomic unit for phylogenetic analysis.

The new sequences were analyzed together with the cyt *b* sequences of Upton & Murphy (1997) to determine to which of the two main maternal lineages they belonged. This data set comprised 555 bp, with *Urosaurus ornatus* and *Petrosaurus mearnsi* as outgroup specimens. Genealogical history was evaluated using maximum parsimony. Heuristic searches were conducted with 10000 random addition sequence replicates. Ten trees were held at each step, retaining minimal trees only. TBR branch swapping was

used saving multiple trees, and zero-length branches were collapsed. Nodal support was assessed with Bremer decay analysis (Bremer 1994) using AutoDecay 4.0.2 (Eriksson 1998) and bootstrap analysis (1000 pseudoreplicates; Felsenstein 1985). In both analyses of nodal support, the heuristic search strategies describe above were used except for the number of replicates being reduced to 10. Each new sequence was determined as part of the northern or southern haplotype clade based on its genealogical association with the specimens used in Upton & Murphy (1997).

Design of lineage-selective primers

Our application for identifying the two maternal groups in *U. stansburiana* was based on each group being a monophyletic assemblage of lineages sharing the same maternal ancestry, very divergent from the other group. We call this approach "maternal ancestry monophyly analysis" (MAMA). We herein wish to emphasize the distinction between maternal and biparental histories, because evidence from data based on nuclear DNA suggests that gene flow is unabated across mtDNA contact zones in many species along the peninsula (Aguirre-León *et al.* 1999; Murphy & Aguirre-León 2002; Lindell *et al.* 2005).

Nucleotide variation among the 15 sequences was surveyed along their entire length (1081 bp) for potential primer locations. Conserved segments and regions with large differences between the two haplotype groups were identified. Three primers were designed for amplification of fragments internal to L16355 and H17415. One primer,

L16742 (5'-GGAACAACCCTAGTTGAATGAAT-3'), was determined for a region fully conserved among all fifteen sequences and both haplotypes. This primer is identical to one of the primers used for internal sequencing. Two lineage-selective primers were designed in regions where all sequences from the two groups differed by at least 5 bp within segments that were 18 bp long: H16929 (5'-GGCTCCTAAAAGGACTTTA-3') was a perfect match for all southern haplotype sequences, and H17115 (5'-GAGTAGGGCAAGTACTCCA-3') was identical to all sequences of the northern haplotype (Table 5.2). These regions had the maximum number of differences observed for a segment of length appropriate for a primer. The three primers provided a clear size difference between the two possible PCR products (228 bp versus 417 bp, primers included). The primers were also designed to have similar annealing temperatures. A nexus file containing the three primers aligned with all sequences has been submitted to TreeBASE (study and matrix accession numbers are S1814 and M3320, respectively).

Haplotype identification

Amplification at lineage-selective PCR conditions using all three primers was optimized for the fifteen specimens originally used for sequencing. The criteria for optimized amplification included: a short total time, annealing temperature yielding an unambiguous band of one of the two possible products, and low amount of enzyme used in reaction. PCR reactions (10 μl) contained 1 μl of template DNA, 0.32 μl of 10 mM dNTPs, 0.4 μl each of the conserved primer and the two lineage-selective primer

Table 5.2. Nucleotide variation within and between the two mtDNA haplogroups and the diagnostic differences at the sites of the two lineage-selective primers. The dots indicate bases identical with the two lineage-selective primers. H16929 is a perfect match for all southern haplotypes, and H17115 is identical to all northern haplotypes.

Position	16929	16930	16931	16932	16933	16934	16935	16936	16937	16938	16939	16940	16941	16942	16943	16944	16945	16946	16947	17115	17116	17117	17118	17119	17120	17121	17122	17123	17124	17125	17126	17127	17128	17129	17130	17131	17132	17133
H16929*	T	A	A	A	G	A	C	C	T	T	T	T	A	G	G	A	G	C	C																			
H17115*																				T	G	G	A	G	T	A	C	T	T	G	C	C	C	T	A	C	T	C
S89	C	.	.	.	.	.	.	.	.	G	C	.	.	.	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S90	C	.	.	.	.	.	.	.	.	G	C	.	.	.	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S91	C	.	.	.	.	.	T	.	.	G	C	.	.	.	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S92	C	.	.	.	.	.	.	.	.	G	C	.	.	.	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S93	C	.	.	.	.	.	.	.	.	G	C	.	.	.	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S95	C	.	.	.	.	.	.	.	.	G	C	.	.	.	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S96	C	.	.	.	.	.	.	.	.	G	C	.	.	.	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S97	C	.	.	.	.	.	.	.	.	G	C	.	.	.	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S159	C	.	.	.	.	.	.	.	.	G	C	.	.	.	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S72	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	G	.	.	T	.	.	A	.	.	.	.	.	T	.	.	A
S73	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	G	.	.	T	.	.	A	.	.	.	.	.	T	.	.	A
S94	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	G	.	.	T	.	.	A	.	.	.	.	.	T	.	.	A
S161	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	T	.	.	A	.	.	.	.	.	T	.	.	A
S162	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	G	.	.	T	.	.	A	.	.	.	.	.	T	.	.	A
S164	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	T	.	.	A	.	.	.	.	.	T	.	.	A

*Lineage-selective primers were designed as the reverse-complement of these sequences.

solutions (each 10 μM), 0.06 μl of 5 U/μl *Taq* DNA polymerase (Boehringer Mannheim)in a 10 mM Tris-HCl and 1.5 mM $MgCl_2$ solution (1 μl of 10× PCR buffer; Boehringer

Mannheim). A thermal cycler [PTC 200 (MJ Research)] with the following conditions was used: 1 step of 94°C for 2 min; 39 cycles of 94°C for 25 s, 48°C for 30 s, and 72°C for 30 s. After the final cycle, a prolonged extension step of 5 min was added. Four microliters of amplified DNA was separated by electrophoresis on a 1% agarose gel and stained with ethidium bromide. DNA bands were visualized on an ultraviolet light table to verify product length and quality. Thirty PCR reactions using the same DNA templates but with only one of the lineage-selective primers available were also conducted as amplification controls.

In addition to the 15 specimens that were sequenced, 132 specimens of *U. stansburiana* from the contact zone were evaluated for lineage identity, in order to assess the quality of the method on a larger sample (n=147).

Results

Sequence variation and genealogical identity

The sequences revealed 12 different haplotypes among the 15 individuals. Genealogically, the data established the existence of two clearly distinct maternal groups, each with very limited within-group variation. Nine (six unique) and six (five unique)

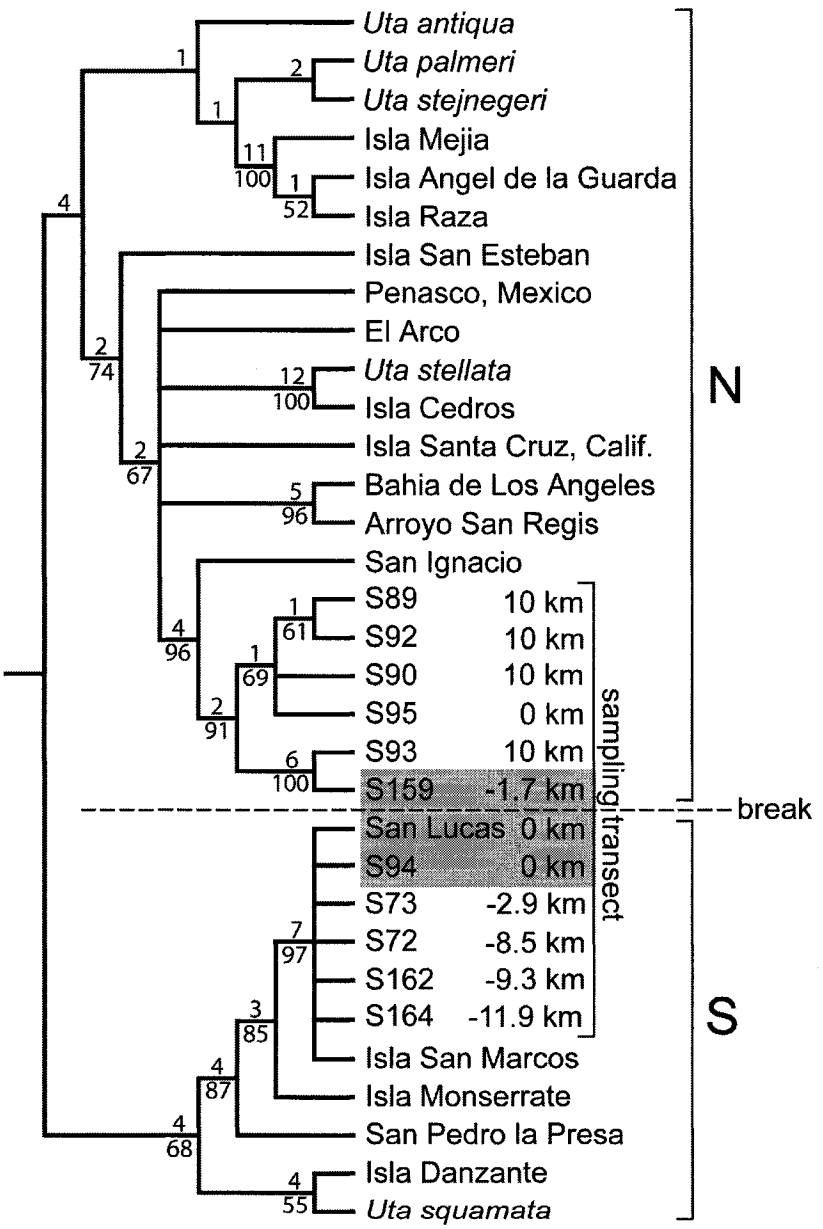
haplotypes grouped together with the northern and southern haplotype clade, respectively (Fig. 5.2). There was a 1.7 km geographical overlap between the divergent lineages just south of San Lucas. Consistent with the findings of Upton & Murphy (1997), extensive nucleotide variation occurred between the two divergent haplotype groups, with 10.4% average sequence divergence. In contrast, sequence divergence within the haplotype groups was low, averaging 0.4% and 0.7% for the northern and southern group, respectively.

Haplotype identification

Amplification consistently produced clear, single products for all 15 specimens initially sequenced (Fig. 5.3). In all cases, product length corresponded to amplification of the haplotype lineage originally identified through sequencing. All control PCRs yielded either a product band of the same quality as under lineage-selective conditions, or no product at all, depending on whether a matching lineage-selective primer was included or omitted in the reaction.

Of the 147 samples evaluated, all but one (99.3%) produced results consistent with the above findings. The inconsistent sample yielded intermediate amounts of both possible products. (A detailed evaluation of the evolution and maintenance of the secondary contact zone will be published separately.)

Figure 5.2. Genealogy of mitochondrial lineages in *Uta stansburiana*, emphasizing the mid-peninsular contact zone. The tree is a strict consensus (1500 MPTs, 488 steps, CI = 0.510, RI = 0.711) of the combined data set spanning 555 bp of cytochrome *b*, comprising the samples of Upton & Murphy (1997) and the specimens used to develop lineage-selective primers in this study. Nodal support is represented by Bremer decay index (DI) and bootstrap (BS) values of 50% or higher above and below the nodes, respectively. Vertical bars named N and S refer to the northern and southern clade, respectively. Horizontal dashed line indicates the mid-peninsular break (contact zone) discussed in text. Approximate location along the sampling transect has been indicated for each new sample as distance away from the San Lucas specimen of Upton & Murphy (1997), with positive values indicating northward direction. Shaded square spans the southernmost sample of the northern haplotype (S159) and northernmost samples of the southern haplotype (San Lucas and S94), hence representing the geographical overlap of the two haplotypes in the contact zone (1.7 km). The consensus has been simplified by omitting sequences that were identical for the 555 bp analyzed; S91 was identical to S90, S96 and S97 were identical to S95, and S161 was identical to S164.


Uta antiqua
Uta palmeri
Uta stejnegeri
Isla Mejia
Isla Angel de la Guarda
Isla Raza
Isla San Esteban
Penasco, Mexico
El Arco
Uta stellata
Isla Cedros
Isla Santa Cruz, Calif.
Bahia de Los Angeles
Arroyo San Regis
San Ignacio
S89 10 km
S92 10 km
S90 10 km
S95 0 km
S93 10 km
S159 -1.7 km
San Lucas 0 km
S94 0 km
S73 -2.9 km
S72 -8.5 km
S162 -9.3 km
S164 -11.9 km
Isla San Marcos
Isla Monserrate
San Pedro la Presa
Isla Danzante
Uta squamata
N
S
break
sampling transect

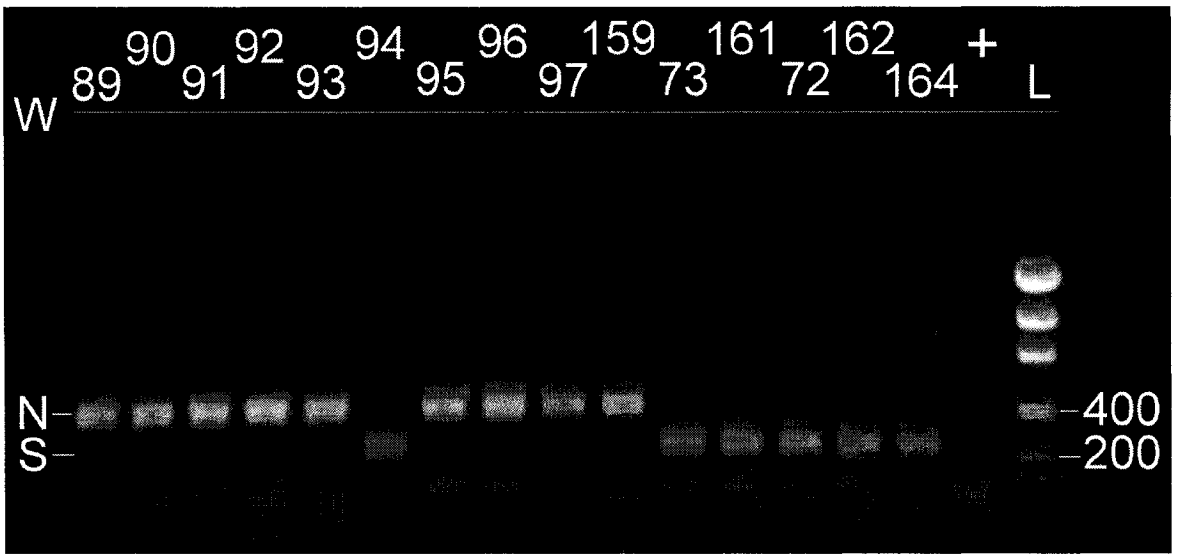


Figure 5.3. Mitochondrial lineage identification based on lineage-selective PCR. Agarose gel separation of PCR products of the fifteen specimens initially sequenced and later used for optimization of lineage-selective PCR conditions. Amplification was performed at lineage-selective PCR conditions using all three primers designed for haplotype identification. Products were loaded following the geographic position of the specimens from north to south along the sampling transect across the contact zone (Table 5.1, Fig. 5.1). Positive control and DNA size ladder (products of lengths 2000 bp, 1200 bp, 800 bp, 400 bp, 200 bp, and 100 bp) were loaded in lanes indicated by + and L, respectively. The line named W shows the position of the loading wells. N and S indicate products corresponding to the northern and southern haplotype, respectively. Note the identification of a southern haplotype in specimen (S94) among geographic neighbours of northern lineage identity, suggesting that the overlap of maternal lineages in the contact zone is at least 1.7 km (the distance between the collection localities for specimens S94 and S159). Each sample well contained 4 μl of PCR products. Samples were run for 40 minutes at 100 volts in a 1% agarose gel.

Discussion

Our approach with lineage-selective primers enabled simple and quick identification of divergent mtDNA lineages in *U. stansburiana*. It was 100% successful in identifying known haplotypes and produced a single, clear, diagnostic band in 99.3% of all samples of unknown haplotype origin. The method has also been adapted for two contact zones in the black-tailed brush lizard (*Urosaurus nigricaudus*) with approximately 11% and 4% sequence divergence in cyt *b* (Lindell *et al.* unpublished data). Lineage-selective amplification always yielded a single, clear product of anticipated lineage-diagnostic size for these contact zones (133 and 114 samples, respectively).

This approach can be useful in analyzing other contact zones, provided that diagnostic nucleotide difference variation is present (Table 5.3). Naturally, an initial phase of lineage identification is necessary, but the main pattern of mtDNA lineage history is usually already known following a genealogical study. Subsequently, a few specimens have to be sequenced for the two divergent lineages to assess nucleotide variation and determine lineage-diagnostic primer sites. Following this development phase, the method may help save time and resources by quickly determining the lineage identity of specimens and eliminate the necessity of extensive sequencing. It can give fast results from which to guide further collection efforts. The PCR assay may even direct ongoing sampling efforts in the field, as it can be performed in simple laboratory settings while conducting fieldwork (Barker 1994).

The contact zone in *U. stansburiana* serves as an illuminating example for the PCR assay described herein. While much is known about the ecology and behaviour of

Table 5.3. Examples of contact zones with divergent mtDNA lineages that are amenable to identification using lineage-selective primers as described herein. Divergences are represented by pairwise proportional sequence divergences (*p*-distances). The examples represent a variety of taxa and contact zones.

Organism	Divergence	Reference
Batrachoseps spp. (Slender salamanders)	5.0–12.4%	Jokusch & Wake (2002)
Bombina bombina and *B. variegata* (Fire-bellied toads)	9.4 ± 1.1%*	Szymura *et al.* (1985)
Carlia rubrigularis (Rainforest skink)	> 15%	Phillips *et al.* (2004)
Macoma balthica (Baltic clam)	≤6.8%†	Luttikhuizen *et al.* (2003)
Mytilus edulis and *M. trossulus* (Blue mussels)	21.4%‡, 17.1%§	Riginos *et al.* (2004) and Riginos & Cunningham (2005)
Phylloscopus brehmii and *P. collybita* (Chiffchaffs)	4.6%	Helbig *et al.* (2001)
Mammals in Baja California	≤12.8%¶	Riddle *et al.* (2000)
Reptiles in Baja California	≤10.4%	Murphy & Aguirre-León (2002) and present study

*Estimated from RFLP analysis.

†K2P model of nucleotide substitution used.

‡Male mtDNA.

§Female mtDNA.

¶Tamura-Nei model of nucleotide substitution used.

the side-blotched lizard (Tinkle 1967; Sinervo *et al.* 2001), nothing is yet known about the evolutionary interactions between these two highly divergent mitochondrial lineages in their secondary contact zone on the peninsula of Baja California. Our approach allowed us to quickly get a more precise estimate of the location of the secondary contact zone than that provided by the genealogy of Upton & Murphy (1997) alone. Henceforth, further directed sampling enables a more detailed analysis of the evolutionary interactions between the northern and southern mtDNA lineages and the dynamics of the seemingly narrow contact zone.

Other methods are available that offer screening of multiple samples in contact zones, including SSCP, RFLP and AFLP. However, the cocktail PCR approach is simpler than all of these; once lineage-selective primers have been developed it only requires amplification and subsequent differentiation of two possible outcomes by using agarose gel electrophoresis. Conversely, SSCP and AFLP patterns can be difficult to interpret even with fairly limited sequence variation. However, lineage-selective amplification cannot differentiate between similar lineages (which e.g. SSCP can), as it requires substantial sequence divergence. Scientists planning a large-scale analysis of a contact zone with less overall divergence may consider developing lineage-selective primers for a more divergent region of mtDNA, such as parts of the D-loop region (Pesole *et al.* 1999). Using lineage-selective primers to determine lineage identity may also be helpful in other research involving deeply divergent lineages, such as the interactions between mtDNA lineages and nuclear DNA in laboratory populations, which require mtDNA lineage identification of large numbers of individuals (Ellison & Burton 2006).

In conclusion, maternal ancestry monophyly analysis employs simple PCR methodology of divergent lineages, enabling quick identification of maternal lineages in contact zones. While an initial evaluation of the genealogical relationships among mtDNA lineages is necessary, the approach can save time and resources and yields quick results that are attainable even in simple laboratory conditions.

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Chapter 6

Characterization of an old mitochondrial DNA contact zone in the black-tailed brush lizard *Urosaurus nigricaudus* and development of lineage-selective primers for maternal ancestry monophyly analysis

Abstract

The peninsula of Baja California of northwestern Mexico has a complex geological history, which has greatly affected the regional biota. Since late Miocene, transpeninsular seaways cut across the peninsula, resulting in temporary range fragmentation of numerous species. Following the retreat of the seaways, populations reunited to form secondary contact zones. In the black-tailed brush lizard (*Urosaurus nigricaudus*), two deeply divergent mtDNA lineages meet in a contact zone across the Isthmus of La Paz. We evaluated the genealogical history and mitochondrial DNA (mtDNA) sequence variation in lizards from this region to provide a better understanding of the genetic interactions across this old contact zone. Our results suggest that mtDNA lineage overlap is limited, despite evidence for ongoing gene flow. Studies on contact zones in other species are needed to evaluate the genetic interactions between reunited populations in

Baja California, and may quickly be pursued using a simple PCR assay for mtDNA lineage identification, as described in this study.

Introduction

Baja California is an approximately 1,300 km long peninsula in northwestern Mexico (Fig 6.1), which has a complex geological history. Since Miocene times, plate tectonic activity, volcanism, and sea level changes have combined to shape the landscape (Carreño & Helenes 2002; Carreño & Smith 2007). The regional biota has been strongly affected by this history. Evidence for this comes from the deeply divergent mitochondrial DNA (mtDNA) genealogies that have been discovered in numerous species (Riddle *et al.* 2000; Murphy & Aguirre-León 2002). These genealogical splits are also geographically clustered in three regions: midway on the peninsula, in the vicinity of Loreto, and on the Isthmus of La Paz. The congruent mtDNA breaks are the result of a common history of temporary isolation, involving transpeninsular seaways that inundated parts of the peninsula (Upton & Murphy 1997). Following the retreat of the seaways, the populations were reunited in secondary contact zones. However, the genetic breaks in mtDNA were left as a legacy of the vicariant history (Lindell *et al.* 2006).

Given the history of cryptic vicariance (Riddle *et al.* 2000), Baja California provides an opportunity to study the genetic interactions between reunited populations and compare the genealogical history of mtDNA with patterns of gene flow and population differentiation in nuclear DNA. Moreover, as the temporary seaways may

have occured as long ago as late Miocene (Lindell *et al.* 2005, in press), it is possible to study the evolution and maintenance of contact zones over long time periods.

The black-tailed brush lizard (*Urosaurus nigricaudus*) is a common phrynosomatid lizard that is found along the full length of the peninsula (Fig 6.1). This species is characterized by a deeply divergent mtDNA genealogy shaped by the complex history of the peninsula (Lindell *et al.* in press). Across the Isthmus of La Paz, two highly divergent mtDNA lineages [11% in cytochrome b (cyt *b*)] meet in a contact zone believed to be the result of a late Miocene seaway (Lindell *et al.* in press). In this study, we investigate the genetic interactions across this contact zone by evaluating the genealogical history of *U. nigricaudus* in this region, and assess sequence variation within and between the two divergent haplogroups. We also develop a simple PCR assay for quick identification of the divergent mtDNA lineages, an approach which can be used to further investigate this and other contact zones (Lindell & Murphy in press).

Materials and methods

Specimen sampling

Thirteen specimens of *U. nigricaudus* [eight from Lindell *et al.* (in press) and five new] were chosen to represent both divergent lineages and assess genetic variation between and within the two haplotype clades meeting on the Isthmus of La Paz (Fig. 6.1; Table 6.1). The specimens were chiefly collected along the main highway (Hwy 1) and

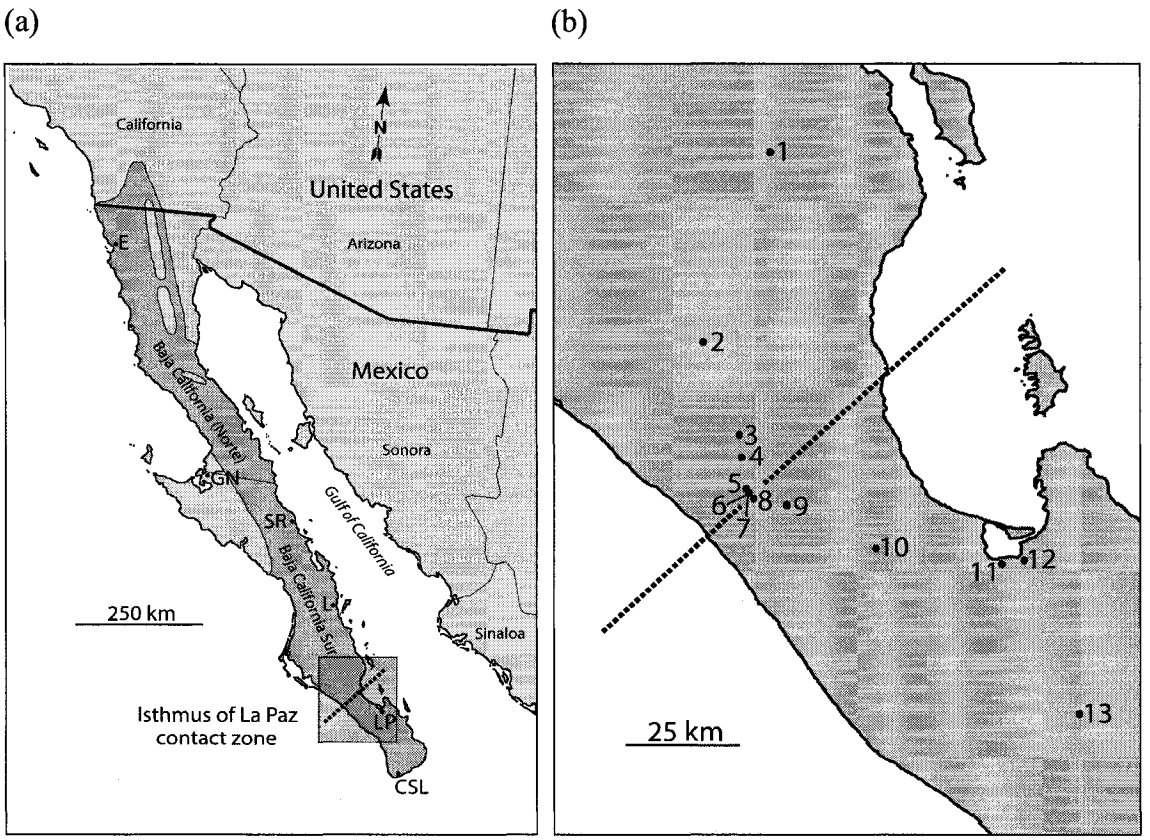


Figure 6.1. Sampling of specimens for mitochondrial lineage identification. (a) Map of Baja California of northwestern Mexico showing the range of *Urosaurus nigricaudus* (shaded area) and the secondary contact zone across the Isthmus of La Paz where two highly divergent mtDNA lineages meet. CSL = Cabo San Lucas, E = Ensenada, GN = Guerrero Negro, L = Loreto, LP = La Paz, SR = Santa Rosalía. (b) Sampling locations for specimens used to assess nucleotide variation within and between the two mtDNA lineages. Samples were collected along a transect (mainly along Hwy 1) across the contact zone. Lineage-selective primers were developed based on lineage-diagnostic sites in the targeted mtDNA segment.

Table 6.1. Information for the 13 specimens of *Urosaurus nigricaudus* evaluated in this study and used to characterize the mtDNA contact zone and develop lineage-selective primers for MAMA. Specimens are listed according to their geographic position from north to south. MtDNA denotes mtDNA haplotype lineage: N and S corresponding to northern and southern haplotype lineage, respectively. Distances given for collection localities refer to distance traveled along Hwy 1. Tissue numbers correspond to field numbers (with voucher specimens being kept in the herpetological collection of the Instituto de Biología, Universidad Nacional Autónoma de México, using the identical number), except for italicized entries, which correspond to voucher catalogue numbers. LACM = Los Angeles County Museum. ROM = Royal Ontario Museum. Distances listed under Collection locality were measured along Highway 1 and are approximate. Sequence accession numbers are listed under GenBank.

Specimen	Collection locality	mtDNA	GPS coordinates	Tissue number	GenBank
1	San Pedro de la Presa	N	Not available	*ROM 45239*	EF653296
2	La Paz (123 km west of)	N	24°27.267'N 111°04.050'W	ROM 35344	EF653297
3	La Paz (91 km west of)	N	24°16.800'N 110°57.767'W	ROM 35340	XXXXXX
4	La Paz (86 km west of)	N	24°14.207'N 110°56.925'W	ROM 37206	XXXXXX
5	La Paz (78.2 km west of)	N	24°10.473'N 110°55.895'W	ROM 37208	EF653298
6	La Paz (77 km west of)	N	24°09.990'N 110°55.458'W	ROM 37028	XXXXXX
7	La Paz (77 km west of)	S	Not available	ROM 37027	XXXXXX
8	La Paz (75.3 km west of)	S	24°09.467'N 110°54.883'W	ROM 35338	EF653301
9	La Paz (67.2 km west of)	S	24°09.283'N 110°50.400'W	ROM 35337	XXXXXX
10	La Paz (43 km west of)	S	24°05.667'N 110°38.433'W	ROM 35332	EF653302
11	La Paz (10 km west of)	S	Not available	ROM 35321	EF653303
12	La Paz	S	Not available	*ROM 23100*	EF653304
13	San Pedro (10 mi south of)	S	Not available	*LACM 128210*	EF653305

represented a range of locations across the contact zone as tentatively described by Lindell *et al.* (in press).

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from small amounts of ethanol-preserved liver or muscle tissue by standard phenol extraction (Sambrook *et al.* 1989). One segment (1094 bp) of the mitochondrial genome was amplified using the primers L16355 (5'-CCATCCAACATCTCAGCATGATGAAA-3') and H17415 (5'-GTCTTCAGTTTTTGGTTTACAAGAC-3'). This region contained most (1045 bp) of cyt *b* plus a 31 bp segment of threonine tRNA.

PCRs (25 μl) contained approximately 150 ng template DNA, 0.75 U *Taq* DNA Polymerase (Boehringer–Mannheim), 320 μM dNTPs, 10 mM Tris–HCl, 1.5 mM $MgCl_2$, and 10 pmol of each primer). A thermal cycler [PTC 200 (MJ Research)] with the following conditions was used: 1 step of 94°C for 2 min; 39 cycles of 94°C for 30 s, 48°C for 45 s, and 72°C for 45 s. After the final cycle, a prolonged extension step of 5 min was added. Two microliters of amplified DNA were separated by electrophoresis on a 1% agarose gel and stained with ethidium bromide. DNA bands were visualized on an ultraviolet light table to verify product quality. The remaining product of each reaction was cleaned using the QIAquick PCR Purification Kit (Qiagen).

Amplified DNA was sequenced using an ABI PRISM 377 DNA Sequencer (Applied Biosystems). Sequencing was performed in both directions with the same

primers used in PCR amplification. In addition, L16676 (5'-tga gga caa ata tcc ttc tga gg-3'; Fu 1999) was used to sequence an internal section of the amplified cyt *b* fragment. The ABI PRISM BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems) was used following the manufacturer's protocols. Sequencing products were cleaned using Centri-Sep Columns (Princeton Separations).

Sequence data analysis

Sequence data were entered into BioEdit 5.0.9 (Hall 1999) and combined with the cyt *b* sequences of Lindell *et al.* (in press). The near absence of insertions and/or deletions (indels) in the sequences made it possible to align these sequences by eye, with the exception of the non-coding region between the cyt *b* and threonine tRNA genes. The combined data set was exported into MacClade 4.05 (Maddison & Maddison 2002) and then exported as a nexus file into PAUP* 4.0b10 (Swofford 2000). Values of pairwise proportional sequence divergences (*p*-distances) were obtained using PAUP*.

The new sequences were analyzed together with those of Lindell *et al.* (in press) to determine to which of the two divergent mtDNA lineages they belonged. This data set comprised 1094 bp, with seven specimens of closely related *Urosaurus* species and three specimens of more distantly related *Petrosaurus* species as outgroup taxa. Genealogical relationships were determined using maximum parsimony in PAUP*. Heuristic searches were conducted with random addition of sequences, one million replicates, retaining minimal trees only, using tree-bisection-reconnection branch swapping, and collapsing

zero-length branches. Nodal support was assessed with Bremer decay analysis (Bremer 1988, 1994) using AutoDecay 4.0.2 (Eriksson 1998) as implemented with PAUP*, and with bootstrap analysis (Felsenstein 1985) in PAUP* using 10000 replicates. In both analyses of nodal support, the heuristic search strategies were identical to the genealogical analysis described above, except that the number of replicates was reduced to 10000 and 100 for Bremer decay and bootstrap analysis, respectively.

Each new sequence was determined as part of the northern or southern haplotype clade based on its genealogical association with the specimens analysed by Lindell *et al.* (in press).

Design of lineage-selective primers

Nucleotide variation among the thirteen sequences was surveyed along their entire length for potential primer sites for maternal ancestry monophyly analysis (MAMA; Lindell & Murphy in press). Conserved segments and regions with large differences between the two haplotype groups were identified. Three primers were designed for amplification of fragments internal to L16355 and H17415. One primer, 16784L (5'-AACGCAACACTAACCCGATT-3'), was designed for a region fully conserved among all thirteen sequences and both haplotypes. Two lineage-selective primers were designed in regions where all sequences from the two groups differed by (at least) 7 and 6 bp, respectively: 17143H (5'-GGGAACTAGCATTAAGATTAG-3') was a perfect match for all northern haplotype sequences, and 17325H

(5'-AGAATCAGAAACAGTGCG-3') was identical to all southern haplotypes (Table 6.2). These regions had the maximum number of differences observed for a segment of length appropriate for a primer. The three primers provided a clear size difference between the two possible PCR products (402 bp versus 581 bp, primers included). The primers were also designed to have similar annealing temperatures.

A nexus file containing the three primers aligned with the sequences used to evaluate genealogical history has been submitted to TreeBASE.

Haplotype identification

Amplification at lineage-selective PCR conditions using all three primers was optimized for the thirteen specimens originally used for sequencing. The criteria for optimized amplification included: a short total time, annealing temperature yielding an unambiguous band of one of the two possible products, and low amount of enzyme used in reaction. PCR reactions (10 μl) contained 1 μl of template DNA, 0.32 μl of 10 mM dNTPs, 0.4 μl each of the conserved primer and the two lineage-selective primer solutions (each 10 μM), 0.06 μl of 5 U/μl *Taq* DNA polymerase (Boehringer Mannheim) in a 10 mM Tris-HCl and 1.5 mM $MgCl_2$ solution (1 μl of 10× PCR buffer; Boehringer Mannheim). A thermal cycler [PTC 200 (MJ Research)] with the following conditions was used: 1 step of 94°C for 2 min; 39 cycles of 94°C for 25 s, 48°C for 30 s, and 72°C for 30 s. After the final cycle, a prolonged extension step of 5 min was added. Four microliters of amplified DNA was separated by electrophoresis on a 1% agarose gel

Table 6.2. Nucleotide variation within and between the two mtDNA haplogroups and the diagnostic differences at the sites of the two lineage-selective primers. The dots indicate bases identical with the two lineage-selective primers. 17143H is a perfect match for all northern haplotypes, and 17325H is identical to all northern haplotypes.

Position	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	5	5	6	6	6	6	2	2	2	2	2	3	3	3	3	3	3	3	3	3	3	3	3	3	4	4	4	5	5	5	5	5	5
	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1
17143H*	C	T	A	A	T	C	T	T	A	A	T	G	C	T	A	G	T	T	C	C	C																											
17325H*																						C	G	C	A	C	T	G	T	T	T	C	T	G	A	T	T	C	T							
1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A	.	.	.	T	.	A	.	.	C	A	.							
2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A	.	.	.	T	.	A	.	.	C	A	.							
3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A	.	.	.	T	.	A	.	.	C	A	.							
4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A	.	.	.	T	.	A	.	.	C	A	.							
5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A	N	N	N	T	.	A	.	.	C	A	.							
6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A	.	.	.	T	.	A	.	.	C	A	.							
7	T	.	.	.	.	T	C	.	T	.	.	A	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.							
8	T	.	.	.	.	T	C	.	T	.	.	A	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.							
9	T	.	.	.	.	T	C	.	T	.	.	A	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.							
10	T	.	.	.	.	T	C	.	T	.	.	A	T	.	.	.	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.							
11	T	.	.	.	.	T	C	.	T	.	.	A	T	.	.	A	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.							
12	T	.	.	.	.	T	C	.	T	.	.	A	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.							
13	T	.	.	.	.	T	C	.	T	.	.	A	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.							

*Lineage-selective primers were designed as the reverse-complement of these sequences.

along with a negative control and a DNA ladder and stained with ethidium bromide. DNA bands were visualized on an ultraviolet light table to verify product length and quality. Twenty-six PCR reactions using the same DNA templates but with only one of the lineage-selective primers available were also conducted as amplification controls.

In addition to the thirteen specimens used to assess nucleotide diversity within and between the two haplotype groups, 120 specimens of *U. nigricaudus* from the contact zone were evaluated for lineage identity, in order to assess the quality of the method on a larger sample.

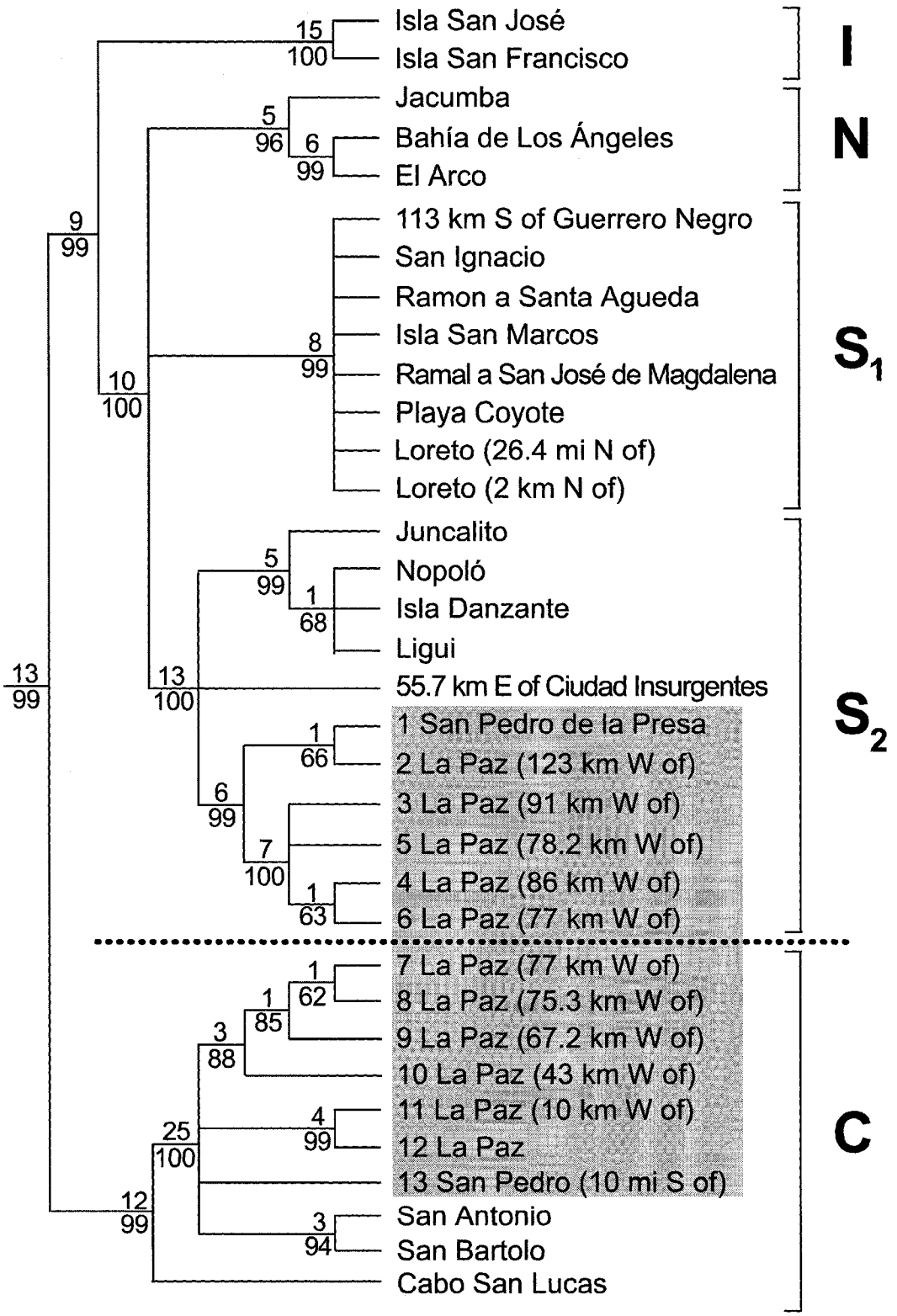
Results

Sequence variation and genealogical identity

Nucleotide sequence length varied due to indels that were chiefly located in the non-coding region between the cyt *b* and threonine tRNA genes. Gaps were inserted to accommodate the alignment of conserved regions, resulting in a total of 1094 aligned positions. Nevertheless, 16 characters (1048-1063) were excluded from further analysis due to uncertain alignment, limiting the data set to 1078 characters.

The sequences revealed unique haplotypes for all thirteen individuals. Genealogically, six and seven haplotypes grouped together with the northern and southern haplotype clade, respectively (Fig. 6.2). There was no geographical overlap between the two haplotype clades, although the two specimens collected 77 km west of

Figure 6.2. Genealogy of mitochondrial lineages in *Urosaurus nigricaudus*, emphasizing the Isthmus of La Paz contact zone. The tree is a strict consensus (308 MPTs, 1010 steps, CI = 0.539, RI = 0.825) of the combined data set spanning 1094 bp of a cytochrome *b* region, comprising the samples of Lindell *et al.* (in press) and the additional specimens sequenced for this study. Nodal support is represented by Bremer decay index (DI) and bootstrap (BS) values above and below the nodes, respectively. Vertical bars named C, I, N, S_1, and S_2 refer to the Cape Region, Isla San José and Isla San Francisco, northern Baja California (including southern California, mid-southern, and deep southern (excluding the Cape Region) clade, respectively, following Lindell *et al.* (in press). Horizontal dashed line indicates the Isthmus of La Paz break (contact zone) discussed in text. Shaded square depicts the 13 specimens used to assess mtDNA lineage identity and nucleotide variation in the contact zone (Table 6.1).


Isla San José
Isla San Francisco
Jacumba
Bahía de Los Ángeles
El Arco
113 km S of Guerrero Negro
San Ignacio
Ramon a Santa Agueda
Isla San Marcos
Ramal a San José de Magdalena
Playa Coyote
Loreto (26.4 mi N of)
Loreto (2 km N of)
Juncalito
Nopoló
Isla Danzante
Ligui
55.7 km E of Ciudad Insurgentes
1 San Pedro de la Presa
2 La Paz (123 km W of)
3 La Paz (91 km W of)
5 La Paz (78.2 km W of)
4 La Paz (86 km W of)
6 La Paz (77 km W of)
7 La Paz (77 km W of)
8 La Paz (75.3 km W of)
9 La Paz (67.2 km W of)
10 La Paz (43 km W of)
11 La Paz (10 km W of)
12 La Paz
13 San Pedro (10 mi S of)
San Antonio
San Bartolo
Cabo San Lucas
I
N
S1
S2
C

La Paz (specimens 6 and 7) revealed one northern and one southern mitochondrial haplotype.

Extensive nucleotide variation occurred between the two divergent haplotype groups, with 11.47% average sequence divergence. In contrast, sequence divergence within each haplotype group was low, averaging 1.36% and 1.48% for the northern and southern group, respectively. Sequences are deposited in GenBank (Table 6.1).

Haplotype identification

Amplification consistently produced clear, single products. In all cases, product length corresponded to amplification of the haplotype lineage originally identified through sequencing. All control PCRs yielded either a product band of the same quality as under lineage-selective conditions, or no product at all, depending on whether the matching lineage-selective primer was included or omitted in the reaction. Lineage-selective amplification yielded a single, clear product of anticipated lineage-diagnostic size for all the additional specimens (n = 120). (A detailed evaluation of the evolution and maintenance of the secondary contact zone will be published separately.)

Discussion

The deep genealogical break across the Isthmus of La Paz is congruent with genealogies of other species, including the little desert pocket mouse (*Chaetodipus arenarius*; Riddle *et al.* 2000), scorpions (*Centruroides exilicauda*; Gantenbein *et al.* 2001) and leaf-toed geckos (*Phyllodactylus*; Murphy *et al.* in preparation). These congruent mtDNA breaks suggest a common vicariant history, possibly resulting from a late Miocene seaway (Lindell *et al.* in press). Our results, combined with the available data on variation in allozyme loci for *U. nigricaudus* (Aguirre-León *et al.* 1999), provide an early insight into the genetic interactions between populations previously isolated by this seaway.

The mtDNA contact zone is sharp; there was no overlap of the divergent mtDNA lineages. While some lineage overlap is likely, the sampling distribution, with several specimens within a short distance, suggests that the width of the mtDNA contact zone is limited to a few kilometers. This is somewhat surprising, given the old age of the contact zone and possibility for female dispersal, and indicates that some form of selection is restricting the dispersal of mtDNA lineages. Such a pattern is compatible with the development of isolating mechanisms and overall population differentiation (Coyne & Orr 2004). However, the variation in allozymes strongly disagrees with this view, and suggests that population differentiation is effectively absent (Aguirre-León *et al.* 1999). Therefore, this contact zone represents a strong case of cytonuclear discordance with ongoing gene flow across a sharp mtDNA break. (Possible explanations for this are developed in Chapter 7.) A similar case of cytonuclear discordance has been found in the

mid-peninsular contact zone of the side-blotched lizard (*Uta stansburiana*; Lindell *et al.* unpublished data).

Additional studies on the genetic interactions of reunited populations are needed to address the evolution and maintenance of the old contact zones in Baja California. The simple mtDNA identification method described herein can provide rapid analysis of numerous samples from contact zones. Given the substantially less sequence variation within than between the two haplogroups in *U. nigricaudus*, mtDNA identification based on lineage-selective amplification is reliable, as suggested by the the consistently clean products for all the *U. nigricaudus* specimens evaluated (n = 133). With more accurate estimates of the location of contact zones of different species resulting from the same vicariance event, it will be possible to evaluate the movement of contact zones over time (Dasmahapatra *et al.* 2002; Secondi *et al.* 2003). Similarly, analysis of different contact zones within the same species will provide a temporal view on the evolution of cytonuclear discordance resulting from the vicariant history of Baja California.

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Chapter 7

Conclusions

Introduction

In this chapter I summarize the main findings of my doctoral research beyond the conclusions of each individual chapter. When evaluated in concert, the distinct studies of my thesis provide a better understanding of the genetic consequences of a complex geological history. The significance of this dissertation falls into three primary areas of research: historical biogeography, cytonuclear discordance, and the evolution of contact zones. I discuss my results in the context of current research, and suggest some opportunities for further investigations.

The historical biogeography of Baja California: an emerging picture

Scientific research involves a continuous cycle of hypothesis testing, as new data are evaluated in relation to current knowledge. Historical biogeography is no exception, and the history of Baja California is constantly being revised and refined. The genealogies of

mitochondrial lineages have recently provided great input to our understanding of this region, in particular with regards to the history of Baja California as a 'peninsular archipelago' (Riddle *et al.* 2000; Murphy & Aguirre-León 2002). No doubt, mitochondrial DNA (mtDNA) can be very useful in historical biogeography, given its nature to form hierarchical trees, which can be evaluated for common patterns among species. In this way, the discovery of congruent genealogical breaks midway on the peninsula, following the initial study on *Uta stansburiana* by Upton & Murphy (1997), quickly led to the widely accepted view of a mid-peninsular seaway of Pleistocene age. Accordingly, much focus in the historical biogeography of Baja California has been given to younger events of Plio-Quaternary times. However, such a time frame contrasts with the scenarios commonly debated in the geological community, which point to a deeper history. Can this apparent discrepancy be resolved, or is the genetic signal of older events simply lost in time?

To provide temporal resolution in historical biogeography, branching patterns among mtDNA lineages must be analyzed along with evidence from other fields. Upton & Murphy (1997) followed this approach and evaluate the genealogy of *Uta stansburiana* in relation to geological data on island formation, specifically the formation of Isla Ángel de la Guarda at 1 Mya (Moore 1973). Given the topology of the *Uta stansburiana* genealogy, Upton & Murphy suggested a mid-peninsular vicariance event at approximately 1 Mya. My research into the genealogical history of *Callisaurus draconoides* and *Urosaurus nigricaudus* in relation to other genealogies and new geological evidence refuted this younger date for the mid-peninsular seaway (Lindell *et al.* 2005, in press) and pointed to a significantly older event of late Miocene age. The

relative lack of evaluation of geological data in studies of genealogical history following Upton & Murphy (1997) may therefore explain some of the temporal discrepancy between the historical scenarios of biogeographers and geologists. For example, Douglas *et al.* (2006), in their assessment of the relative importance of vicariance and climate change in shaping genealogical history in rattlesnakes (*Crotalus* spp.), only included three primary references on geological data, two of which were severely outdated.

My work implies that mtDNA genealogies evaluated in concert with data from geology and related fields have an important role to play in the emerging view of the history of Baja California and the Gulf of California. In fact, genetics has the potential to guide research efforts in geology, by suggesting regions of particular interest (A. Carreño, personal communication.) Following the deep historical biogeography suggested by the genealogies of *C. draconoides* and *Urosaurus nigricaudus*, it now also appears possible to address older events associated with the early evolution of the Gulf of California (Carreño & Smith 2007). This may be feasible by reconstructing the phylogenetic relationships among species with a long history in the region, such as leaf-toed geckos (*Phyllodactylus* spp., Murphy *et al.* unpublished data), tree lizards (*Urosaurus* spp.), and organisms belonging to other classes. The deeper time frame suggested is also of importance to evolutionary research that is based on historical biogeography, such as the dynamics of contact zones, their geographic clustering (Remington 1968; Swenson & Howard 2005), and the effects of climate change on genetic history (Lindell *et al.* 2006; Swenson 2006).

Cytonuclear discordance: when maternal and biparental histories disagree

A striking result of my research is the disparity between the genetic differentiation in DNA representing maternal and biparental histories. While the deep divergences in mtDNA genealogies reflect a history of temporary vicariance along the peninsula of Baja California, variation in allozyme loci suggest very low levels of population differentiation across mtDNA breaks (Lindell *et al.* 2005, in press). Such cytonuclear discordance[4] has recently also been found in *Drosophila mojavensis* (Ross & Markow 2006), *D. pachea* (Pfeiler *et al.* 2007), and *Uta stansburiana* (Lindell *et al.* in preparation), based on data from faster evolving microsatellite DNA loci. The limited population differentiation in characters encoded in nuclear DNA suggests that gene flow is strong along the peninsula, a pattern that appears to be common (Murphy & Aguirre-León 2002).

How can the cytonuclear discordance along the peninsula be explained? In general, it appears that the duration of the temporary seaways was long enough to generate divergence among mtDNA lineages, yet insufficient to drive differentiation in the nuclear genome. This difference can be attributed to the faster rate of evolution and lower effective population size of mitochondria in comparison to nuclear DNA (Moore 1995). Nuclear gene flow later resumed when the populations were reunited, yet the divergences in mtDNA were left as a genetic trace of the temporary isolation events,

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[1] 'Cytonuclear discordance' describes a situation in which the evolutionary history resolved based on DNA from organelles in the cytoplasm (mitochondria or chloroplasts) differs from that of the nucleus. The term is often used when discussing geographic patterns of genetic variation.

because mitochondrial lineages do not recombine. Conversely, little, if any, isolating mechanisms developed in the nuclear genome between populations during allopatry. While it may be argued that nuclear DNA lineages have yet to reach reciprocal monophyly, this appears unlikely given the considerable age that has passed since the vicariance events and strong evidence of ongoing gene flow between reunited populations.

The cytonuclear discordance along the peninsula of Baja California implies that mtDNA divergence is a poor approximation of intraspecific population differentiation. Inferences about population differentiation must instead, at least partially, be based on biparentally inherited characters. Similarly, mtDNA genealogies may mislead decisions about species status. For example, should an insular population with divergent mtDNA and evidence of nuclear differentiation be disqualified as a distinct species, simply because there is gene flow between populations representing deeper genealogical structure on the mainland (cf. *Crotalus atrox*; Castoe *et al.* 2007)? Undoubtedly, discordance between mtDNA and nuclear DNA will continue to be a hotly debated topic of relevance to taxonomic practice (Funk & Omland 2003; Rubinoff & Holland 2005).

The evolution and maintenance of contact zones

Contact zones have long been hailed as 'natural laboratories for evolutionary studies' (Hewitt 1988), or 'windows on evolutionary process' (Harrison 1990), providing a valuable setting for studies on selection, adaptation, reproductive isolation, and

speciation. The analysis of the genetic interactions among reunited populations in secondary contact zones[5] also provides further insights into the discordance between mitochondrial and nuclear DNA.

Given the history of cryptic vicariance of Baja California, numerous species are characterized by secondary contact zones where highly divergent mitochondrial lineages meet. Because these contact zones are unusually old, they can help us understand the evolution and maintenance of contact zones over long time periods. Results from *Uta stansburiana* (mid-peninsula) and *Urosaurus nigricaudus* (Isthmus of La Paz) suggest that these contact zones are very narrow with exceptionally strong cytonuclear discordance (Lindell & Murphy in press; Lindell *et al.* in press). In fact, data based on extensive sampling across the contact zones indicate that mtDNA lineage overlap is restricted to only a few kilometers, while population differentiation is effectively absent according to analyses of microsatellite DNA and allozyme loci (Lindell *et al.* unpublished data).

Discordance between mtDNA and other characters in contact zones is often explained by the peculiar inheritance of mtDNA, meaning it is less closely linked, on average, with nuclear genes than nuclear genes are with each other (Barton & Jones

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[5] Broadly speaking, 'contact zone' and 'hybrid zone' are two terms that are commonly used to describe a geographic area where two populations, which show some level of difference based on one or more heritable characters, meet. The term 'contact zone' tends to be favored when it is clear that the interacting entities are in secondary contact, following a temporary isolation event. Given the cryptic vicariant history of Baja California, I favour the use of 'contact zone' in this thesis.

1983; Barton & Hewitt 1985). This often leads to increased introgression of mtDNA (Chan & Levin 2005). The contact zones of Baja California therefore represent a contrasting case with very restricted mtDNA introgression coupled with ongoing gene flow and lack of reproductive isolation. I discuss some hypotheses for the evolution of this type of discordance below.

Sex-biased dispersal can lead to restricted movement of mtDNA lineages in relation to nuclear gene flow, if female dispersal is limited in comparison to males (Melnick & Hoelzer 1992). In *Uta stansburiana*, females disperse less than males (Doughty *et al.* 1994), although the difference is not large (average dispersal distance from hatching site to adult home range is 53 and 84 feet for females and males, respectively; Tinkle 1967). However, even if females disperse less than males, such a disparity alone cannot explain the limited overlap of mitochondrial lineages in the contact zones. Given that the mid-peninsular contact zone in *Uta stansburiana* formed millions of years ago, and that this species has a short generation time (9.5 months; Turner *et al.* 1970), millions of generations of female lizards are expected to have caused considerable overlap of the divergent mitochondrial lineages. A similar scenario can be argued for the old contact zone across the Isthmus of La Paz in *Urosaurus nigricaudus*. This suggests that some other factor is selecting against mtDNA lineage dispersal.

Cytonuclear discordance may result from Haldane's rule, which postulates that hybrids of the heterogametic sex are more likely to be inviable or sterile than the homogametic sex (Haldane 1922; Orr 1997). However, as available evidence suggests that males are heterogametic in phrynosomatid lizards (Pennock *et al.* 1969; Sites *et al.*

1992), Haldane's rule can not explain the restricted dispersal of maternal lineages in these lizards.

While mtDNA was initially held as a neutral marker, it is now clear that various types of selection operates on mtDNA (Rand 2001). For example, in recent years numerous studies have investigated the coevolution between the mitochondrial and nuclear genomes (Blier *et al.* 2001; Rand *et al.* 2004). Polypeptides encoded by mitochondrial and nuclear genes form large enzyme complexes in the membranes of mitochondria, which are responsible for cellular respiration. This metabolic process is the main source of energy production in eukaryotic cells. Accordingly, selection for coevolution of mitochondrial and nuclear genes ('cytonuclear coevolution') is strong, and the disruption of coadapted gene complexes can have severe effects on hybrid fitness (Ellison & Burton 2006; Harrison & Burton 2006). A mitochondrial lineage dispersing across a contact zone may therefore be selected against if it encounters nuclear genes that are coadapted to a very divergent mtDNA type. This implies that nuclear genes under selection for cytonuclear coevolution also show restricted introgression across the contact zone, despite general flow of nuclear DNA. Such selective gene flow has been documented in other contact zones (Bengtsson 1985; Martinsen *et al.* 2001). Cytonuclear coevolution therefore has the potential for affecting the evolution and maintenance of contact zones following initial divergence of mitochondrial lineages (Dakin & Asmussen 2005; Hofman & Szymura 2007). The results of my doctoral research on phrynosomatid lizards in Baja California provide an opportunity to test this and other hypotheses on the evolution and maintenance of contact zones.

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