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LOMA LINDA UNIVERSITY  
School of Science and Technology  
in conjunction with the  
Faculty of Graduate Studies

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Phylogeography and Conservation of the Arroyo Toad (*Bufo californicus*)

by

Robert Edward Lovich

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A Dissertation submitted in partial satisfaction of  
the requirements for the degree of  
Doctor of Philosophy in Biology

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March 2009

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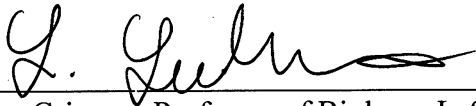
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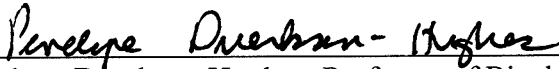
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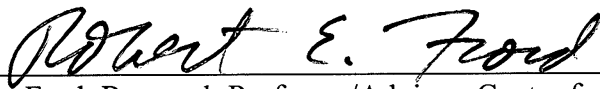


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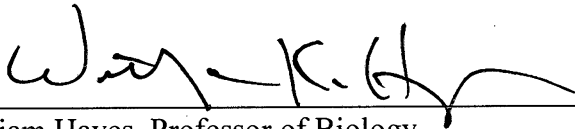
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## ABSTRACT OF THE DISSERTATION

Phylogeography and Conservation of the Arroyo Toad (*Bufo californicus*)

by

Robert Edward Lovich

Doctor of Philosophy, Graduate Program in Biology

Loma Linda University, March 2009

Dr. Ron Carter, Chairperson

The Arroyo Toad (*Bufo californicus*) is an endangered species found in rivers of southern California, USA and northern Baja California, México. It has experienced population declines throughout its range, as a result of urbanization, hydrologic alterations, and overall habitat loss. As part of the Recovery Plan for this species, it was determined that more research was needed to assist in management decisions. One of those research needs was to better understand the intraspecific genetic variation within the Arroyo Toad. I conducted a rangewide genetic study of the Arroyo Toad to uncover lineages and genetic variation among and between all major watersheds. This information is essential to our understanding of the species by providing data to determine recovery units independent of geographic location, delimit metapopulations, identify closely related populations for the potential augmentation of declining or extinct populations, assess dispersal between watersheds, and quantify genetic diversity throughout its distribution. In the course of this project, I also identified a more precise range of the species in Baja California, México, along with the issues and challenges facing its conservation in the poorly known southern portion of its range.

## CHAPTER ONE

### INTRODUCTION

#### **Arroyo Toad**

##### Biology and Ecology

The Arroyo Toad, *Bufo californicus*, inhabits rivers and streams of coastal southern California, from Monterey County southward into northern Baja California México (Figs. 1 and 2). It occurs primarily in second- to fifth-order low-gradient streams with sandy alluvial substrate and shallow water pools (Sweet, 1992, 1993; Sweet and Sullivan, 2005). Its specialized requirements reveal a patchy and poorly understood distribution that is limited by urban development and other anthropogenic modifications to river corridors throughout its range. Generally, it inhabits sandy streambeds overgrown to *Populus fremontii*, *Platanus racemosa* or *Salix spp.* These streams typically have adjacent pools where the toads may sit in the shallow water while partially exposed.

Several life history characteristics of *B. californicus* make it unique among most bufonids: eggs are laid at calling sites of males; larvae are cryptically colored, non-toxic, and solitary; larvae feed on interstitial organic material; and juvenile dispersal occurs only after the wet season ends (Sweet, 1992, 1993, Sweet and Sullivan 2005). Eggs are laid from March to July, dependent upon sufficient weather conditions and annual

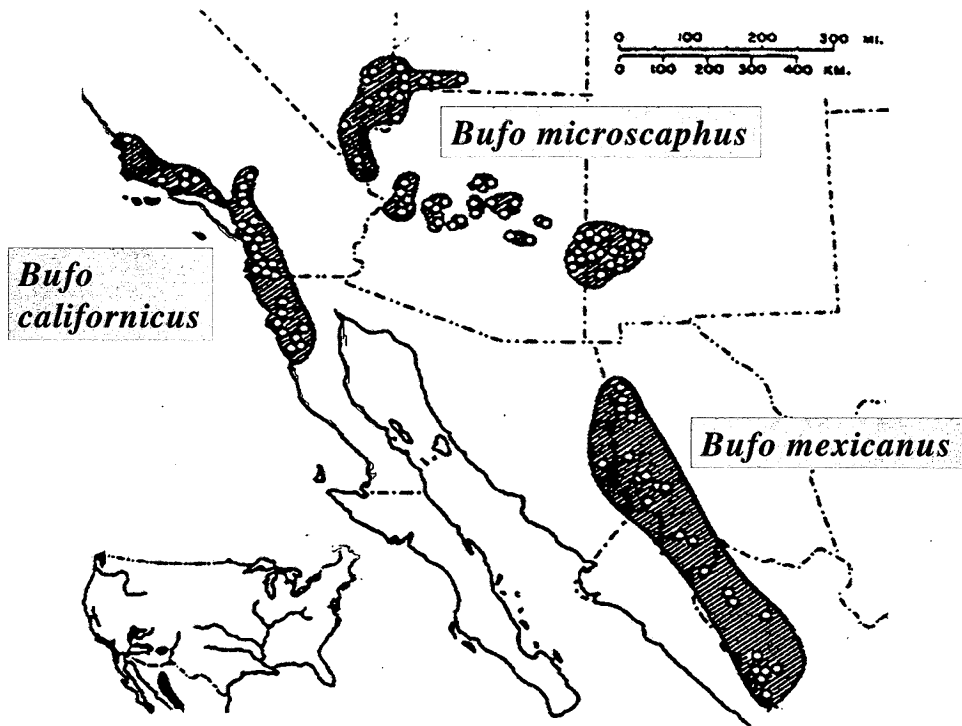


Figure 1. Distribution map of the three species of the “*microscaphus*” complex, all of which were formerly subspecies of *Bufo microscaphus* (sensu Price and Sullivan 1988).

rainfall. The breeding season may be extended in exceptionally wet years. Eggs are small, dark, and laid in strings along the edges of pools with mild current velocities.

Larvae develop over an extended period of 65–85 days. The lengthy larval period makes them extremely susceptible to mortality during this time (Sweet, 1992, 1993, Sweet and Sullivan 2005).



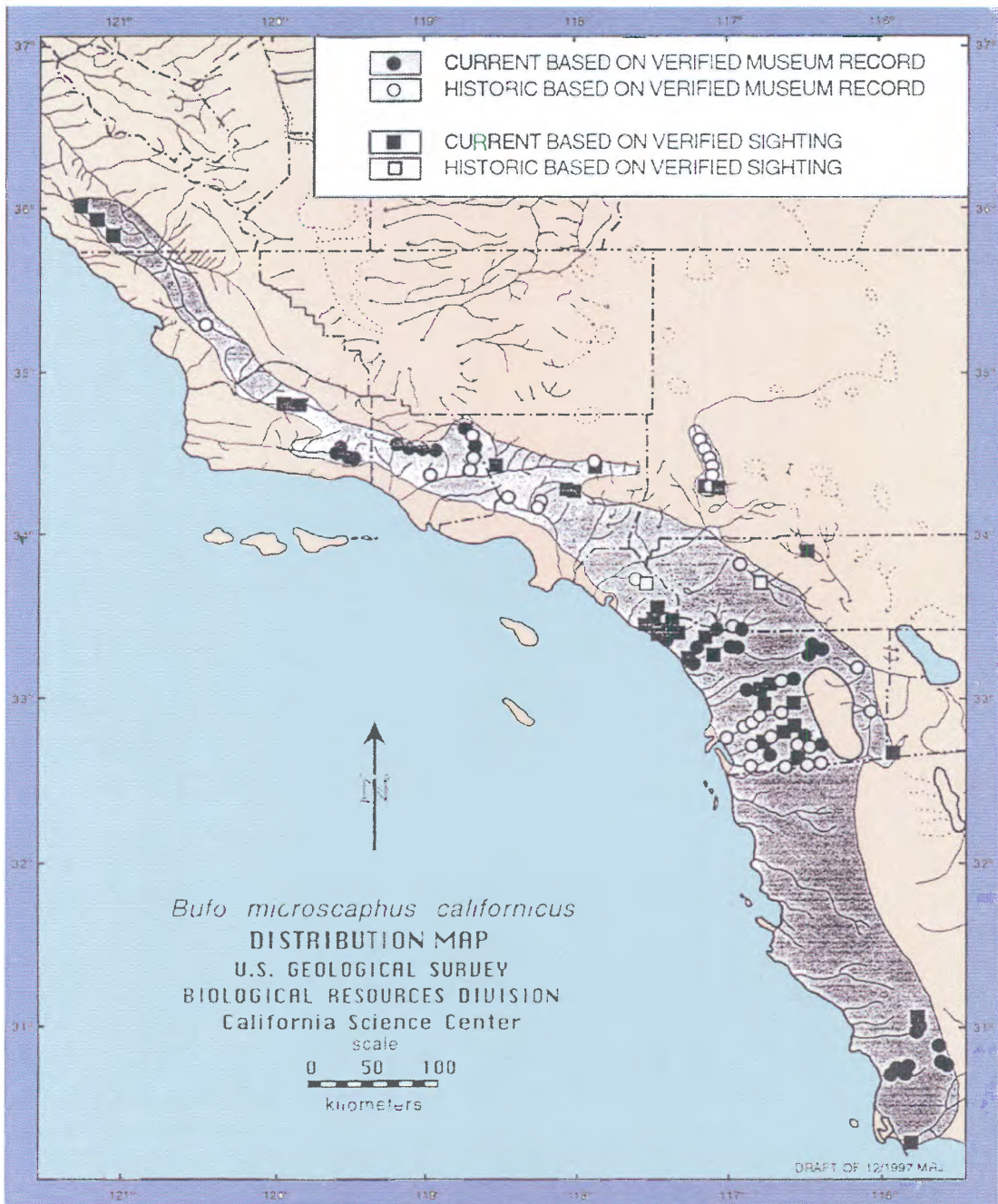


Figure 2. Approximate range map for *Bufo californicus* as depicted in USFWS (1999). Note the gap in distribution depicted for Baja California, México. Many rivers in northern Baja California are now known to be occupied by *B. californicus*.



Figure 3. *Bufo californicus* from Rio Santo Domingo, Baja California, México. Photo by Robert Lovich.

environmental conditions. Females search for mates only when carrying eggs and they do this by moving along pool margins to seek calling males. Calling starts approximately one hour after sunset and may continue for several hours. Egg-laying is generally in or near the site where the “suitor” was first calling.

The specialized life history of *B. californicus* requires the presence of certain habitat characteristics such as low stream gradient, coarse material, sandy refugia and low disturbance potential. They are found within riparian zones where deposition of coarse sediments is stored and streams are seasonally intermittent (Sweet, 1992, 1993, Sweet and Sullivan 2005).

*Bufo californicus* is relatively small (5–8 cm snout-vent length), olive green or gray to light brown in coloration, with dark spots and small warts (Fig. 3). A prominent white “v-shaped” stripe crosses the top of the head between the eyes. The ventral portion is buff-white and often lacks spots. Its call is a long trill lasting 4–10 seconds. Locomotion is generally in the form of hopping (Sweet, 1992, 1993, Sweet and Sullivan 2005).

*Bufo californicus* tadpoles are uniformly black in coloration from hatching to Gosner stage 26 (10-12 mm total length; Sweet 1992). By Gosner stage 30, the dorsum becomes tan with dark crossbars on the tail base, an irregular black stripe on the tail, and a white venter. *B. californicus* larvae distribute themselves evenly throughout the pools they inhabit, relying on cryptic coloration to avoid predation, and foraging on detritus and microbial mats (Sweet, 1992)

After metamorphosis, juvenile Arroyo Toads forage diurnally (Sweet, 1992, 1993, Sweet and Sullivan 2005). When they become developed enough to dig burrows, they conceal themselves by day and forage nocturnally in terrestrial habitats as stream water levels recede. Burrows are dug in sandy areas that are often highly localized and contain high toad densities. Shade and moisture are a preferred component of burrowing locations as well. Adult toads typically move farther inland to burrow. They bury themselves 5–10 cm down in the interface between dry and damp sand. Not only do they burrow as part of a daily cycle, with most activity occurring in nocturnal fashion, but they burrow seasonally to over-winter as well.

Dispersal from natal areas occurs the year after birth. On average, sexual maturity is reached in one year by males and in two years by females, depending upon



Figure 3. *Bufo californicus* from Rio Santo Domingo, Baja California, México. Photo by Robert Lovich.

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## Distribution

*Bufo californicus* inhabits cismontane rivers and streams draining to the Pacific Ocean along the west coast of North America, from Monterey County, California (Salinas River), to Northern Baja California, México (Rio Santa Maria; Gergus et al., 1997a), with transmontane populations inhabiting a few rivers that drain to the Mojave Desert from the north side of the Transverse Ranges (i.e., San Bernardino and San Gabriel Mountains) of Southern California (Price and Sullivan, 1988; Grismer 2002; Mahrtdt et al. 2003, 2003, 2004; Mahrtdt and Lovich, 2004; Lovich et al., in press). A few populations were reported from the Sonoran Desert drainages of Riverside and San Diego Counties (Patten and Myers 1992, USFWS 1994), but those records proved to be misidentifications of *B. boreas* and *B. punctatus* by museum specimen analysis and focused surveys of reported localities (Ervin et al. in prep., pers. obs.). Recent work on this species (Mahrtdt et al. 2003, 2003, 2004; Mahrtdt and Lovich, 2004; Lovich et al., in press) indicates that many of the pre-existing maps and detailed descriptions of the distribution of *B. californicus* are in need of revision. Northern and southern localities are frequently missed or incorrectly referenced, and circumscribed ranges include broad areas not occupied by *B. californicus*. Given the fact that *B. californicus* is an endangered species in the United States, management and conservation efforts would best be directed by a detailed and accurate understanding of the range of the species.

## Historic Versus Present Status

Historically, this species was documented as being abundant in areas that it inhabited (Sanders, 1950; Stebbins, 1951; and Cunningham, 1962). Since historic times, change has been the only constant with *Bufo californicus*. Numbers for this species have

declined from historic observations. Jennings and Hayes (1994) are most commonly cited as documenting a decline of 76% of the populations throughout the range of the species. Virtually no riparian habitats within the range of *Bufo californicus* are without some form of anthropogenic modification within the last 100 years. For instance, changes to the hydrology of rivers resulting from urbanization of the Los Angeles area has completely eliminated the species from any localities between the uppermost reaches of the Los Angeles River and the Santa Ana River far to the south (Fig. 2). The same can be said for the Tijuana River, which, like the rivers of Los Angeles, has seen significant alterations, channelization, concreted stretches of river, invasion by non-native species, and widespread pollution (Pers. Obs.).

Evidence indicates that *Bufo californicus* has declined throughout much of its range, both in numbers and in area occupied (Jennings and Hayes 1994, Sweet and Sullivan 2005, Sweet 1992, 1993). Following declines through the 1990's, several new populations have been found as a result of increased search efforts, leading to a belief that the numbers and range of the species may rebounding from their previous lows (Clayton 2006, and Sweet and Sullivan 2005). In Baja California, surveys have identified several newly recognized populations and the first records of the species in the Rio Las Palmas, Rio El Zorillo, Rio Santo Tomas (Mahrtdt et al. 2002, 2003, Mahrtdt and Lovich 2004, Lovich et al. Ch. 4).

## Listing and Protections

### United States

As a result of the documented declines that occurred, *Bufo californicus* was listed as endangered in the United States under the Endangered Species Act (ESA) in 1994 (USFWS 1994). The Species is also endangered under the California Endangered Species Act (CESA) (California Fish and Game Code §2050). The Global Amphibian Assessment lists the species as endangered (IUCN, Conservation International, and NatureServe 2008).

### México

In contrast to the United States, México has no protections for this species under the Norma Oficial Mexicana (NOM-059-ECOL-2001, Poder Ejecutivo Federal. 2002), or any other Mexican laws or regulations. The only protections that *Bufo californicus* receives are those granted to populations that occur within the lands of the Parque Nacional Sierra San Pedro Mártir. All other populations are vulnerable to land use and urbanization throughout their range in northern Baja California. Only one other National Park, the Parque Nacional Ciudad Constitución, occurs in northern Baja California, and *Bufo californicus* has not been documented within its borders, although it is found downstream of rivers whose headwaters are within the Parque (Lovich et al. Ch. 4).

## Prior Genetic Analyses

### Allozyme Variation

Gergus (1994, 1998) looked at relationships among members of the *Bufo microscaphus* complex using allozymes. His work showed support for recognition of *Bufo microscaphus californicus* as a full species, and also elevated to full species *Bufo m. microscaphus* and *Bufo m. mexicanus*. No studies prior to Gergus (1994, 1998) had used genetic information to identify the relationships among members of the “*microscaphus*” complex. This research clarified relationships among the recognized subspecies of *Bufo microscaphus*, following a relatively confusing taxonomic history for the species in the complex (see Sweet and Sullivan 2005, Price and Sullivan 1988). Only three specimens of *Bufo californicus* were used in Gergus’ study, all from widely dispersed locations. Gergus’ work showed that *Bufo californicus* had the most polymorphic loci of the three “*microscaphus*” members evaluated.

### Microsatellite Variation

Shanahan (1999) studied microsatellite variation in *Bufo californicus*, analyzing 81 individuals from several tributaries of three rivers that flow across Marine Corps Base Camp Pendleton, including the San Mateo, San Onofre, and Santa Margarita Rivers, as well as five individuals from Lake Castaic in Los Angeles County. Findings of Shanahan’s analysis of nine microsatellite loci indicated high levels of variation in *Bufo californicus*. All nine loci analyzed were highly polymorphic, displaying 10 – 19 alleles. Twenty-four alleles were discovered, with six being unique to Lake Castaic. Samples



between Camp Pendleton's Rivers and those at Lake Castaic were significantly different, as were samples from each of the three river watersheds sampled at Camp Pendleton.

### MtDNA Sequence Variation

Pauly et al. (2004) sequenced 2,500 base pairs of mtDNA from the 12s and 16s genes for 56 species of toads to determine the historical biogeography of Nearctic toads. A single individual of *Bufo californicus* was sequenced as part of this study, from Kitchen Creek in the Tijuana River system of the United States. Results of this study indicated that *Bufo californicus* is most closely related to, but distinct from, the Arizona Toad (*Bufo microscaphus*). *Bufo californicus* was nested within recognized Nearctic toads, and evidence supports the idea of a single colonization of *Bufo* from the Neotropics.

### Rangewide Variation

Besides genetic variation mentioned above, other aspects of *Bufo californicus* have been documented as variable. *Bufo californicus* inhabits a relatively wide latitude that includes several different habitat types. It has been observed that timing of breeding appears to vary at different elevations and/or latitudes with respect to temperature and rainfall (= breeding pools). Typically, *Bufo californicus* breeds and its young develop between February and July of a given year (Sweet 1992, Sweet and Sullivan 2005), but Welsh (1985) found juvenile *Bufo californicus* at several high elevation locations ( $\leq$  8,200 feet) in the Sierra San Pedro Mártir as late as July and August. Cunningham (1962) noted breeding later in the year at higher elevations, where thermal and metabolic

requirements of ectothermic *B. californicus* necessitate a strategy to breed later in the year when temperatures are warmer.

Call variation has been documented within and among populations of *Bufo californicus*, and between it and *Bufo microscaphus* and *Bufo mexicanus* (Sullivan 1992, Gergus et al. 1997b). The magnitude of variation observed in measured call duration, dominant frequency, and pulse rate among *B. californicus* was similar to that found between species in the “*microscaphus*” complex (Gergus et al. 1997b). This has important implications as Gergus et al. (1997b) showed support for species boundaries being influenced by call variation. Since *B. californicus* has wide variation in the call variables measured, there is further need for research to determine whether or not variation in calls is an indicator of, or matches with lineages within *B. californicus*.

### **Approach of Studies**

The approach of this study was to determine the intraspecific relationships of *Bufo californicus* using sequence data from ND1 and Dloop regions of mitochondrial DNA. Samples taken from throughout the range of the species in both the United States and México were analyzed using multiple techniques and statistical comparisons to generate gene trees from which relationships could be elucidated.

### **Significance of Study**

This study is significant because it is the first to analyze range-wide molecular variation in *Bufo californicus*. It also represents an important contribution to the recovery of the species under the Recovery Plan (USFWS 1999). Results of this study provide an independent interpretation of the recovery units described within the Recovery Plan

(USFWS 1999). In the Recovery Plan, the recovery units were defined as “geographically proximal populations separated from other units by distributional gaps with an unspecified distance.” Results of this analysis are used to test the boundaries of the recovery units and their population make-up. This information provides valuable insight into whether these recovery units should be managed as independent units or treated as an interconnected system. It also provides insight into the use of traditional conservation planning techniques, with and without incorporation of molecular genetic information.

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## CHAPTER TWO

### PHYLOGEOGRAPHY OF THE ARROYO TOAD (*Bufo californicus*)

#### **Abstract**

The Arroyo Toad, *Bufo californicus*, is an endangered bufonid found in rivers draining to the Pacific Ocean in California, USA, and Baja California, México, as well as a few drainages flowing into the Mojave Desert. This study analyzed mitochondrial DNA sequence variation across the range of the species. The ND1 gene and Dloop regions of mtDNA were sequenced (859 and 451 bp, respectively), and aligned sequences were analyzed using maximum likelihood, maximum parsimony, and Bayesian inference. Despite the conservation status of *B. californicus*, a phylogeny had never before been constructed for this species. Herein, DNA sequence data are compared across its range to determine gene relationships and infer evolution. Two well supported clades are resolved for northern and southern populations, excepting a few northern haplotypes being identified within the range of the southern group. Phylogeography, congruence with other regional studies, and biogeographic comparisons are explored, and provide further evidence for the complex biodiversity of the southern California and northern Baja California region. These findings are important to better understand relationships among extant populations of the endangered Arroyo Toad.

## Introduction

The Arroyo Toad (*Bufo californicus*) is one of three closely related species, formerly designated as subspecies of one another in the “*microscaphus*” complex (Gergus 1998, Fig. 1); the other two members of the “*microscaphus*” complex are *Bufo microscaphus* and *Bufo mexicanus*. These three allopatric species inhabit alluvial stretches of rivers in northern México and the southwestern United States (Price and Sullivan 1988). The Arroyo Toad (*Bufo californicus*) is a small to medium-size (5-8 cm snout vent length) burrowing toad primarily distributed in cismontane riparian habitats from Monterey County, California, USA southward to Rio Santa Maria near San Quintin in northwestern Baja California, México (Grismer 2002). A few populations also occur in transmontane desert drainages of the San Gabriel and San Bernardino Mountains of California, USA. Since the original description of *B. californicus* (Camp 1915), aspects of the biology of populations in the United States have received considerable attention (Myers 1930; Miller and Miller 1936; Cunningham 1962; Price and Sullivan 1988; Sweet 1991, 1993; Gergus 1998). *Bufo californicus* was once a relatively common toad in coastal central and southern California but has since been extirpated from approximately 76% of its historic range (Sweet 1991, Jennings and Hayes 1994). The causes for such severe declines in *Bufo californicus* populations are summarized by Jennings and Hayes (1994) and include alteration of streamside habitats, hydrologic change, off-road vehicle activity, urban development, sand mining, cattle grazing, and introduction of non-native species. It generally inhabits riparian and oak woodland habitats in the north to maritime succulent scrub in the south. Disjunct populations occur near headwaters and streams of the Sierra de Juárez and Sierra San Pedro Mártir. Given the well documented and global



decline of *Bufo californicus*, as well as other amphibians (Pechmann and Wilbur 1994, Green 1997, Pechmann and Wake 1997, Duellman 1999, Vredenburg and Wake 2007), specific information on current status and distribution is needed to effectively conserve and manage *B. californicus* populations. A gap in information regarding genetic relationships among populations of *Bufo californicus* existed prior to this study.

## Materials and Methods

### Sampling

The sampling design used in this study centers on representation of major watersheds from throughout the distribution of *Bufo californicus*. Twenty four samples from throughout the range of the species were analyzed (Appendix I). Tissue sampling represents the major watersheds, and many of the currently known populations of *Bufo californicus* (Figure 4). Sampling allows for analysis of intraspecific variation. Several samples were gathered from many of the collecting localities, but given the rarity of the species there were locations where only one sample was available. To avoid problems associated with heteroplasmy, genetic data from more than one individual was generated.

Different collection methods were employed per permissions given via permits that authorized collection of *Bufo californicus*. In the United States where *Bufo californicus* is endangered, most tissue samples were removed from the animal by clipping a toe between the first and second phalanges. Tissue samples were stored in ethanol and frozen as soon as possible, usually within hours of collection. In addition to tissue sampling, specimens collected in the field were measured, weighed, and photographed. Some samples were the result of road-kill collection, or tadpole collection,

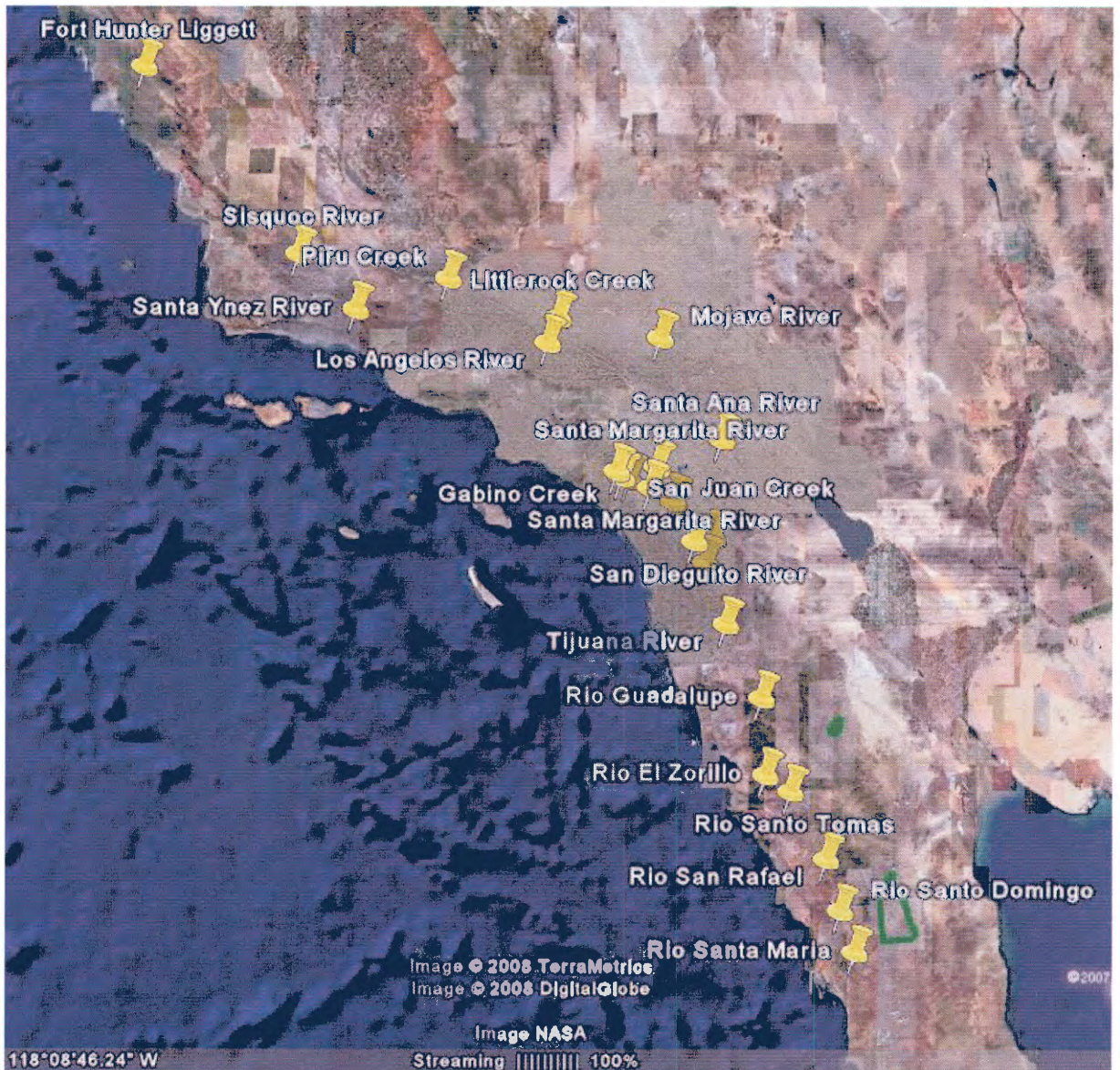


Figure 4. Map showing collecting localities for samples used in this study.

in both cases individuals were vouchered as dead material. Specimens taken from Baja California, Mexico were taken using the above-described methods, or vouchered as whole specimens with tissues taken during the vouchering process and according to accepted protocols outlined in Altig (1980).

Because the relationships among the toads within the “*americanus*” group are unresolved (Gergus 1994, 1998), outgroups were selected to best meet the criteria outlined in Maddison et al. (1984) and Watrous and Wheeler (1981). Therefore, I am using *Bufo woodhousii* from Salton City, CA, and *Bufo microscaphus* from two localities on the Upper Colorado River in UT, and *Bufo microscaphus* from Yavapai County, AR.

In the last two decades, the use of mitochondrial DNA sequences to analyze intra- and interspecific relationships (Avice 1987, Avice et al., 1987; Hillis et al., 1996a), has matured consummate with the technological advances allowing generation and analysis of large amounts of data (Hillis 1996). Consequently, it is currently possible to undertake complex phylogenetic studies, using thousands of base pairs of nucleotide sequence data, with reasonable hope of recovering genetic relationships from populations or samples with a high degree of accuracy (Hillis 1996, Huelsenbeck and Rannala 1997, Rannala et al. 1998, Soltis et al., 1998). Despite the advances in molecular systematics and the theoretical issues concerning the use of phylogenetic methods at or below the species level, a number of issues are still being considered. Hudson (1990) emphasized the importance of distinguishing between a pedigree, where diploid individuals are traced historically and the number of ancestors grows as one proceeds back through time, and a gene tree, where the absence of recombination results in a divergent, branching history. The use of non-recombining replicators (= haplotypes) is analogous to looking at species lineages in a phylogeny. Therefore, when constructing hypotheses of relationships from clonally inherited sequences of mitochondrial DNA (= gene trees), it is generally understood that individual organisms (or their haplotypes) are used as terminals. In

comparison, species (= lineages) are used as terminals when reconstructing a phylogeny (Davis and Nixon 1992, DeSalle and Vogler 1994).

### Laboratory Protocols

This study utilized mitochondrial genes from Dloop and ND1 for phylogenetic analysis. Numerous regions have been analyzed via PCR and/or subsequent sequencing, and due to homogeneity of mtDNA across the range of the species, variation is low (~1%) even in those sequences that have been found to have variation. Table 1 shows the primers used in the study. Preliminary analyses using several geographically-disparate samples showed ND1 and Dloop as gene regions of comparatively high genetic variation. For these two regions, sequence data has been generated using PCR and DNA sequencing techniques for 24 (ND1) and 21 (Dloop) samples from major rivers/watersheds occupied by *Bufo californicus* (Table 2).

Table 1. Sequences of primers used in the PCR and sequencing reactions.

Name	Gene	Sequence	Reference
BW16s	ND1	5'-ATT TTT TCT AGT ACG AAA GGA C-3'	Masta et al. (2002)
B-IIIE	ND1	5'-GCA CGT TTC CAT GAA ATT GGT GG-3'	Masta et al. (2002)
DloopH	Dloop	5'-GTC CAT AGA TTC AST TCC GTC AG-3'	Greg Pauly (UT Austin)
DloopJ	Dloop	5'-CTA ACG TTT CAC GAAGAT GGA A-3'	Greg Pauly (UT Austin)

Table 2. Specimens sequenced for ND1, Dloop, and combined ND1 and Dloop analyses.

<b>Sequenced Specimens</b>		
<b>ND1</b>	<b>DLOOP</b>	<b>COMBINED</b>
BuBo_DS053		
BuBo_DS077		
BuMi_AG75	BuMi3	BuMiAG75C
BuMi_TC9	BuMi2	BuMiTC9C
BuMi_LP1	BuMi	BuMiLP1C
BuWo1	BuWo1	BuWo1C
DS002	DS002	DS002C
DS004	DS004	DS004C
DS008	DS008	DS008C
DS019	DS019	DS019C
DS025	DS025	DS019C
DS028	DS028	DS028C
DS029	DS029	DS029C
DS031	DS031	DS031C
DS032	DS032	DS032C
DS035		
DS041	DS041	DS041C
DS049	DS049	DS049C
DS062	DS062	DS062C
DS063	DS063	DS063C
DS064	DS064	DS064C
DS066	DS066	DS066C
DS067	DS067	DS067C
DS070	DS070	DS070C
DS074	DS074	DS074C
DS078	DS078	DS078C
DS087	DS087	DS087C
DS089	DS089	DS089C
DS094		
DS104		

Mitochondrial DNA sequence data were collected using techniques outlined in Hillis et al. (1996*b*) and Palumbi (1996*b*). Thermocycler conditions are listed in Table 3. Gene fragments were prepared with molecular equipment at Loma Linda University. All

sequences were generated with an Applied Biosystems, Inc., Fluorescent Automated Sequencer 377 located at the DNA Sequencing Facilities at California State University Northridge.

Table 3. PCR conditions used.

Primer Combination	Gene	Thermocycler Conditions
DloopH and DloopJ	Dloop	120s(94°C); 30s(94°C), 40s(48°C), 90s(72°C) for 35 cycles; 420s(72°C)
BW16s and B-IIIE	ND1	120s(94°C); 30s(94°C), 40s(48°C), 90s(72°C) for 35 cycles; 420s(72°C)

### Tree Reconstruction

The computer program Sequencher 3.0 (Gene Codes Corporation 1995) was used to verify peak calls and align sequences. Pairwise alignments were first assembled for all ingroup samples and then combined with the alignment of assembled outgroup taxa. The ND1 mtDNA sequences were aligned using information on codon structure, and compared with samples from other studies found in GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>). The Dloop region of mtDNA is noncoding, and thus sequence data could not be verified for codon structure. Alignment of this region of mtDNA could only be verified by comparison with other published sequences found in GenBank.

Aligned sequences were exported from Sequencher 3.0 as a NEXUS file, formatted for phylogenetic analysis using MacClade 3.01 (Maddison and Maddison 1992), and executed in PAUP\* (version 4.0b2a; Swofford 1999). Formatting in

MacClade 3.01 includes codon position assignment. In PAUP\*, base-pair composition, pair-wise variation, codon position variation, and total variation were calculated.

All phylogenetic analyses were performed using PAUP\* (version 4.0b10; Swofford 1999). The combined DNA sequence data set was analyzed using unweighted (= equally) parsimony analysis on only phylogenetically informative characters. Shortest trees were sought using heuristic searches with 50 random addition sequence replicates per search and tree bisection-reconnection (TBR) branch swapping. Homoplasy levels (e.g., character incongruence) were evaluated with the consistency index (Kluge and Farris 1969) and retention index (Farris 1989, 1990). Because the consistency index is affected by uninformative characters, these were excluded from the calculations. Support for individual clades was evaluated using non-parametric bootstrapping (Felsenstein 1985), using 5,000 pseudoreplicates per analysis with five random addition sequences per pseudoreplicate. The proposed cut-off for “strongly supported” is a bootstrap value of approximately 70% or higher (based on Hillis and Bull 1993; but see their caveats). Gaps in DNA sequences are treated as an alternate character state in parsimony analyses rather than as missing data, assuming that insertions and deletions also represent evolutionary changes. Thus, no gaps were included.

Maximum likelihood was used to compare the relative likelihoods of trees from the parsimony analyses, to compare the goodness-of-fit of different models of sequence evolution to the observed data and to search for optimal likelihood trees. Due to the limitations of large number of samples and computer software limitations, the likelihood analysis was facilitated by reducing samples that are identical to a single known representative.

Shortest trees were generated with an unweighted parsimony analysis, one of which was used to analyze six nested models of increasing complexity (loosely following Huelsenbeck and Crandall 1997, Sullivan et al. 1997, Wiens and Hollingsworth 2000): (1) Jukes-Cantor (JC; Jukes and Cantor, 1969; assuming equal rates of change for transitions and transversions and equal base frequencies), with no invariable sites, and no among-site rate variation; (2) Kimura two parameter (K2P; Kimura 1980; assuming different rates of change for transitions and transversions and equal base frequencies), with no invariable site or among-site variation; (3) Hasegawa-Kishino-Yano (HKY85; Hasegawa et al., 1985); different rates for transitions and transversions and unequal base frequencies) with no invariable sites or among-site rate variation; (4) HKY85 with some sites assumed to be invariable but equal rates of change assumed at variable sites (=HKY85 + I; Hasegawa et al., 1985); (5) HKY85 with some sites assumed to be invariable and variable sites assumed to follow a gamma distribution (=HKY85 + I +  $\Gamma$ ; Gu et al., 1995); and (6) general time reversible (GTR; Yang, 1994; assuming a different rate for all six classes of substitutions), with some sites assumed to be invariable, and variable sites assumed to follow a gamma distribution (= GTR + I +  $\Gamma$ ). Specific model parameters for likelihood analyses were estimated from the data using PAUP\* (e.g., base frequencies, transition-transversion ratios, proportion of invariable sites, gamma distribution shape parameters). Using maximum likelihood, the goodness-of-fit of different models to the observed data was evaluated by comparing likelihoods for different models for the same tree. Statistical significance of differences of the models were evaluated using the likelihood-ratio test statistic  $-2\log\Lambda$  (the difference between the negative log likelihoods for the two models, multiplied by two), which should



approximate a chi-square distribution, with the degrees of freedom equal to the differences in the number of parameters between the two models (Yang et al. 1995). A sequential Bonferroni correction was employed because multiple tests were performed (Rice 1989). The best-fitting model was used in a heuristic search to find the overall best likelihood topology.

All Bayesian phylogenetic analyses were conducted using MrBayes v. 3.1.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). These consisted of paired runs of 4 Markov Chain Monte Carlo analyses each, using default settings and iterated for  $6 \times 10^6$  generations sampled every 500 generations. The stationarity of ln-likelihood (ln L) scores of reconstructed trees was evaluated using the methods of Geweke (1992), Heidelberger and Welch (1983), and Raftery and Lewis (1992a; 1992b) as implemented with default settings in the R package BOA v. 1.1.5-2 (Smith 2005). All trees sampled before stationarity were discarded from subsequent analyses, including the construction of 50% majority-rule (MrBayes “half-compatible”) consensus trees.

### Outgroup Selection

Outgroup choice has been shown to affect tree rooting positions in phylogenetic analyses (e.g. Messenger and McGuire 1998, Hollingsworth 1999). Outgroups used in this analysis were selected using closest related toads from the “*Bufo americanus* complex” per Masta et al. (2002) and Pauly et al. (2004). These outgroup species included *Bufo microscaphus* and *Bufo woodhousei* from within the “*Bufo americanus* complex,” and *Bufo boreas* was included as a more distantly related outgroup for ND1 analysis (Appendix I). Opportunistically gathered outgroup sequence data was obtained

from GenBank from the submissions of Stöck et al. (2006), and Zhang et al. (2008) for Dloop comparison. All analyses were subjected to an unconstrained outgroup topology.

### Confidence and Signal

The computer program Sequencher 3.0 (Gene Codes Corporation 1995) is used to verify peak calls and align sequences. Pairwise alignments were first assembled for all ingroup samples and then combined with the alignment of assembled outgroup taxa. Aligned sequences are then exported from Sequencher 3.0 or Clustal W as a NEXUS file, formatted for phylogenetic analysis using MacClade 3.01 (Maddison and Maddison 1992), and executed in PAUP\* (version 4.0b2a; Swofford, 1999). Formatting in MacClade 3.01 includes codon position assignment and transition versus transversion weighting using Sankoff stepmatrices (Sankoff and Rousseau, 1975). In PAUP\*, base-pair composition, pair-wise variation, codon position variation, and total variation were calculated.

Maximum likelihood was used to compare the relative likelihoods of trees from the parsimony analyses, to compare the goodness-of-fit of different models of sequence evolution to the observed data, and to search for optimal likelihood trees. Due to the large number of samples and computer software limitations, the likelihood analyses were unable to run to completion within the time period proposed for this study. Therefore, the total number of taxa was judiciously pruned of identical samples. Identical samples were pruned from the data set as judged by the shortest trees from the parsimony analyses, and raw sequence data (Graybeal 1998, Hillis 1998; Poe 1998).

A major question in the analysis of diverse data is what constitutes a “data set” (e.g., Kluge and Wolf 1993, Chippindale and Wiens 1994). In this study, it is proposed

that the data from representative mtDNA gene regions be analyzed separately as well. The various molecular data sets also were combined and analyzed together. By analyzing the data separately, I was able to detect if any single gene region conflicts with another. The combination of conflicting data sets is controversial (see reviews by Bull et al., [1993], de Queiroz et al. [1995] and Huelsenbeck et al. [1996a]). If the molecular data produces conflicting topologies, the combining of these data is simply to examine the effects of this practice, rather than using the combined-data tree as the best estimate of this toad's phylogeography. All characters were weighted equally in the combined analyses.

Nonparametric bootstrap analysis was used to evaluate confidence within phylogenetic trees (Felsenstein 1985) using 1000 bootstrap replicates with the fast-heuristic search option in PAUP\*. Only bootstrap values of over 50% are reported and support of 70% or greater is believed to represent a strongly supported clade corresponding to a 95% confidence interval (Hillis and Bull 1993).

The ingroup data set was examined for phylogenetic signal using the  $g_1$  statistic (Hillis and Huelsenbeck 1992). The  $g_1$  statistic measures the skewness of the distribution of random trees (10,000 random trees in this analysis) to discern phylogenetic information relative to random noise. Critical values for random data were derived by extrapolating values from table 2 of Hillis and Huelsenbeck (1992).

## Results

Results of this phylogenetic analysis of respective ND1, Dloop, and combined ND1 and Dloop analyses are represented in the form of phylogenetic gene trees (Figs. 5-13). These trees depict the relationships between the individuals used in the analysis and

reveal rangewide intraspecific relationships. Non-parametric bootstrap values, homoplasy indices, branch lengths, and apomorphy lists assisted in interpreting the reliability and strength resolved relationships.

### Phylogenetic Signal

The  $g_1$  analysis indicated that the data set used in this study contained phylogenetic signal (as opposed to random data) based on significantly left-skewed  $g_1$  values at  $P < 0.01$  (Hillis and Huelsenbeck 1992). For ND1, the lengths of 10,000 randomly-sampled trees resulted in a  $g_1$  value of  $-3.60$  for ingroup and outgroup taxa, and  $-1.01$  for ingroup taxa alone. For Dloop, the lengths of 10,000 randomly sampled trees resulted in a  $g_1$  value of  $-2.43$  for ingroup and outgroup taxa, and  $-0.84$  for ingroup taxa alone. For combined analysis, the lengths of 10,000 randomly sampled trees resulted in a  $g_1$  value of  $-2.85$  for ingroup and outgroup taxa, and  $-0.67$  for ingroup taxa alone. The critical  $g_1$  value for 500 four-state characters (i.e., DNA characters) from 25 or more taxa is  $-0.09$ . ( $P = 0.01$ ; Hillis and Huelsenbeck 1992). The figure of 25 ingroup taxa was used to estimate phylogenetic signal. Therefore, the  $g_1$  values in this study indicate that the data set was significantly more structured than random data.

### DNA Sequences Data

The ND1 data consisted of 859 aligned nucleotide positions from the ND1 gene. This aligned sequence did not include primer nucleotide positions. The ND1 sequence data contained a total of 138 parsimony-informative characters including ingroup and outgroup taxa, of which 5 characters were parsimony informative within the ingroup. The total aligned sequence includes 28.6% adenine (A), 24.3% cytosine (C), 13.3%

guanine (G), and 33.8% thymine (T). Locality information for all samples referenced is shown in Appendix I.

The Dloop sequence data consisted of 451 aligned nucleotide positions from the Dloop region of mtDNA. This aligned sequence did not include primer nucleotide positions. The ND1 sequence data contains a total of 37 parsimony-informative characters including ingroup and outgroup taxa, of which 6 characters are parsimony informative within the ingroup. The total aligned sequence included 30.8% adenine (A), 11.9% cytosine (C), 21.4% guanine (G), and 35.8% thymine (T).

The combined sequence data consisted of 1309 aligned nucleotide positions from the ND1 gene and Dloop region of mtDNA. This aligned sequence did not include primer nucleotide positions. The combined ND1 and Dloop sequence data contains data set contained a total of 85 parsimony-informative characters including ingroup and outgroup taxa, of which 12 characters are parsimony informative within the ingroup. The total aligned sequence includes 29.5% adenine (A), 20.2% cytosine (C), 15.8% guanine (G), and 34.5% thymine (T). Outgroup relationships were not analyzed in this study, and are excluded here.

Identical samples were reduced to a single specimen for many analyses to allow for more efficient data analysis. Identical sequences for each gene region, and for the combined sequence data, are indicated in Table 4.

Table 4. Identical sequences for each respective gene region sequenced and geographic locality.

ND1	DLOOP	Combined ND1 and Dloop
<b><u>Southern Central Coast</u></b>		
DS002	DS002	DS002C
	DS004	
DS029	DS029	DS029C
DS032	DS032	DS032C
	DS062	
<b><u>Northern</u></b>		
DS063		
DS049	DS049	
DS064	DS064	
DS066		
<b><u>Southern</u></b>		
	DS008	
DS019	DS019	DS019C
DS067		
DS070	DS070	DS070C
DS078	DS078	DS078C
DS087	DS087	DS087C
DS089	DS089	DS089C

## Maximum Parsimony Analysis

### ND1

The unweighted maximum parsimony analysis for ND1, using all variable characters, produced a most-parsimonious tree of 182 steps. The consensus tree had a rescaled consistency (excluding uninformative characters) index of 0.88 and a retention index of 0.95. Results from the strict consensus tree indicated support for a few groups within *Bufo californicus* (Fig. 5).

A 100% bootstrap value supported all ingroup samples including DS104 as an individual representing a basal lineage from the Rio Santo Domingo. A 56% bootstrap support value was given to all ingroup samples other than DS104. Samples other than DS104 were comprised of two major groups, one composed of samples DS002, DS004, DS029, DS032, and DS062 from central southern California and supported by a 42% bootstrap value. All remaining samples were weakly supported as a group. Within this group, a 95% bootstrap value supported the terminal group of DS041, DS035, DS049, DS063, DS064, and DS066, which were all from the northernmost range of *Bufo californicus*.

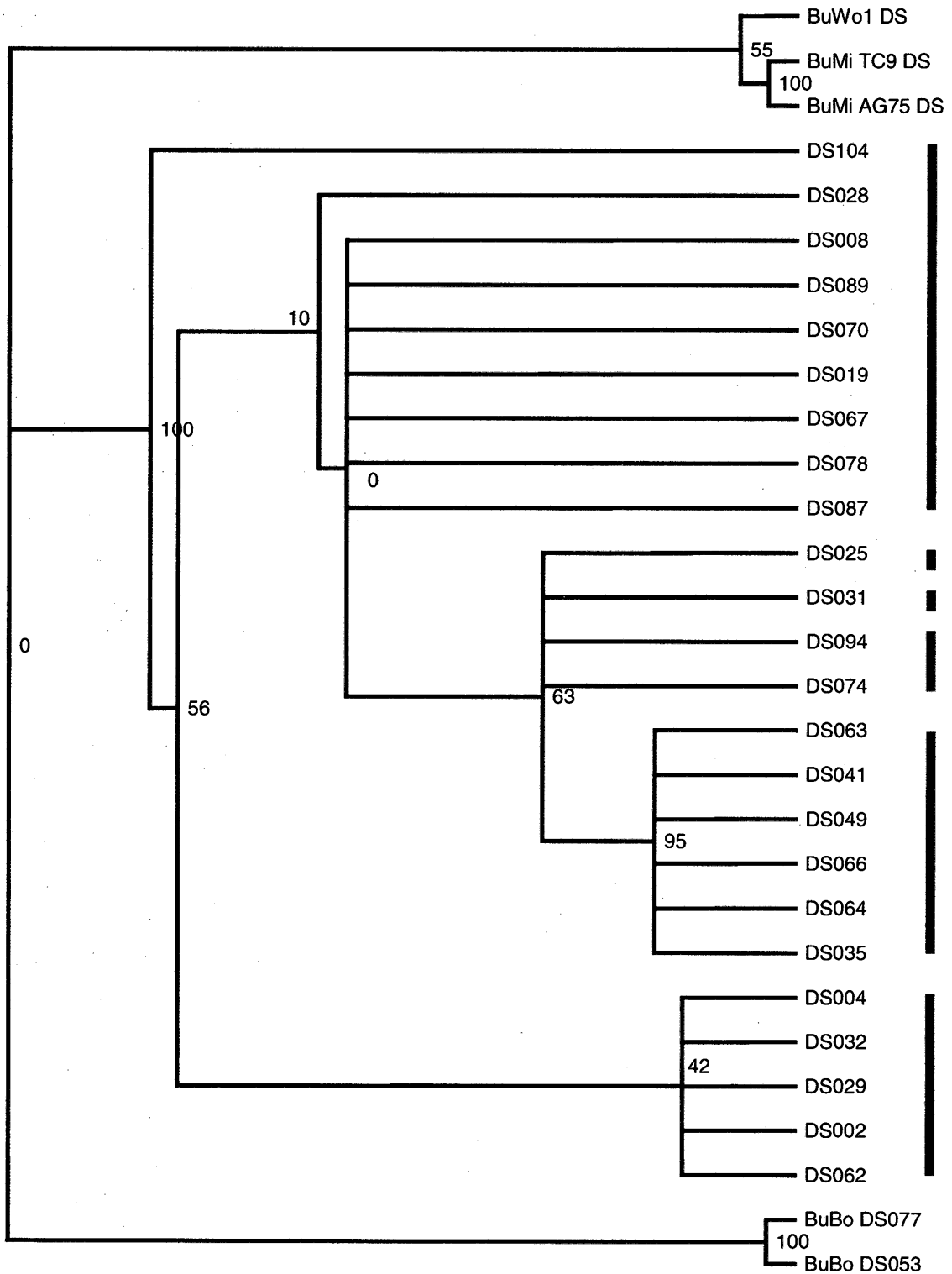


Figure 5. Maximum parsimony analysis gene tree for ND1 data. BuMi-, BuBo-, and BuWo- are outgroup samples. Bootstrap support values are indicated at respective nodes. Blue samples are from southern localities, and red samples are from northern localities.



## Dloop

The unweighted maximum parsimony analysis for Dloop, using all variable characters, produced a most-parsimonious tree of 61 steps. That tree had a rescaled consistency index (excluding uninformative characters) of 0.91 and a retention index of 0.96. Identical samples were reduced to a single sequence (Table 3, Fig. 6), and multiple samples indicated as a single node.

Results from the maximum parsimony bootstrapping analysis (Fig. 6) showed six samples from the northern range of the species as an unresolved polytomy (DS031, DS041, DS049, DS064, DS063, and DS066). Two other groups, including all remaining samples, were supported by 86% and 65% bootstrap values. Similar support exists for groups within *Bufo californicus* as that shown in the ND1 maximum parsimony analysis (Fig. 5). Sixty four % support was shown for samples DS025 and DS074 from San Dieguito River, USA, and Rio San Rafael, México, respectively. All other samples were supported with an 86% likelihood value.

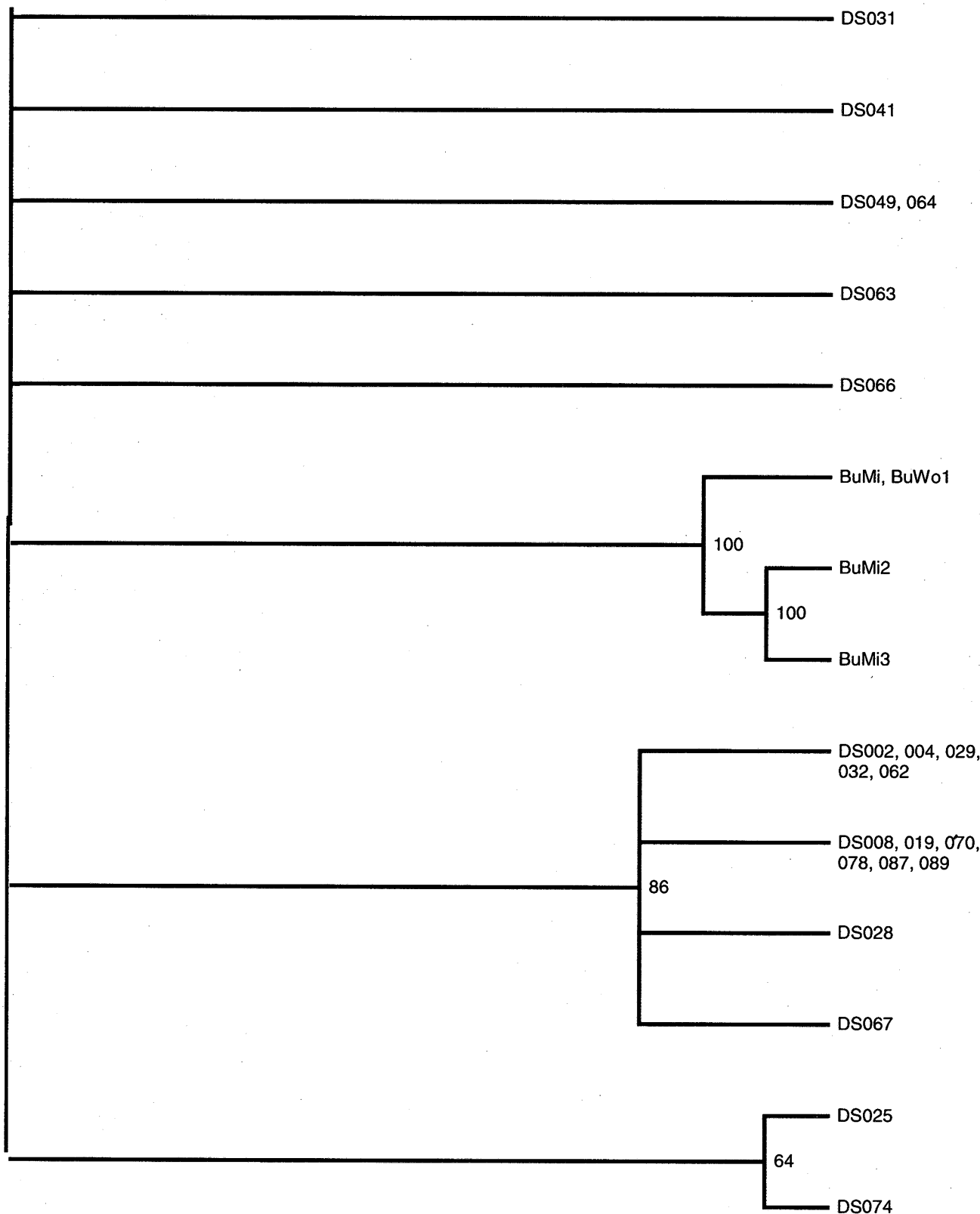


Figure 6. Maximum parsimony analysis gene tree for Dloop data. BuMi- and BuWo- are outgroup samples. Bootstrap support values and identical samples are indicated at respective nodes. Blue samples are from southern localities, and red samples are from northern localities.

## Combined ND1 and Dloop

The unweighted maximum parsimony analysis for combined ND1 and Dloop, using all variable characters, produced a most-parsimonious tree of 138 steps. That tree had a rescaled consistency (excluding uninformative characters) index of 0.95 and a retention index of 0.98. Results from the strict consensus tree indicated support for a few groups within *Bufo californicus* (Fig. 7).

Two groups, including all samples, were supported by 67% and 73% percent. The group supported by 73% included samples from the north (DS031, DS041, DS049, DS064, DS063, and DS066) that were further supported by an 87% bootstrap value (DS031 from the Mojave River) and another group supported by a 61% bootstrap value (including samples DS025 and DS074 from San Dieguito River, USA, and Rio San Rafael, México, respectively). The group supported by a 67% bootstrap value includes all samples south of the northern samples, along with strong (80% bootstrap value) support for samples from the central southern rivers (DS002, DS004, DS029, DS032, DS062).

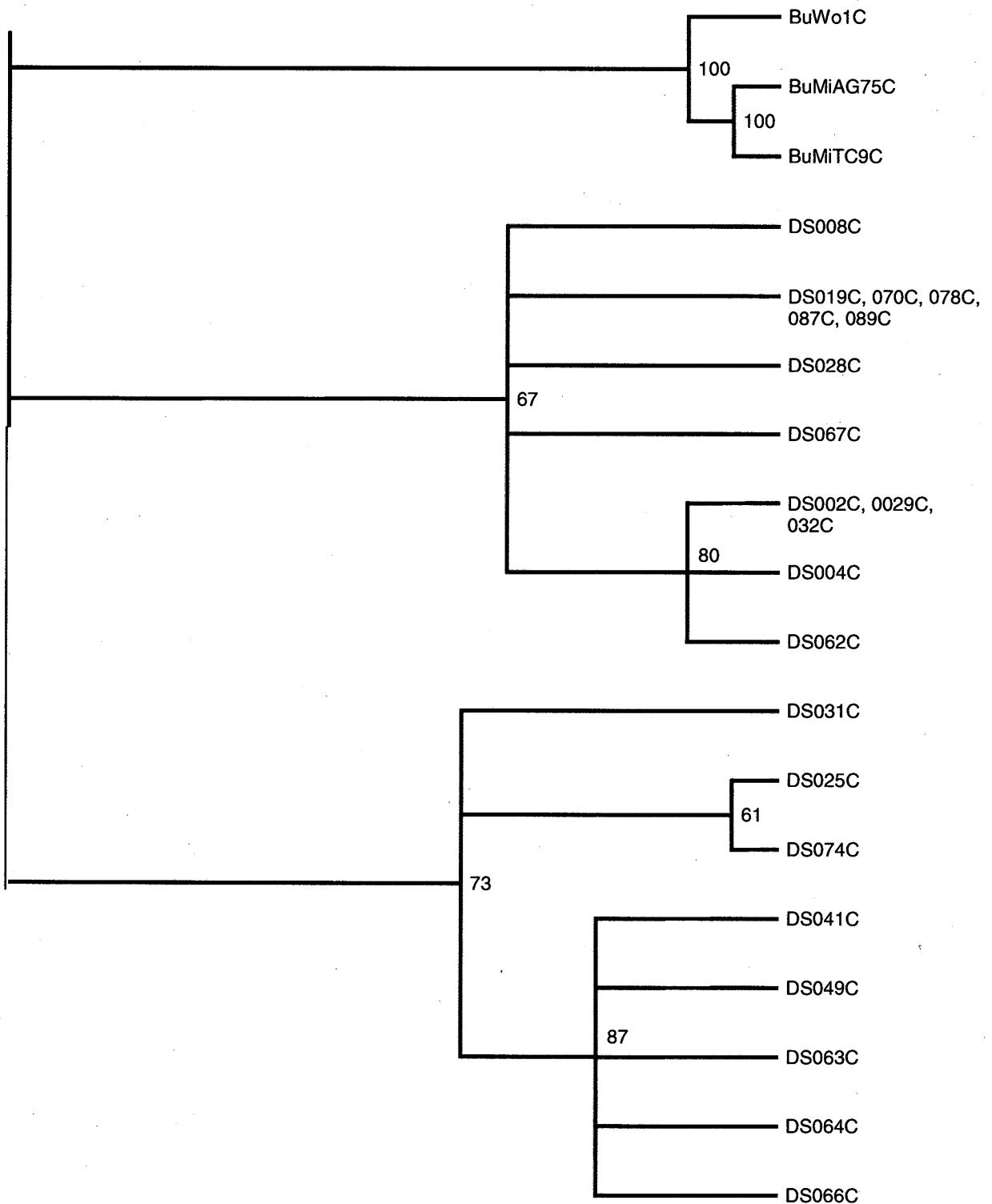


Figure 7. Maximum parsimony analysis gene tree for combined ND1 and Dloop data. BuMi- and BuWo- are outgroup samples. Bootstrap support values and identical samples are indicated at respective nodes. Blue samples are from southern localities, and red samples are from northern localities.

## Maximum Likelihood Analysis

### ND1

The maximum likelihood analysis for ND1, using all characters, produced one optimal reconstruction and the resulting strict consensus tree indicated support for a few groups within *Bufo californicus* (Fig. 8). The most parsimonious trees had a consistency index of 0.58 and a retention index of 0.78.

The maximum likelihood analyses support DS104 from the Rio Santo Domingo as being basal to all ingroup members of the tree. A 54 likelihood score supported all other ingroup relationships, which were comprised of several groups. Eight samples were unresolved (DS008, DS019, DS070, DS078, DS087, DS089, DS067, and DS028). A likelihood score of 52 supported the southern central California group, including DS002, DS004, DS029, DS032, and DS062. A 66 likelihood score supported samples DS025, DS031, DS074, and DS094 from as far north as the Mojave River (DS041) to the southernmost sample (DS094). Greatest support (96) was given to a northern group of samples including DS035, DS041, DS049, DS063, DS066, and DS064.

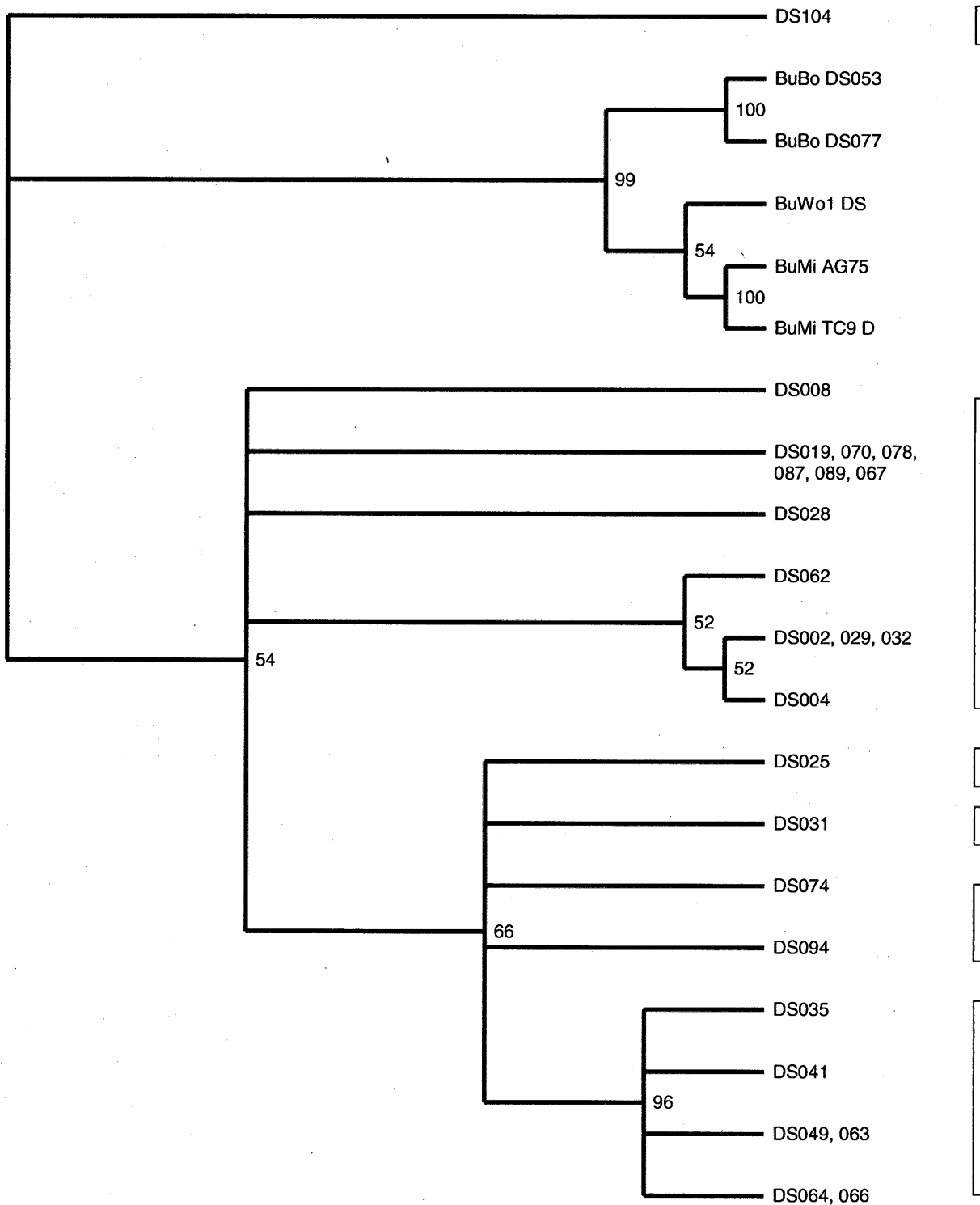


Figure 8. Maximum Likelihood analysis gene tree for ND1 sequence data. BuMi-, BuBo- and BuWo- are outgroup samples. Likelihood support values and identical samples are indicated at respective nodes. Blue samples are from southern localities, and red samples are from northern localities.

## **Dloop**

The maximum likelihood analysis for Dloop, using all characters, produced one optimal reconstruction (Fig. 9). The most parsimonious tree has a consistency index of 0.58, and a retention index of 0.78. Results from the strict consensus tree indicate similar support for groups within *Bufo californicus* as that shown in the ND1 ML analysis (Fig. 8), but reduced to three major groups. A likelihood score of 55 supports ingroup samples DS041, DS049, DS063, DS064, and DS066, along with outgroup samples. A likelihood score of 67 supports a group of samples including DS031, DS025, and DS074 from Mojave River, San Dieguito River and Rio San Rafael respectively (App. I). Greatest support is seen with a likelihood score of 88 for all remaining samples from southern California, USA and Baja California, México.

## **Combined ND1 and Dloop**

The maximum likelihood analysis for ND1 and Dloop, using all characters, produced one optimal reconstruction. The most parsimonious tree has a consistency index of 0.95, and a retention index of 0.98. Results from the strict consensus tree indicate support for a few groups within *Bufo californicus* (Fig. 10).

A 64 likelihood score supports nearly all samples from southern California and Baja California, with a likelihood score of 82 given to those samples from the rivers of central southern California (DS002, DS004, DS029, DS032, and DS062). A likelihood score of 77 supports all northern samples (DS035, DS041, DS049, DS063, DS066, and DS064; 95 likelihood score), and DS031, DS025, and DS074 from Mojave River, San Dieguito River and Rio San Rafael respectively.

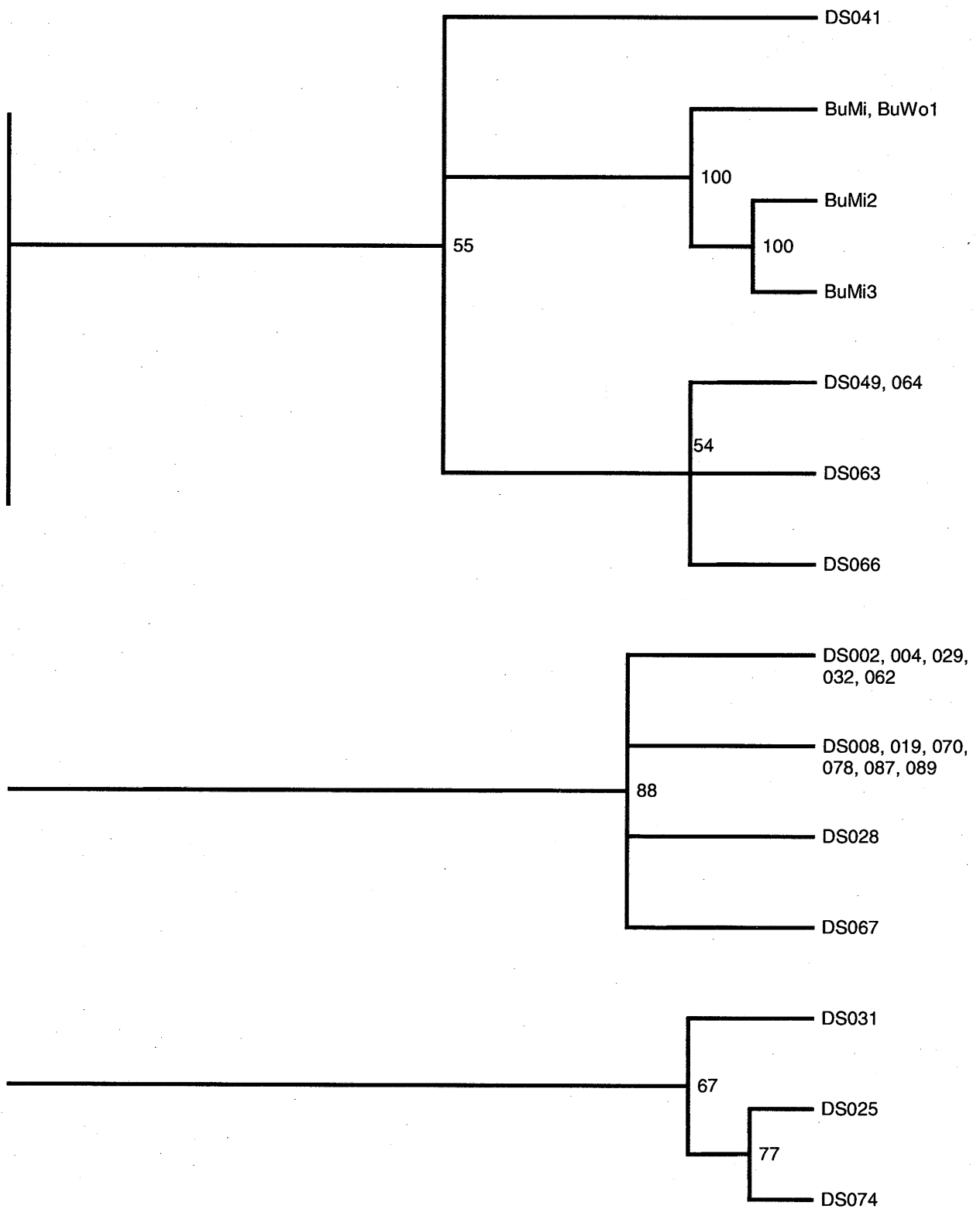


Figure 9. Maximum likelihood analysis gene tree for Dloop sequence data. BuMi- and BuWo- are outgroup samples. Bootstrap support values and identical samples are indicated at respective nodes. Blue samples are from southern localities, and red samples are from northern localities.



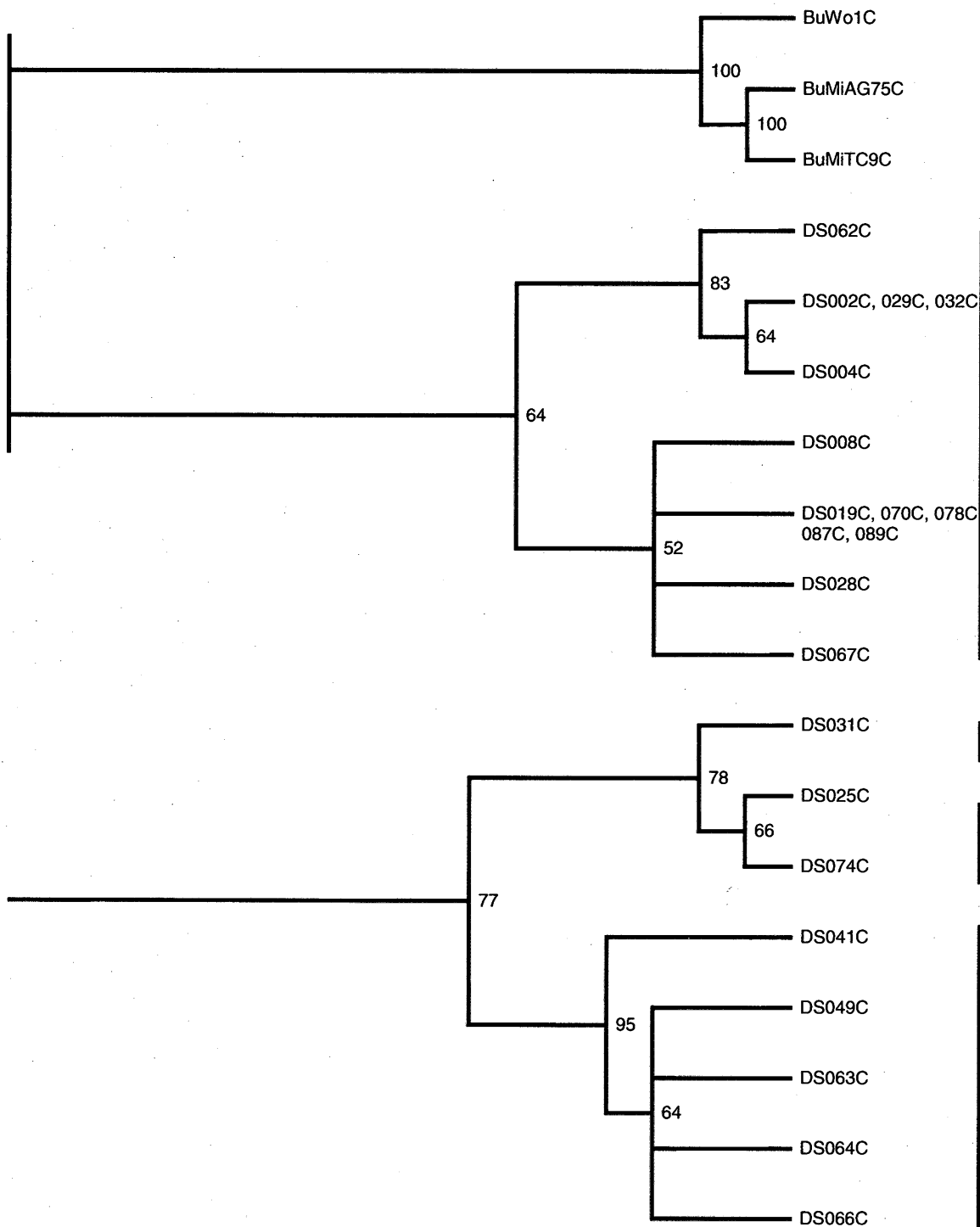


Figure 10. Maximum likelihood analysis gene tree for combined ND1 and Dloop sequence data. BuMi- and BuWo- are outgroup samples. Bootstrap support values and identical samples are indicated at respective nodes. Blue samples are from southern localities, and red samples are from northern localities.

## Bayesian Analysis

### ND1

The unweighted Bayesian analysis for ND1, using all variable characters, produced a single optimal tree. Outgroups are basal to all ingroups. Results from the strict consensus tree indicate support groups within *Bufo californicus* (Fig. 11).

Basal ingroup samples include good support (1.0) for the coastal southern California group of DS004, DS002, DS029, DS032, and DS062. Bayesian likelihood score of 0.57 supports all remaining samples. Within all of the remaining samples, a score of 0.89 supports DS025, DS031, DS074, and DS094. High support is also shown for a northern group of samples including DS035, DS041, DS049, DS063, DS064, and DS066.

### Dloop

The unweighted Bayesian analysis for Dloop, produced a single optimal tree. (Fig. 12). Samples from southern California and Baja California form an unresolved basal polytomy. Additionally, a likelihood score of 0.99 supports outgroup samples, northern ingroup samples (DS041, DS049, DS063, DS064, DS066), and DS031, DS025, and DS074 from Mojave River, San Dieguito River and Rio San Rafael respectively (App. I). Within this branch, support for samples DS031, DS025, and DS074 is indicated by a 0.99 Bayesian likelihood score, northern samples and outgroup samples by 0.70.

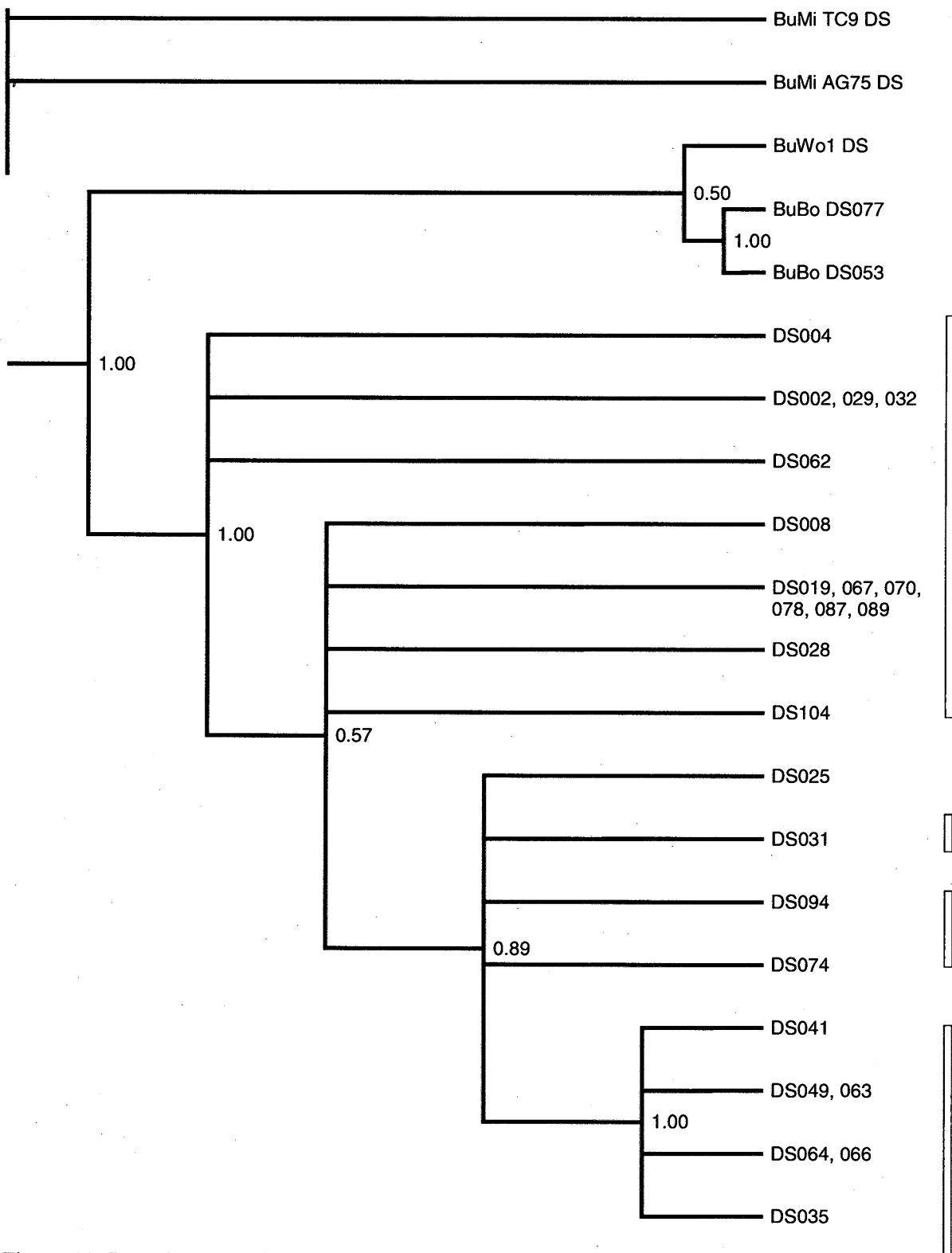


Figure 11. Bayesian analysis gene tree for ND1 sequence analysis. BuMi-, BuBo-, and BuWo- are outgroup samples. Posterior probability values and identical samples are indicated at respective nodes. Blue samples are from southern localities, and red samples are from northern localities.

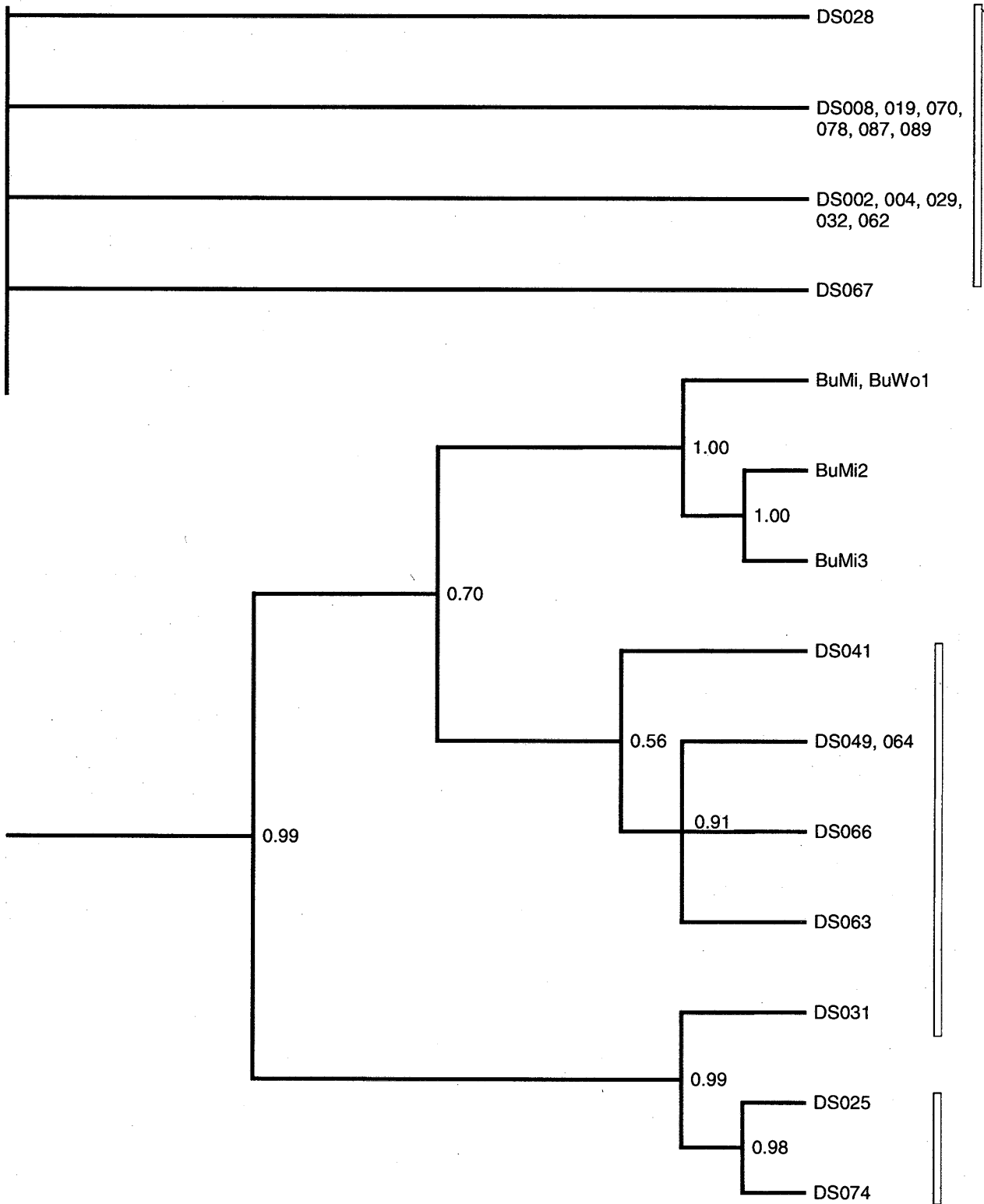


Figure 12. Bayesian analysis gene tree for Dloop sequence analysis. BuMi- and BuWo are outgroup samples. Posterior probability values and identical samples are indicated at respective nodes. Blue samples are from southern localities, and red samples are from northern localities.

## Combined ND1 and Dloop

The unweighted Bayesian analysis for ND1 and Dloop produced a single optimal tree. Outgroups are basal to all ingroups, which are well supported with a 1.0 likelihood score. Results from the strict consensus tree indicate support two groups within *Bufo californicus* (Fig. 13).

Within the ingroup, two major groups are supported by a 0.60 and 0.88 likelihood scores, and correspond to southern California and Baja California, and northern samples respectively. A likelihood score of 0.99 supports DS031, DS025, and DS074 from Mojave River, San Dieguito River and Rio San Rafael respectively (App. I), which are also supported by the 0.88 score with northern samples.

## Discussion

Analysis of the sequence data for *Bufo californicus* showed that there was phylogenetic signal and structure evident, which was for the most part congruent among the methods and gene regions used. Resulting gene trees depicted the relationships between the individuals used in the analysis and revealed interpopulational affinities. The gene tree relationships from all analyses reconfirmed that *Bufo californicus* is an exclusive lineage from outgroup specimens, except for Dloop, which placed outgroup samples with northern *Bufo californicus* samples in two of three analyses conducted (ML and Bayesian).

A few major patterns appeared in this study, and with congruence among trees generated using different analyses (MP, ML, and Bayesian). Among all trees, there was a well supported southern group, and a northern group that included three samples

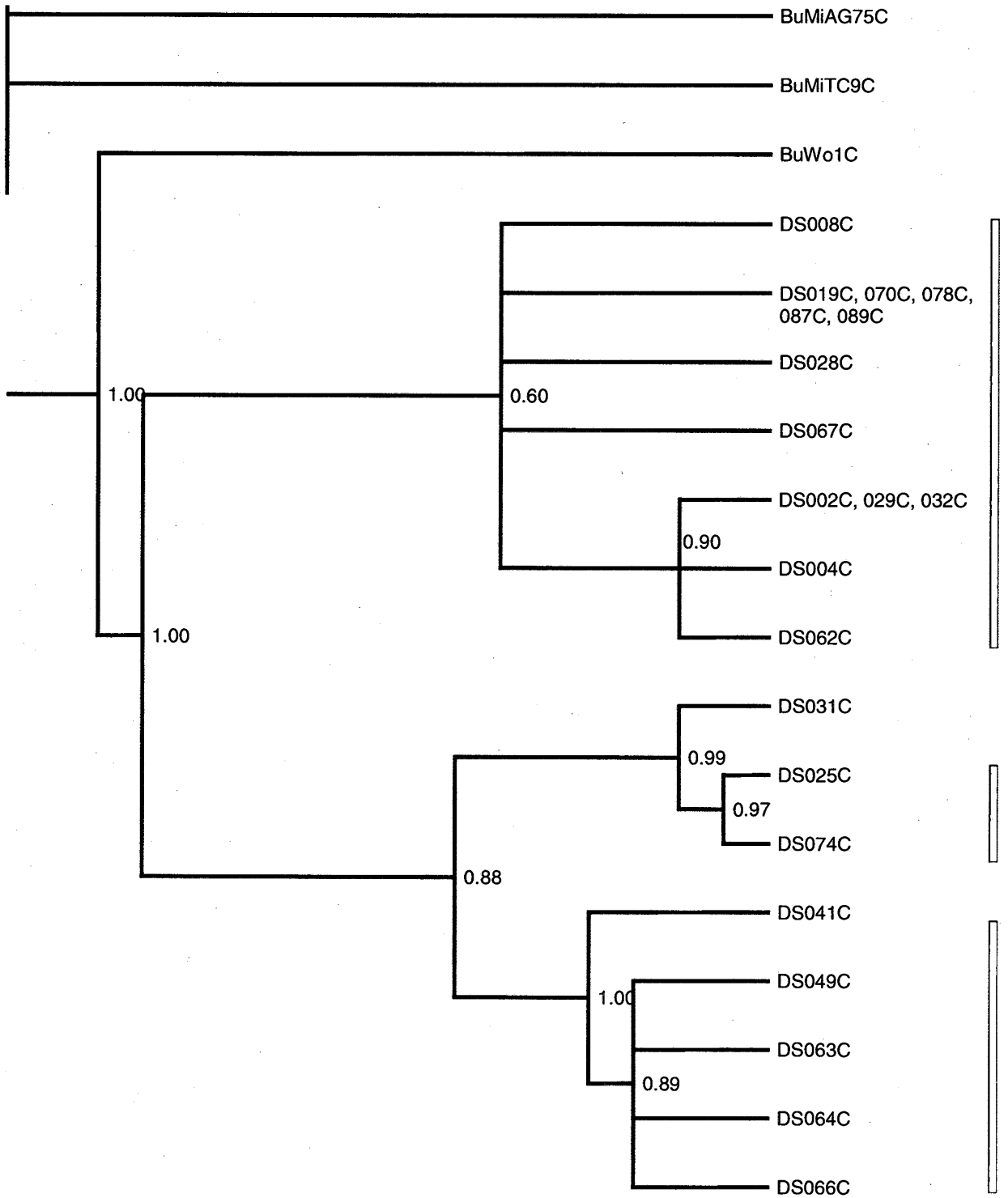


Figure 13. Bayesian analysis gene tree for combined ND1 and Dloop sequence analysis. BuMi- and BuWo are outgroup samples. Posterior probability values and identical samples are indicated at respective nodes. Blue samples are from southern localities, and red samples are from northern localities.

from the Mojave River, San Dieguito River, and Rio San Rafael. Within the southern group, strong support was shown for a central southern group from southern California. Wherever their placement lay within respective trees, strong support existed for these groups.

### Phylogeographic Patterns

Haplotypes recovered in this study reveal congruence with watersheds. All of the samples from the northern group are concordant with the rivers draining from the Coast and San Gabriel Mountains. The Northern Group include samples from the San Gabriel Mountains and northwestward to Fort Hunter Liggett. These samples span the Transverse Ranges (San Gabriel Mountains) and Coast Ranges of southern California. These ranges were separated from other members historically by the San Andreas Fault, and Los Angeles Basin. Other groups from the San Bernardino Mountains of southern California and southward into Baja California are also well supported as a result of their sharing of the Peninsular Ranges and/or proximity of their drainages to one another.

A well supported group is found in those samples from central southern California, from the Santa Ana River southward to the Santa Margarita River. Samples from the Santa Ana River south to the Rio Santa Margarita are all basal, with some *Bufo californicus* north and south of this group being more closely related to one another than to those from central southern California. Samples from the Santa Ana River southward to the Santa Margarita River are very closely related, and are nested within the southern group.

An interesting pattern is seen in samples DS031, DS025, and DS074 from the Mojave River, San Dieguito River, and Rio San Rafael respectively. All three of these

samples are widely separated from one another, and are more closely related to members of the northern group. Given that congruence is seen with support for northern and southern groups in all analyses, it is likely that the presence of “northern” haplotypes in southern group rivers are artifacts of multiple colonization events of coastal California and Baja California by *B. microscaphus*. Further analysis may be necessary to unravel this pattern along with that seen in DS104 from the Rio Santo Domingo. DS104 was only sequenced using ND1, and was placed as basal to all *Bufo californicus* in all analyses except the Bayesian analysis of ND1.

The well supported groups of *Bufo californicus* seen in this study cover a wide range of habitats, latitude, and mountain ranges. Previously, they were hypothesized to have originated in the Colorado River via the Mojave River when it flowed to the Colorado during the Pleistocene (Gergus 1994, 1998). Data from this study do not support the Mojave River origins for the species, as all analyses nested the Mojave River sample within those from the north, and not basal in any analysis. Other hypotheses are evidenced by the results. The most interesting of these is an “out of Baja” hypothesis via dispersal from the Sierra San Pedro Mártir and the Rio Santo Domingo that would require rafting on the Baja Peninsula as it broke off of mainland México and migrated. In the maximum parsimony analysis for ND1, sample DS104 is basal to all other groups, and is from the Rio Santo Domingo. This major river drains the highest mountain range in Baja California, and has a long history of occupation by riparian species. Namely, this river is the only river that the endemic fish *Onchorhynchus mykiss nelsoni* occupied historically (Ruiz-Campos et al. 2000), and is also one of the only rivers in Baja California with robust populations of the Red-legged Frog (*Rana draytonii*: Welsh, 1988). Despite the



support for this hypothesis shown by sample DS104 being basal to all samples, other Baja California rivers sampled do not necessarily support this hypothesis. Haplotypes from other Baja California rivers, both north and south of the Rio Santo Domingo, are nearly identical to one another, and are more closely related to samples from the northernmost location of the Salinas River than to DS104 from the Rio Santo Domingo.

### Phylogeographic Comparison

Whereas a number of studies have been conducted on the phylogeography of various vertebrates found in California (summarized in Calsbeek 2003), few covered the area of southern California or northern Baja. Amphibian phylogeographies from southern California or Baja California are scarce. Shaffer et al. (2004) looked at the phylogeography of *Rana aurora* and found significant support for southern California and Baja California members being closely related, and recommended that they be recognized as a separate species from Red-legged Frogs to the north. The two lineages defined in this study are congruent with a north-south split in lineages, similar to that seen in Shaffer et al. (2004). Phillipsen and Metcalf (2007) analyzed *Pseudacris cadaverina* phylogeography from the United States, where it is broadly sympatric with *Bufo californicus*. Similar to Phillipsen and Metcalf (2007), results of this study indicate that geographic features and watersheds have influenced the genetic variation in *B. californicus*. Both *P. cadaverina* and *B. californicus* share well supported northern and southern lineages, with some overlap in central, and central southern California groups of *P. cadaverina* and *B. californicus* respectively.

Gergus et al. (1997) found that call variation within *B. californicus* was significant, and that some populations exhibited call characteristics more similar to *B.*

*microscaphus* than to other members of *B. californicus*. Notably, Gergus et al. (1997) found that samples from the Rio Santo Domingo were significantly different from samples from the Santa Ynez and Santa Clara Rivers in all call variables measured. Gergus et al. (1997) also implicated call variation as a contributor to species boundaries in the “*microscaphus*” complex. Their results with call variation are concordant with lineages in this study as having variation from north to south, at least between the Rio Santo Domingo and the Santa Ynez and Santa Clara River populations. Further study may reveal *B. californicus* is comprised of more than a single species lineage, possibly congruent with gene lineages shown in this study.

### Regional Biogeography

*Bufo californicus* is one of many species among the amphibians and reptiles in southern California who share aspects of their biogeographic history (Savage 1960). Grismer (1994, 2002) recognized *B. californicus* as being part of the Chaparral-Madreaan Woodland biogeographical complex, and ecologically part of the “Northwestern Mesophilic Group” that is confined to northern Baja California and the southern half of California. Gergus (1994, 1998) hypothesized that *Bufo californicus* and Arizona Toads were derived from a common ancestor that dispersed widely among the Gila and Colorado Rivers, making it as far as the then-mesic coastline of southern California and Baja California. Subsequent aridification of this region led to the current range of Arroyo Toads in both coastal draining rivers and desert rivers draining from the same mountains (San Bernardino and San Gabriel) in transmontane southern California. The lack of Arroyo Toads in Sonoran Desert drainages may be the result of a recent expansion to southern watersheds, or from displacement by the Red-spotted Toad (*Bufo punctatus*).

Persistence of aquatic herpetofauna in desert rivers has been noted for several other mesophilic reptile and amphibian species in southern California and Baja California (Grismer & McGuire 1993; Lovich & Meyer 2002). Phylogenetic relationships among *Bufo californicus* indicate that their range may have experienced a postglacial withdrawal to the north and west as the deserts developed, similar to sympatric *Pseudacris cadaverina* (Phillipsen and Metcalf 2002). If the features found in the Transverse Ranges (e.g. Mojave River) contain ancestral lineages of *Bufo californicus* (sensu Gergus 1994, 1998), we would expect them to be basal to all members. This was not the case as reflected in the analyses, although populations may have retreated from the Transverse Ranges to coastal areas as temperatures decreased and the pine belt descended post-pleistocene. Phillipsen and Metcalf (2002) hypothesized that a Pleistocene exodus from the Transverse Ranges would also explain the phylogenetic pattern within *Pseudacris cadaverina*, wherein northern and southern haplotypes descended from haplotypes in the Santa Ana Mountains, which in turn descended from Transverse Range haplotypes. Compared with the Transverse Ranges, the Santa Ana Mountains are much lower in elevation and, due to the maritime influence of the nearby Pacific Ocean, probably experienced more moderate temperatures during the Pleistocene. No fossil evidence of *Bufo californicus* exists to further illuminate its origins.

#### MtDNA Variation

Although good support for and congruence was seen in the phylogenetic analysis, relatively little variation existed among and between some samples. This was best characterized by the identical mtDNA sequence data for individuals from different watersheds (Table 3). *Bufo californicus*, as a member of the “*Bufo americanus*” complex,

is characterized by relatively low levels of mtDNA variation. This study found that enough variation exists for well-supported and more or less consistent substructuring across the range of the species. Levels of variation seen are the product of a complex history of geologic events and vicariance in riparian habitats. The low levels of mtDNA sequence variation (Appendix IV) that are found in *Bufo californicus* may be associated with the ecology of *Bufo californicus*. The bulk of our understanding of this species has come about through studies of them in riparian habitats and during the breeding season (generally March-June). Detection of *Bufo californicus* is maximized when they congregate in breeding pools for mating opportunities, and can be found in large congregations. Outside of the breeding season, *Bufo californicus* exhibits long periods of dormancy when buried into soft sandy substrate or leaf litter. With this understanding, our comprehension of *Bufo californicus* biology is relatively lean outside of the mating season. Despite the fact that *Bufo californicus* has been found quite distant from the rivers and streams they commonly inhabit, our understanding about their dispersal is very shallow. *Bufo californicus* are known to occupy upland habitats in Cristianitos Creek (Tributary of San Mateo Creek) as far as 1175 meters from the edge of Riparian habitat (Holland and Sisk 2000). The analyses of this study indicate low levels of variation in maternally inherited genes. This is an indication that *Bufo californicus* is either a recent arrival and dispersed rapidly into the habitats they now occupy, or they move between watersheds far more frequently than we understand. Either explanation supports the idea of overland dispersal often enough to have maintained the low levels of genetic variation we see in the mtDNA.

## Conclusions

Support for the *Bufo californicus* as an exclusive lineage is seen in all ND1 and combined ND1 and Dloop analyses, but not in maximum likelihood or Bayesian analysis of Dloop sequence data. Congruence is seen among all gene regions for northern and central/southern groupings, within which strong support is seen for samples from the Santa Ana River to the San Luis Rey River.

There is a strong correlation between the well-supported haploclades and geographic patterns linked to watershed connectivity. Barriers to dispersal, in the form of changing environmental conditions since the Pleistocene, have contributed to the relationships evidenced in this study. The hypothesis that *Bufo californicus* had its origins in the Mojave River is not supported by this study, and a more likely scenario is connectivity to the Colorado River through rivers farther south, or from Baja California.

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## CHAPTER THREE

### CONSERVATION GENETICS OF THE ARROYO TOAD (*Bufo californicus*)

#### **Abstract**

Present conservation of *Bufo californicus* does not rely on a comprehensive understanding of haplotypes within the species, or their relation to Recovery Units as defined by the United States Fish and Wildlife Service. Herein, mitochondrial DNA evolution from the ND1 and Dloop regions are analyzed to determine relationships within and among major watersheds throughout the range of *Bufo californicus*. Well supported groups and genetic structure are evident, and recommendations are made to enhance conservation of the respective haplotypes within *Bufo californicus*. Each of the strongly supported groups identified in this study is an evolutionarily distinct unit that should be protected and prioritized accordingly in management and conservation actions throughout the range of the species.

#### **Introduction**

Genetic patterns and variation can reveal much about a species when trying to bolster conservation efforts. This is especially true for *Bufo californicus*, which is listed as endangered in the United States (USFWS 1994), after declining significantly in range and numbers (Jennings and Hayes 1994). In spite of the attention that this species has received as one in need of conservation, and its protection by federal and myriad other laws in the USA, our understanding of its intraspecific relationships remains weak. Given

the well-documented and global decline of amphibians (Pechmann and Wilbur 1994, Green 1997, Pechmann and Wake 1997, Duellman 1999, Vredenburg and Wake 2007), specific information on relationships is needed to effectively conserve and manage *B. californicus* populations. Genetic relationships among and between populations of *Bufo californicus* have until this time been a major gap in our knowledge of this species.

*Bufo californicus* is a small to medium-sized (5-8 cm snout-vent length) burrowing toad primarily distributed in cismontane riparian habitats from Monterey County, California, USA, southward to Rio Santa Maria, near San Quintin in northwestern Baja California, México (Grismer 2002). A few populations also occur in transmontane desert drainages of the San Gabriel and San Bernardino Mountains of California, USA. Since the original description of *B. californicus* (Camp 1915), aspects of the biology of populations in the United States have received considerable attention (Myers 1930; Miller and Miller 1936; Cunningham 1962; Price and Sullivan 1988; Sweet 1992, 1993; Gergus 1998). *B. californicus* was once a relatively common toad in coastal central and southern California, but has since been extirpated from approximately 75% of its historic range (Sweet 1992, Jennings and Hayes 1994). The causes for such severe declines in *B. californicus* populations are summarized by Jennings and Hayes (1994), and include alteration of streamside habitats, hydrologic change, off-road vehicle activity, urban development, sand mining, cattle grazing, and introduction of non-native species. Consecutive years of drought may have also threatened stressed populations. These threats and associated population declines prompted the U. S. Fish and Wildlife Service (1994) to list the species as endangered. This protection fostered additional regulatory and other related research interest with respect to conservation, distribution, habitat

preference, and dispersal patterns (Beaman et al. 1995; Campbell et al. 1996; Barto 1999; U. S. Fish and Wildlife Service 1999; Ramirez 2000; Holland and Sisk 2000; Griffin and Case 2001; Brown and Fisher 2002; Atkinson et al. 2002, Sweet and Sullivan 2005).

In contrast to protections in the United States (USFWS 1994), approximately one-third of the range of this species is within Baja California, México, where it is currently unprotected by government legislation (SEMARNAT, Poder Ejecutivo Federal 2002). In northwestern Baja California, *B. californicus* occurs mainly along the Pacific versant in the southernmost coastal streams of the California Phytogeographic Province (Grismer 2002). It generally inhabits riparian and oak woodland habitats in the north to maritime succulent scrub in the south (Linsdale 1932, Tevis 1944, Welsh 1988, Grismer 1994a, b, and Gergus et al. 1997b). Additional data on distribution and habitat were presented on recently discovered populations of *B. californicus* in Baja California (Mahrtdt et al. 2002, 2003; Dominquez-Torres, J. and E. Mellink 2003; Mahrtdt and Lovich 2004).

The Recovery Plan for the species (USFWS 1999) recommends that determining "...genetic differences and similarities within and among populations..." is a priority. Herein, we examine organelle DNA (mtDNA) from throughout the range of the species. As a result of this study, assumptions about conservation units and species homogeneity are replaced by a true understanding of the genetic variation across the range of *Bufo californicus*.

## **Materials and Methods**

Mitochondrial DNA sequence variation was analyzed for samples from throughout the range of *Bufo californicus* (Chapter 2). The ND1 gene and Dloop region

were sequenced and analyzed using maximum parsimony, maximum likelihood, and Bayesian methods, both independently, and as combined data sets.

### Prior Genetic Studies

Prior genetic studies focused on allozyme analysis of a few samples for higher taxonomic work on the “microscaphus” complex (Gergus 1994, 1998). Shanahan (1998) analyzed microsatellite markers across populations of *Bufo californicus* within three watersheds in San Diego County and one in Los Angeles County. Both of these prior efforts, although useful and informative, did not provide rangewide sampling or a comprehensive strategy for understanding relationships within the species. Prior assumptions for conservation planning relied upon scant genetic information and the legal protections that exist for all *Bufo californicus* in the United States under the Endangered Species Act. The less developed landscapes of Baja California have served as a refugia for the species in the form of intact habitats, but human population growth and regional planning chart a different course for Baja California, México. Well resolved intraspecific relationships of *Bufo californicus* are first explored in this study.

### Conservation Units

*Bufo californicus* was listed as an endangered species in 1994 (USFWS 1994) in the United States. The subsequent Recovery Plan for the species listed *Bufo californicus* as being comprised of three distinct conservation units in California, comprised of northern, southern, and desert units (USFWS 1999). The Northern Recovery Unit includes populations from Monterey, San Luis Obispo, Santa Barbara, and Ventura Counties, and the coastal slopes of Los Angeles County. The Southern Recovery Unit

encompasses populations in the coastal drainages of Orange, San Bernardino, Riverside, and San Diego Counties. The Desert Recovery Unit includes *Bufo californicus* populations and habitats on streams and rivers that drain the northern and eastern slopes of the San Gabriel, San Bernardino, and peninsular mountain ranges in Los Angeles, San Bernardino, Riverside, San Diego, and Imperial Counties. Since the Desert Recovery Unit was established, populations referenced in desert drainages of San Diego and Riverside Counties have been proven to represent misidentifications of *Bufo boreas* and *Bufo punctatus* (Ervin et al. In Prep.), thus restricting *Bufo californicus* in the Desert Recovery Unit to only the desert slopes of the Transverse ranges (e.g. Mojave River, San Bernardino County and Littlerock Creek, Los Angeles County, California).

Federal law and protections for *Bufo californicus* under the Endangered Species Act (ESA; USFWS 1994) essentially protect only those populations in the United States, and have very little direct impact on populations in México. The Endangered Species Act only applies to other countries for import or export of species listed, and activities that have an origin in the United States but which may impact species listed under the ESA. Thus, the recovery units defined in the Recovery Plan (USFWS 1999) have little relevance on Mexican populations. All Mexican populations are presumably referred to the southern recovery unit in the Recovery Plan (USFWS 1999). The NOM-059 (Poder Ejecutivo Federal, 2008) reflects those species protected under Mexican federal law, and the arroyo toad was proposed on this list as recently as 2008. There are no recovery units defined in the NOM-059, and the species is protected equally throughout its range in México, with no other distinctions made regarding individual populations or geographic regions.



## Results

Resulting gene trees from ND1 and Dloop mtDNA sequence analyses (Chapter 2) revealed that the Recovery Units set forth in the Recovery Plan for the Arroyo Toad (USFWS 1999) do not completely match those haplotypes identified by the gene trees (Figs. 5-12). The Northern Recovery Unit is the best supported among the three recovery units, with strong congruence and support in nearly all analyses. Excepting the inclusion of the desert sample from Littlerock Creek in Los Angeles, the Northern Recovery Unit is essentially supported by the results. Numerous identical sequences were also identified (Table 4), from both gene regions sequenced as part of this study. Identical sequences represent gene flow between and among watersheds, as the likelihood of unique haplotypes evolving independently and reverting to an identical character state across the range of the species is highly unlikely.

The Southern Recovery Unit is partially congruent with results, except that the desert sample from the Mojave River was nested with samples from the Southern Recovery Unit only in the ND1 data. The Dloop and combined analyses indicated that the Mojave River sample (DS031) was more closely related to the northern, and to the Rio San Rafael and San Dieguito River populations, than to southern *Bufo californicus*.

The Desert Recovery Unit is incongruent with the results, in that Mojave Desert samples from the Mojave River and Littlerock Creek are nested within Northern or Southern recovery units, depending on gene regions analyzed (Figs. 5-12). Combined analyses (Figs. 12-13) indicate southern samples well supported as nested within the northern group. Remaining members of the Desert Recovery Unit have proven to be

misidentifications, as no *Bufo californicus* occur in the desert drainages in San Diego or Riverside Counties (Ervin et al., In Prep.).

Mitochondrial DNA gene trees reveal more substructuring than was hypothesized previously. Northern and southern recovery units are supported by sequence data, as are those haplotypes from the central southern group including populations from the Santa Ana River southward to the Santa Margarita River. Other well-supported groups that defy the currently established recovery units are the three samples from the Mojave River, San Dieguito River, and Rio San Rafael (Figs 7, 9-10, 12-13).

Northern group haplotypes have been identified as occurring in southern localities at Boden Canyon, CA, USA and the Rio San Rafael, Baja California, México. These populations within southern localities indicate historical overlap in range between the two lineages that comprise the arroyo toad (Figs. 6-13). All samples from Mexico are nested within the southern group for all combined analyses, excepting those samples from the Rio San Rafael, which are more closely related to the northern group populations.

## Discussion

Mitochondrial DNA sequence data analysis provides an independent interpretation of the recovery units described within the Recovery Plan (USFWS 1999). In the Recovery Plan, the recovery units were defined as “geographically proximal populations separated from other units by distributional gaps with an unspecified distance.” The results of this analysis tested the boundaries of the recovery units and found that the previously identified northern and southern recovery units are partially congruent with findings of the mtDNA sequence data analysis. This information supports recognition of the independent genetic lineages that *B. californicus* is comprised

of. Future conservation and management actions will need to incorporate and address the distinct genetic haplotypes in order to maintain the genetic variation, and avoid genetic “contamination” should any type movement of individuals occur from one watershed to another, or captive propagation occur with the hope of enhancing or re-establishing populations. Relatively low levels of sequence variation indicate recent connectivity of populations within supported groups, and indicate that populations have not been isolated by one another long enough for reciprocal monophyly to have taken place, as *Bufo californicus* is recognized as only a single species (Gergus 1998). This finding is important in the sense that any conservation or enhancement of riparian habitats within the range of *B. californicus* must consider establishment of overland linkages for gene flow. Historic upland habitat connections are for the most part lost as a result of urbanization across the range of the species. Retaining any connectivity between watersheds is an element which may be largely impractical given the anthropogenically fractured and altered landscape inhabited by *B. californicus*. However, identification of upland habitat connections between extant populations of *B. californicus* is the next great challenge in recovering this species from its endangered status.

*Bufo californicus* has also been documented to disperse between watersheds (Holland and Sisk 2000). Genetic information shows that identical sequences are shared between watersheds (Table 3) that are widely separated. Likewise, some samples defy relatedness by distance, and are the result of historic barriers to gene flow. The evidence that arroyo toads crossed from one watershed to another has not been incorporated into conservation planning for the species. In fact, conservation has relied on independent riparian corridors with little overland connectivity between them. Watersheds throughout

the range of *B. californicus* are treated independently, and overland/upland connections between them are lost. This evidence needs to be incorporated into management decisions involving the designation of critical habitat, protected areas, and the expectation of populations to recover once potential or pre-existing detrimental factors have been mitigated.

These results provide valuable information in decisions involving the restoration of declining or extinct populations. If the augmentation of any population is deemed necessary, identifying genetically related populations for use in reintroductions is important to maintain “true” haplotypes within these toads. Minimizing the genetic disturbance caused by relocating animals would presumably increase the chance that the restoration of declining or extinct populations will be successful, as environmental selection favors haplotypes adapted to most identical environmental conditions. The closest haplotypes by distance should contain the best genetic make-up to cope with the selectional pressures within their relocated environment. Further genetic analyses across the range of the species have uncovered greater genetic diversity and distinct haplotypes that require conservation. Future focused conservation efforts should be placed on upon the protection of extant haplotypes, and enhancing potential gene flow naturally via historic corridors between watersheds.

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## CHAPTER FOUR

### THE DISTRIBUTION, THREATS, AND CONSERVATION STATUS OF THE ARROYO TOAD (*Bufo californicus* CAMP) IN BAJA CALIFORNIA, MÉXICO

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#### Abstract

The arroyo toad (*Bufo californicus*) is a federally protected species that has been relatively well studied in its northern range in California, USA. However, the distribution and population status of the species in its southern range in Baja California, México is poorly known. This study reviews the historical and present distribution of the arroyo toad (*B. californicus*) in northwestern Baja California, México based on museum and published records and results from recent surveys (2001-2006). Fourteen major drainage systems were surveyed in northwestern Baja California, representing all rivers

within the Mediterranean habitats of northwestern México. Previous records exist for seven of the 14 drainages; our surveys detected *B. californicus* in ten drainages.

Populations of *B. californicus* were found in three new drainages where no historical records previously existed. Currently, the species is known to inhabit a total of ten drainages in Baja California. A published record for the Rio El Rosario is herein considered doubtful. Regional pressures from development, land conversion, non-native species, sand mining and other human activities are contributing to a loss of riparian habitat and serve as potential threats to the species survival. Regulatory protection and conservation status in Baja California is discussed.

*Key words:* Arroyo Toad; Baja California; *Bufo californicus*; conservation status; distribution; invasive species

### **Introduction**

The Arroyo Toad (*Bufo californicus*) is one of three closely related species, formerly designated as subspecies of one another in the “microscaphus” complex (Gergus 1998). The other two being *Bufo microscaphus* and *Bufo mexicanus*. These three species range throughout the sandy and alluvial riparian habitat of northern México and the southwestern United States (Price and Sullivan 1988). Given the well documented and global decline of amphibians (Pechmann and Wilbur 1994, Green 1997, Pechmann and Wake 1997, Duellman 1999, Vredenburg and Wake 2007), specific information on current status and distribution is needed to effectively conserve and manage *A. californicus* populations.

The Arroyo Toad (*Bufo californicus*) is a small to medium-size (5-8 cm snout vent length) burrowing toad primarily distributed in cismontane riparian habitats from Monterey County, California, USA southward to the Rio San Simon (=Rio Santa Maria) near San Quintin in northwestern Baja California, México (Grismer 2002). A few populations also occur in transmontane desert drainages of the San Gabriel and San Bernardino Mountains of California, USA. Since the original description of *B. californicus* (Camp 1915), aspects of the biology of populations in the United States have received considerable attention (Myers 1930; Miller and Miller 1936; Cunningham 1962; Price and Sullivan 1988; Sweet 1991, 1993; Gergus 1998). *Bufo californicus* was once a relatively common toad in coastal central and southern California but has since been extirpated from approximately 75% of its historic range (Sweet 1991, Jennings and Hayes 1994). The causes for such severe declines in arroyo toad populations are summarized by Jennings and Hayes (1994) and include alteration of streamside habitats, hydrologic change, off-road vehicle activity, urban development, sand mining, cattle grazing, and introduction of non-native species. Consecutive years of drought may have also threatened stressed populations. These threats and associated population decline prompted the U. S. Fish and Wildlife Service (1994) to list the species as endangered. Federal legal protections fostered additional regulatory and other related research interest with respect to conservation, distribution, habitat preference and dispersal patterns (Beaman et al. 1995; Campbell et al. 1996; Barto 1999; Griffin 1999; Griffin et al. 1999; U. S. Fish and Wildlife Service 1999; Ramirez 2000; Holland and Sisk 2000; Griffin and Case 2001; Brown and Fisher 2002; Atkinson et al. 2002, Sweet and Sullivan 2005).

In contrast, one-third of the range of this species is within Baja California, México where it is unprotected by government legislation (SEMARNAT, 2002). In northwestern Baja California, the arroyo toad occurs mainly along the Pacific versant in the southernmost coastal streams of the California Phytogeographic Province (Grismer 2002). It generally inhabits riparian and oak woodland habitats in the north to maritime succulent scrub in the south. Disjunct populations occur near headwaters and streams of the Sierra de Juarez and Sierra San Pedro Martir. Reports on the distribution and habitat of *B. californicus* in Baja California is based on Linsdale (1932), Tevis (1944), Welsh (1988), Grismer (1994a , 1994b), Gergus et al. (1997), and a species account by Grismer (2002). Subsequently, data on distribution and habitat were presented on recently discovered populations of *B. californicus* in Baja California (Mahrtdt et al. 2002, 2003; Dominquez-Torres, J. and E. Mellink 2003; Mahrtdt and Lovich 2004).

The effects of urbanization in the absence of regulatory protection may result in future declines of *Bufo californicus*. Efforts in conservation and riparian management have not been widely implemented in northwestern Baja California, one of the fastest growing regions in México. Due to the population declines of *B. californicus* in southern California (Sweet and Sullivan 2005), our objective was to examine the historic and present distribution of this species in Baja California, México, provide baseline information on cumulative threats occurring in riparian habitats, and discuss conservation status.

## Methods

### Study Area

The region of northwestern Baja California extends 356 km south from the US-Mexico international border to the town of El Rosario. It is bounded by the Pacific Ocean on the west and, as a part of this study, the Pacific slopes of the Sierra de Juarez and the Sierra San Pedro Martir to the east. This mountain range lies approximately 60 km east of the Pacific Ocean. The range in elevation is sea level to approximately 2200 m. From the city of Tijuana south to Ensenada, development is occurring at an accelerated pace. A four-lane coastal highway (Hwy. 1) connects the two cities. From Ensenada south to the small town of El Rosario, Hwy. 1 is a two-lane paved road located near the Pacific Ocean.

The study area lies within the California Phytogeographic Province (Grismer 2002), and comprises five major vegetation communities: Coniferous Forest (>1830 m), Chaparral (1220 m – 2120 m), Coastal Sage Scrub (<1220 m), Oak Woodland, and Riparian Woodland (*sensu* Welsh 1988). Plant communities at higher elevations, inland foothills and mesas and drainages are largely undeveloped with the exception of numerous “ranchos” engaged in subsistence farming and cattle ranching. At lower elevations, coastal plains and drainages have been impacted by human population, widespread grazing, and large-scale commercial agriculture. Some drainages have remained minimally disturbed with the exception of widely distributed, highly invasive, non-native plant species *Tamarix* spp. and *Arundo donax*. Several dominant native plant species occur in drainages throughout northwestern Baja California and include willows (*Salix lasiolepis*, *S. laevigata*), sycamores (*Platanus racemosa*), cottonwoods (*Populus*

*fremontii*) and oaks (*Quercus agrifolia*, *Quercus* spp.). In some drainage systems both riparian and oak woodlands occur extensively as mixed phase plant community. The dominant understory vegetation comprises wild lilac (*Ceanothus* sp.), coffeeberry (*Rhamnus californica*), scrub oak (*Quercus dumosa*), chamise (*Adenostoma fasciculatum*), toyon (*Heteromeles arbutifolia*), mule-fat (*Baccharis salicifolia*), arrow weed (*Pluchea sericea*), mugwort (*Artemisia douglasiana*), rose (*Rosa minutifolia*, *R. californica*), and goldenbush (*Ericameria* sp.). Xerification is evident in drainage systems south of the Rio Santo Tomas and includes cholla and prickly pear cactuses (*Opuntia* spp.), Spanish bayonet (*Yucca whipplei*), and pitaya agria (*Machaerocerus gummosus*) (Roberts 1981). The Rio El Rosario is the only stream in our study area where cardons (*Pachycereus pringlei*) first appear, a species indicator of the central desert regions of Baja California.

For the purpose of this study, we identified 14 major drainage systems in northwestern Baja California (Figure 14). Several streams and their tributaries within these systems are semi-perennial and rarely reach the Pacific Ocean except in years of heavy rainfall and occasional flooding. The winter and summer rains and snow melt in the Sierra de Juarez and Sierra San Pedro Martir also provide runoff to the major coastal streams. In nearly all lower valleys, stream flow is interrupted by extensive agriculture. The Rio El Rosario is the southern-most semi-perennial stream to receive runoff from the Sierra San Pedro Martir.

The climate in northwestern Baja California is generally cooler and wetter than in southern Baja California. Mean annual precipitation varies from 26 cm in Tijuana (32° 31') to less than 10 cm in El Rosario (30°02' (Hastings 1964). Quantitative weather data

for the mountain region is sparse. Welsh (1988) report a mean annual precipitation of 17.8 cm in the Sierra San Pedro Martir and the highest annual precipitation for northern Baja California of 33.4 cm in the Sierra de Juarez. Nearly all rainfall occurs in the winter months. In the mountains, afternoon thunder showers occur from late June – September.

Native aquatic amphibian species occurring in the northwestern region include the western toad (*Bufo boreas*), red-spotted toad (*Bufo punctatus*), western spadefoot (*Scaphiopus hammondi*), Pacific treefrog (*Pseudacris hypochondriaca*), California treefrog (*Hyla cadaverina*), and red-legged frog (*Rana draytonii*). *Bufo punctatus* is found only as far north as the Rio El Rosario drainage on the Pacific Side of the peninsula. Disjunct populations of *R. draytonii* occur in the Rio Santo Domingo drainage and tributaries, the Rio San Jose, and streams in the Sierra San Pedro Martir (Grismer 2002). Aquatic reptile species include western pond turtle (*Actinemys marmorata*) and two-striped garter snake (*Thamnophis hammondi*). Introduced stream species include red swamp crayfish (*Procambarus clarkii*), and dominant fish species bluegill (*Lepomis cyanellus*) and mosquitofish (*Gambusia affinis*) (Ruiz-Campos et al. 2000). The bullfrog (*Lithobates catesbiana*) occurs in disjunct populations from the Rio Tijuana south to the Rio El Rosario drainage.

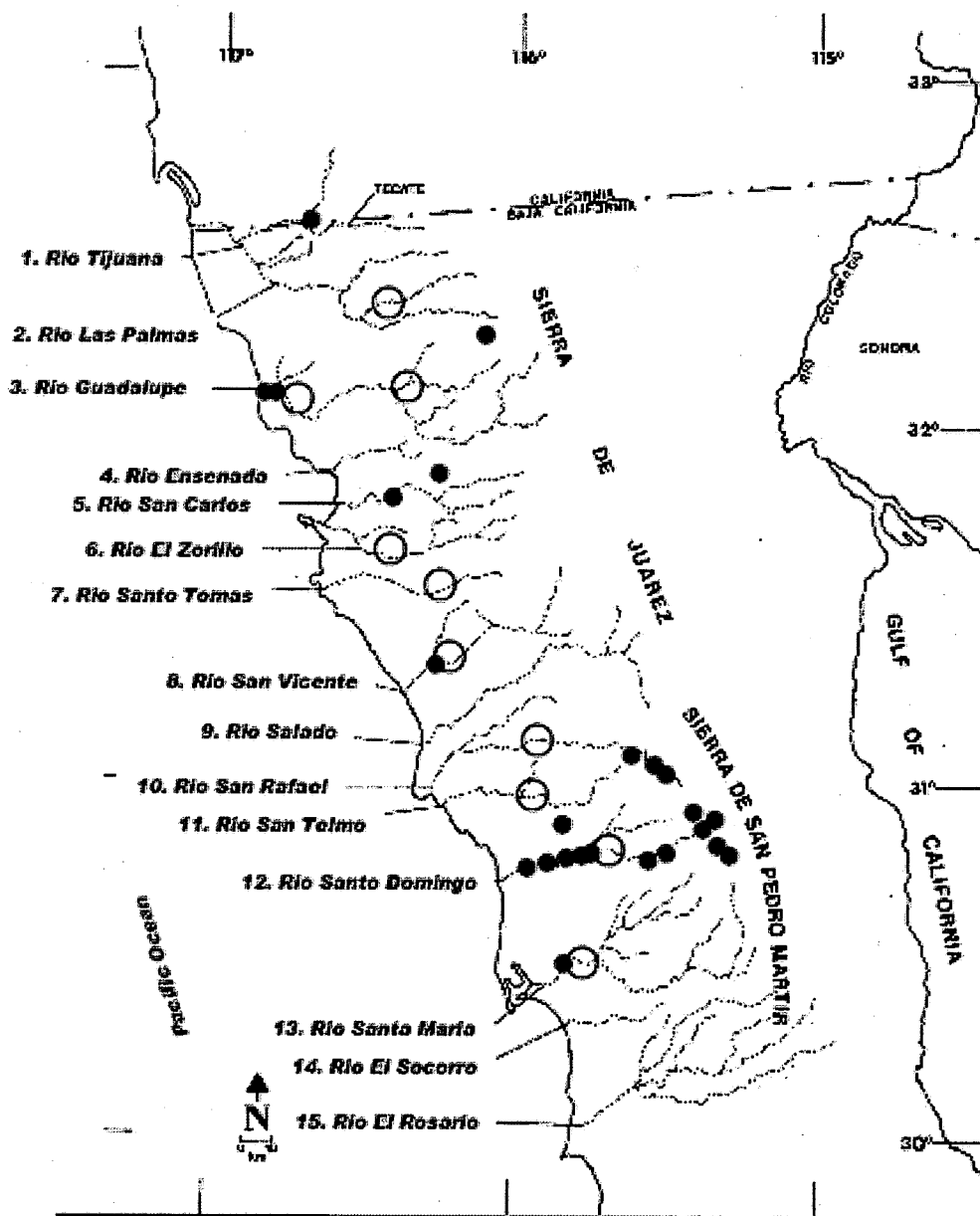


Figure 14. Major drainage systems surveyed for *Bufo californicus* and non-native aquatic species of northwestern Baja California, México. Closed circles represent historic localities, and open circles represent locality records of *B. californicus*. Numbers designate stream survey sites for this study (see Table 5).



### *Data compilation and stream surveys*

Twenty institutions were queried for *B. californicus* specimens from Baja California, México. Of these, six institutions provided locality records (Appendix 7). Published accounts were also summarized to supplement historical museum records. Fourteen major drainage systems (Fig. 1; see Murvosh and Allen 1994) were surveyed between April 2001 and August 2006 in northwestern Baja California from the Rio Tijuana south to Rio El Rosario and eastward to the foothills of the Sierra San Pedro Martir (Table 5). No rivers were surveyed south of the Rio El Rosario, since climate, annual precipitation, vegetation, and habitat transitions markedly to become the Central Desert of Baja California where suitable habitat and vouchered records for the arroyo toad do not occur. The Rio El Rosario is well known as the southern terminus of Mediterranean-type habitats on the mainland of western North America. The rivers surveyed ranged in watershed size from 334 km<sup>2</sup> to 4925 km<sup>2</sup>, and in length from 35 km to 138 km (Table 5). Several smaller coastal arroyos and streams (i.e. Cañada el Rosarito, Cañon el Descanso, Cañon el Carmen, Arroyo San Jacinto, Arroyo Seco) were examined, and eliminated from additional surveys based on the lack of suitable habitat features including <3% stream-slope gradient, sandy banks for burrowing, shallow pools for breeding, and access to upland habitat for over wintering and foraging (Atkinson et al. 2003).

Table 5: Survey sites, dates surveyed and findings for *B. californicus* in watersheds of northern Baja California. Coordinates represent start point of survey reach. Watershed names follow Murvosh and Allen (1994) unless otherwise noted. Watershed size (km<sup>2</sup>) is from Blásquez (1959). Watershed length is maximum distance (km) from headwaters to Pacific Ocean determined from Baja California Almanac (2003). See map (Fig. 1) for numeric designation of survey site localities.

Watershed	Survey Site	Coordinates	Date(s) Surveyed	<i>B. californicus</i>		Watershed Length (km)	Watershed (km <sup>2</sup> )
				present (+), absent (-)			
Rio Tijuana	1	32.50482°N, 116.59078°W	1-Apr-02	-		81	4925 <sup>(*)</sup>
Rio de Las Palmas	2	32.32502°N, 116.52541°W	23-Mar-02	+		128	-
		32.32580°N, 116.52521°W	16-May-05	+			
Rio Guadalupe	3	32.14293°N, 116.64025°W	8-Apr-01	+		117	2278
		32.14481°N, 116.49480°W	22-Mar-02	+			
		32.10052°N, 116.80344°W	16-May-05	+			
Rio Ensenada	4	31.93433°N, 116.49338°W	May-02	-		39	384
Rio Maneadero (San Carlos)	5	31.70007°N, 116.49461°W	1-Apr-02	-		61	1840
		31.82446°N, 116.25260°W	16-May-02	-			
Rio El Zorrillo	6	31.63739°N, 116.46583°W	5-Apr-02	+		61	-
		31.63773°N, 116.46771°W	17-May-05	+			
Rio Santo Tomás	7	31.62693°N, 116.47525°W	5-Apr-02	-		78	565
		31.54012°N, 116.22174°W	28-Mar-04	+			
Rio (Arroyo) San Vicente	8	31.33415°N, 116.25184°W	17-May-02	+		86	1728
		31.33392°N, 116.25322°W	17-May-05	+			
Arroyo Salado	9	31.25567°N, 116.05834°W	25-May-02	-		81	-
Rio San Rafael	10	31.03622°N, 116.02361°W	25-May-02	+		112	1621
		31.02802°N, 116.02525°W	18-May-05	+			
		31.10895°N, 116.62464°W	15-Aug-05	+			
Rio San Telmo (Rio San Jose)	11	30.92794°N, 115.73311°W	14-Apr-05	+		104	1424
Rio Santo Domingo	12	30.77387°N, 115.82180°W	28-May-01	+		95	1541
		30.81578°N, 115.65411°W	12-Aug-05	+			
Rio Santa María (Arroyo San Simón)	13	30.51182°N, 115.81264°W	15-Apr-05	+		114	1916
Rio (Arroyo) Socorro	14	30.34551°N, 115.77516°W	25-Jan-02	-		86	-
Rio El Rosario	15	30.15695°N, 115.54717°W	14-Mar-03	-		117	1538
		30.04932°N, 115.73341°W	15-Mar-03	-			
		30.15847°N, 115.54462°W	15-Apr-05	-			

(\*) denotes watershed size for the Rio Tijuana and Rio Las Palmas

Twenty three excursions were made to collect field data, including approximately 500 person-hours of focused survey time and daytime reconnaissance surveys. Survey locations were determined according to historic records, habitat suitability, and review of topographic maps (Baja California Almanac 2003). Daytime reconnaissance was made to access overall habitat quality, environmental conditions, and dominant vegetation. Nighttime surveys were employed following the pre-determination of habitat suitability and consisted of random encounter surveys (Heyer et al. 1994) and transects along a minimum reach of 300 m of suitable riparian habitat. For each survey location, the habitat was photographed and potential anthropogenic threats, non-native species, native aquatic species, and dominant plant species were recorded. The presence of *Bufo californicus* was detected by both visual and aural encounters and all life stages were documented. Individuals were measured (SVL), weighed (gm), photographed and/or collected and GPS coordinates were recorded. All individuals were sexed when possible.

## Results

Between 2001 and 2006 we collected locality information on specimens of *B. californicus*. Museum records and literature queries are indicated in Appendix 7, and indicate that *B. californicus* occurred in seven of fourteen major streams of Northwestern Baja California prior to this study (Table 6). Surveys reconfirmed seven, and added three drainages (Mahrtdt et al. 2003, Mahrtdt and Lovich 2004) previously undocumented (Table 6). Populations of *B. californicus* presently occur in ten of the fourteen streams surveyed (Table 6; Figure 14). The species was not found in the Mexican waters of the Rio Tijuana, a river which has the northern 1/3 of its watershed draining from California, USA. All historic records and voucher specimens within the Rio Tijuana occur in the

Table 6. Historical and present distribution of *Bufo californicus* and associated anthropogenic threats and non-native aquatic species occurring in watersheds of northwestern Baja California, Mexico. See Map (Fig. 1) for location of watersheds and streams.

Watershed	<i>Bufo californicus</i>		Threat <sup>2</sup>						Non-native Species <sup>3</sup>				
	Historic	Present <sup>1</sup>	Ag	Da	Gr	Ro	Sm	We	PRCL	GAAF	LECY	RACA	XELA
Tijuana-Las Palmas		x	x	x	x	x	x	x	x	x	x	x	x
Guadalupe	x	x	x		x	x	x	x	x*	x*	x**	x	
Ensenada					x	x	x	x	x*				
Maneadero (Arroyo San Carlos)	x	x	x		x	x	x	x	x	x		x	
El Zorillo		x	x		x	x			x	x			
Santo Tomas		x	x		x	x		x	x	x	x**		
San Vicente	x	x	x		x	x		x	x	x			
Salado			x		x	x							
San Rafael	x	x	x		x	x		x					
San Telmo (Rio San Jose)	x	x	x		x	x		x		x*x**	x**		
Santo Domingo	x	x	x		x	x	x	x		x*x**	x**		
Santa Maria (Arroyo San Simón)	x	x	x		x	x		x		x**			
Socorro			x		x	x		x					
El Rosario		x*	x		x	x		x		x**		x	

<sup>1</sup>Present: this study, 2001-2006.

<sup>2</sup>Threat abbreviations: Ag (Agriculture); Da (Dam); Gr (Grazing); Ro (Road); Sm (San Mining); We (Water Extraction).

<sup>3</sup>Non-native species abbreviations: PRCL (*Procambarus danieli*); GAAF (*Gambusia affinis*); LECY (*Lepomis cyanellus*); RACA (*Rana caferblana*); XELA (*Xenopus laevis*).

\* Data from Domínguez-Torres and Mellink (2003).

\*\* Data from Ruiz-Campos et al. (2000).

United States north of the International Border (R. Lovich pers. obs.). Age classes of all *A. californicus* observed varied by locality, with adults being the dominant class detected, followed by tadpoles, egg masses, and metamorphs (Table 6).

A total of six documented anthropogenic threats in the form of sand mining, water extraction, agriculture, roads, dams, and grazing are presented in Table 6. The Rio Tijuana-Rio Las Palmas had all six recorded threats, and was the only drainage system with a municipal dam. Grazing and roads were the most common threats, and sand mining was more prevalent in northern drainages than those in the south. The Rio El Zorillo and Rio San Rafael had the fewest threats (Table 6). While the number of threats in each stream ranged from three to six, the scale (e.g. areal extent, intensity) of threats differed within categories. For example, dirt roads are common in all watersheds surveyed, while paved roads are relatively scarce, and become increasingly so south of urban centers of Tijuana and Ensenada, respectively. Also, subsistence agriculture is common to nearly all populated areas. Large-scale industrial agriculture is relatively widespread with large wine-producing vineyards in the upper Rio Guadalupe and Rio Santo Tomas, and numerous crops that dominate the San Quintin Plain between the mouths of the Rio San Rafael southward to the Rio Santa Maria. Agricultural areas along the San Quintin Plain will soon give way to a mega-harbor to facilitate transport shipping into Baja California, and the United States (San Diego Union Tribune 2008).

The occurrence of non-native species based on our observations and data taken from Ruiz-Campos et al. (2000) and Dominguez-Torres and Mellink (2003) is shown in Table 2. *Gambusia affinis* was the most commonly encountered aquatic vertebrate occurring in all major streams. *Lepomis cyanellus* occurred in five streams;

*Procambarus clarkii* occurred in all but one (i.e. Rio Las Palmas) of the northernmost streams from the Rio Tijuana to the Rio San Vicente. Two non-native anurans, *Xenopus laevis* and *Rana catesbiana* were found in two and four streams, respectively. *Xenopus laevis* was found only in the Rio Las Palmas (Mahrtdt et al. 2002), whereas *R. catesbiana* was more widespread and occurred as far south as the Rio El Rosario. *Xenopus laevis* are also documented from the tributaries of the Rio Tijuana on the U.S.-Mexico border in southern San Diego County, California (Espinoza 1989), and may also occur in drainages of the Municipality of Tijuana, Baja California (Tinsley and McCoid 1996). There was a general correlation between proximity to the more populated cities of northern Baja California and positive detection of non-native species.

### Discussion

The distribution of *Bufo californicus* was until now depicted as largely disjunct (USFWS 1999); however our data suggests that the species is more widely distributed as populations throughout most major Pacific Coast river drainages of northern Baja California. These non-contiguous populations are the result of widespread degradation of upland and riparian habitats from several anthropogenic causes. The physical conditions of riparian habitat throughout the range of *B. californicus* vary considerably among rivers. The Rio Tijuana, lower Rio Las Palmas, Rio Ensenada, and Rio San Carlos have been altered dramatically as a result of development and land conversion. Other streams such as the upper Rio Guadalupe, Rio Santo Tomas, Rio San Vicente, Rio San Rafael, Rio San Telmo, Rio Santo Tomas, Rio Santo Domingo, and Rio Santa Maria have undeveloped headwaters with increasingly more developed and urbanized areas downstream. In concert with rapid human development of the Pacific coastal region,

urban areas also occur where Mexican Highway 1 crosses respective streams. These locations generally have rapidly growing human populations and infrastructure which requires a continuous water supply directly from the adjacent watersheds.

Based on habitat characteristics (e.g. lack of suitable substrate, small watershed size,) it is likely that the Rio Ensenada and Rio Socorro, do not represent suitable habitat for *B. californicus*. Although Dominguez-Torres and Mellink (2003) reported *B. californicus* from Cañada el Morro (el Descanso), and Rio El Rosario, extensive surveys of these drainages do not corroborate their observations. The Rio El Rosario lies at the division of the California Phytogeographic Province and the Central Desert of Baja California, and is inhabited by *Bufo punctatus*, a xerophilic species not found in any other Pacific-draining rivers north of the Rio El Rosario (Grismer 2002). Competition with *B. punctatus* and/or the lack of suitable habitat, average annual rainfall (< 10 cm) and infrequent episodic flooding are likely reasons for the absence of *B. californicus* in the Rio El Rosario. Upstream, the Rio Los Martires, one of several tributaries of Rio El Rosario, was a perennial source of water during 2002-2006 surveys. In addition to *B. punctatus*, surveys revealed the presence of tadpole and metamorph *Scaphiopus hammondii* and *Hyla regilla*.

Supplying enough water to support human population growth and agriculture is a considerable challenge for local government municipalities, and is also creating challenges for maintenance of riparian habitats. Groundwater pumping using diesel generators is becoming widespread, with a noticeable increase in systems being installed at many of the rivers visited between 2002 and 2006. Competition for water has put a premium on management of this limited resource. Minnich et al. (2000) suggested the

addition of dams to solve the problem of water deficiency in the northwestern region. However the construction of year-round water sources, thereby flooding lotic habitats, may threaten *B. californicus* (Sweet 1991, 1993; Campbell, et al. 1996). Dams would also serve as refugia for non-native species that require a permanent water supply. All these activities can potentially affect the viability of riparian drainages as habitat for *Bufo californicus*. During the course of our survey work from 2001 to present, we witnessed additional wells and delivery systems being built in major coastal rivers (e.g. Rio Santa Maria, Rio Guadalupe, Rio Santo Domingo, Rio El Rosario). Ruiz-Campos et al. (2000) commented that widespread agriculture in cismontane watersheds may have long term deleterious effects on the aquatic and semi-aquatic fauna of northern Baja California. Water extraction has even been observed to 'dry down' pools within a few hours, thus causing direct mortality of arroyo toad tadpoles by prolonged exposure to air (B. Haase pers. comm.).

Agricultural land use has resulted in the apparent extirpation of *B. californicus* from their southernmost locality (Gergus et al. 1997) in the alluvial floodplain of the Rio Santa Maria. At this locality, riparian habitat has been directly converted to commercial planting on an enormous scale. After four years of field work and above normal winter rains, a breeding population of *B. californicus* was located on April 15, 2003 in the Rio Santa Maria seven km upstream from the Gergus et al. (1997) site. Breeding activity was also observed in *Spea hammondi* and *Bufo boreas* within 10 m of breeding *B. californicus* at this same time and location. Because of its widespread nature, its implementation directly within riparian habitats, and its reliance on water pumped from



the rivers, agriculture has reduced the occupied habitat for *B. californicus* , and little coastal habitat remains for the *B. californicus*.

The sand mining industry is impacting the Rio Guadalupe, Rio Las Palmas, Rio Ensenada, and other smaller coastal arroyos by providing the necessary raw materials to support some of California's construction industry. Sand and rock are extracted in large volumes affecting the physical and hydrological characteristics of the arroyos and eliminating riparian habitat. Despite public opposition to this activity (Peñuelas, 2004), the Mexican government has issued concessions to extract 900,000 m<sup>3</sup> of sand from the Rio Guadalupe from August 2004 -March 2008 (Gortázar, 2004). Considering the dependence of *B. californicus* on riparian sand flats, the viability of northern Baja California populations are threatened by sand mining. According to the United States Endangered Species Act, the legality of importing sand in Baja California to California is questionable, because sand extraction is directly affecting a species which is protected.

It is widely known that non-native species left unmanaged can result in the reduction in population numbers and diversity of native species (Lannoo 2005). Non-native species were present in nearly all river drainages (Table 6) with the exception of Rio Socorro. These species include, in decreasing order of relative abundance, *Gambusia affinis*, *Lepomis cyanellus*, *Procambarus* sp., *Rana catesbiana*, and *Xenopus laevis*. *Rana catesbiana* is common in the Rio Tijuana, Rio Maneadero and Rio El Rosario. This species has also been shown to prey upon and adversely affect populations of native anurans (Moyle 1973, Pearl et al. 2004). *Rana catesbiana* was introduced to California to replace diminishing numbers of *Rana draytonii* as a food source (Jennings and Hayes 1985) and *X. laevis* were used for pregnancy testing in the 1940's and 1950's (Crayon

2005) before the advent of over-the-counter testing products. *Gambusia sp.* is likely found widely throughout rivers in Baja California as a result of their use as vector control to reduce breeding of mosquitos. The presence of *Gambusia affinis* in large numbers in most rivers in the region may have a direct threat on the survivorship of *B. californicus* larvae (Goodsell and Kats 1999). *Procambarus sp.* has been linked to the absence of breeding success in *B. californicus* (Brehme et al. 2006. Ruiz-Campos et al. (2000) There has also been reported stocking of *Micropterus salmoides* to promote sportfishing, and the stocking of *Onchorhynchus mykiss nelsoni* in streams other than its native Rio Santo Domingo between 1929 and 1941. However, there is no direct evidence suggesting that *O. m. nelsoni* preys upon egg and larval stages of *B. californicus* anywhere within its natural range. This may be due, in part, to minimal or no overlap in the microhabitat of *B. californicus* and *O. m. nelsoni* (R. Fisher, pers. comm.). Considering the widespread establishment of non-native species in nearly all rivers in northern Baja California, their control or eradication should be a high priority for conservationists and resource managers.

The decline in populations of *B. californicus* have already occurred (Jennings and Hayes 1994), including the elimination the species from the Mexican waters of the Rio Tijuana. Given the endangered status of the species in the United States, in concert with evidence of declines in México per this study, some level of riparian and species-specific management and conservation should be considered. Increases in human population pressures and continued loss of *B. californicus* populations will, through time, reduce opportunities for conservation and enhancement of existing populations, as well as the potential for reintroduction of the species. The loss of populations will likely result in

further reduction of the genetic variation found in this species *B. californicus* is an indicator of a healthy riparian ecosystem, and its loss from areas within its range does not bode well for riparian ecosystem integrity in the California Phytogeographic Region.

This study has shown that arroyo toads are found in more rivers than were previously known in Baja California, and has also identified a number of potential threats to the species. These threats are no different from those that exist in the United States (USFWS 1999), including the non-native species. Planned and continuing growth of human population and associated infrastructure will further reduce available riparian habitat for *B. californicus* and other species. Conservation efforts and research should focus on extant populations to further increase our knowledge of the arroyo toad, along with the southernmost rivers of the Mediterranean region of México.

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APPENDIX A

MATERIAL EXAMINED FOR PHYLOGENETIC ANALYSIS

Abbreviations are as follows: Marine Corps Base Camp Pendleton (MCBCP), Multiple Species Conservation Plan (MSCP), United States Forest Service (USFS).

Species	Sample ID	Country	Sequence	Watershed	Land Status
<i>Bufo boreas</i>	BuBoDS053	MX	Baja California: La Misión: Rio Guadalupe	Rio Guadalupe	
<i>Bufo woodhousei</i>	BuBoDS077	USA	California: San Diego: Warner Springs	San Luis Rey River	
<i>Bufo woodhousei</i>	BuWo1DS	USA	California: Imperial Co.: Salton City	Salton Sea	
<i>Bufo microscaphus</i>	BuMiTC9	USA	Utah: Taylor's Creek: Kolob Canyon	Colorado River	
<i>Bufo microscaphus</i>	BuMiAG75	USA	Arizona: Yavapai: Vicinity of Dewey	Gila River	
<i>Bufo californicus</i>	DS002	USA	California: Orange: San Juan Creek	San Juan Creek	Private, County, USFS
<i>Bufo californicus</i>	DS004	USA	California: San Diego: San Mateo Creek	San Mateo River	MCBCP
<i>Bufo californicus</i>	DS008	USA	California: San Diego: Cottonwood Creek	Tijuana River	County MSCP
<i>Bufo californicus</i>	DS019	USA	California: San Diego: San Luis Rey River	San Luis Rey River	Private, Indian, County, USFS
<i>Bufo californicus</i>	DS025	USA	California: San Diego: Boden Canyon	San Dieguito River	Private, County MSCP
<i>Bufo californicus</i>	DS028	USA	California: Orange: Gabino Creek	San Mateo River	Private
<i>Bufo californicus</i>	DS029	USA	California: San Diego: Santa Margarita River	Santa Margarita	MCBCP
<i>Bufo californicus</i>	DS031	USA	California: San Bernardino: Mojave River	Mojave River	Private, USFS
<i>Bufo californicus</i>	DS032	USA	California: Riverside: Santa Rosa Plateau	Santa Margarita	TNC
<i>Bufo californicus</i>	DS035	USA	California: Monterey: San Antonio Creek	Salinas River	Fort Hunter Liggett
<i>Bufo californicus</i>	DS041	USA	California: Santa Barbara: Santa Ynez River	Santa Ynez	USFS
<i>Bufo californicus</i>	DS049	USA	California: Ventura: Piru Creek	Santa Clara River	USFS, Private
<i>Bufo californicus</i>	DS062	USA	California: Riverside: Bautista Creek	Santa Ana River	USFS, Private
<i>Bufo californicus</i>	DS063	USA	California: Santa Barbara: Sisquoc Creek	Santa Maria River	USFS, Private
<i>Bufo californicus</i>	DS064	USA	California: Los Angeles: Littlerock Creek	Littlerock Creek	USFS, Private
<i>Bufo californicus</i>	DS066	USA	California: Los Angeles: Tujunga Creek	Los Angeles River	USFS
<i>Bufo californicus</i>	DS067	MX	Baja California: Guadalupe: Rio Guadalupe	Rio Guadalupe	Private, Municipal
<i>Bufo californicus</i>	DS070	MX	Baja California: La Grulla: Rio El Zorillo	Rio El Zorillo	Private, Municipal
<i>Bufo californicus</i>	DS074	MX	Baja California: Potrero: Rio San Rafael	Rio San Rafael	Private, Municipal
<i>Bufo californicus</i>	DS078	USA	California: San Diego: Santa Ysabel Creek	San Dieguito River	USFS, Private
<i>Bufo californicus</i>	DS087	USA	California: San Diego: San Vicente Reservoir	San Diego River	Private, Indian, County, USFS
<i>Bufo californicus</i>	DS089	MX	Baja California: Santo Tomas: Rio Santo Tomas	Rio Santo Tomas	Private, Municipal
<i>Bufo californicus</i>	DS094	MX	Baja California: Santa Maria: Rio Santa Maria	Rio Santa Maria	Private, Municipal
<i>Bufo californicus</i>	DS104	MX	Baja California: Santo Domingo: Rio Santo Domingo	Rio Santo Domingo	Private, Municipal

## APPENDIX B

### ND1 MTDNA SEQUENCE DATA (859 BASE PAIRS) ANALYZED AS PART OF THIS STUDY

BuMi\_TC9\_DS

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BuMi\_AG75\_DS

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BuWo1\_DS

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DS019

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DS067

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DS078

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DS087

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DS063

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ATTTTTTCTCGCGAATACGCTAATATTCTTATAATAAACACAATTTCTGCTGTTATTTTTCTTAGGATCCT  
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CTCTGAG

DS041

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CCACCCCTCTTTCTTATCATTGACAACAACATCACTAATATTTAAATCAGCCATTCTATCCATAGTTTTT  
CTCTGAG

DS049

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CCACCCCTCTTTCTTATCATTGACAACAACATCACTAATATTTAAATCAGCCATTCTATCCATAGTTTTT  
CTCTGAG

DS066

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CTCTGAG

DS064

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CCACCCTTCTTTTCTTATCATTGACAACAACATCACTAATATTTAAATCAGCCATTCTATCCATAGTTTTT  
CTCTGAG

DS035

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GCCCTAATTGGAGCCCTTCGAGCAGTAGCACAAACAATCTCTTATGAGGTTACCTTAGCCCTTATTCTCCT  
ATGCACAATTCTTCTATCAGGAACTTCTCTCTTCAAACTTCAGTATTACTCAAGAACCTTTATGACTTA  
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CCACCCTTCTTTTCTTATCATTGACAACAACATTACTAATATTTAAATCAGCCATTCTATCCATAGTTTTT  
CTCTGAG

DS025

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CCACCCTTCTTTTCTTATCATTGACAACAACATCACTAATATTTAAATCAGCCATTCTATCCATAGTTTTT  
CTCTGAG

DS031

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CCACCCTTCTTTTCTTATCATTGACAACAACATCACTAATATTTAAATCAGCCATTCTATCCATAGTTTTT  
CTCTGAG

DS094

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CTCTGAG

DS074

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CTCTGAG

DS028

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CTCTGAG

DS004

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CTCTGAG

DS032

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CTCTGAG

DS029

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CTCTGAG

DS002

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CTCTGAG

DS062

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CCACCCTTCTTTTCTTATCATTGACAACAACATCACTAATATTTAAATCAGCCATTCTATCCATAGTTTTT  
CTCTGAG

BuBo\_DS077

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CTTTGAG

BuBo\_DS053

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CCACCCTTCTTCTACTGTCATTTACTACAACATCATTAATATTTAAAATCGGCCGCCCTTTCTATAGTATTT  
CTTTGAG

## APPENDIX C

### DLOOP MTDNA SEQUENCE DATA (451 BASE PAIRS) ANALYZED AS PART OF THIS STUDY

DS028

GTCAGATGCCTCCTGAAGCGGGATTACTGCCACTCTTGTGTTAGGTCCCTTGGAGATTAAGATCTCCAAGT  
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CAAAGTTAATGAGGATATAAGTTAGATTAATGTTTGATCTATTACTGTTGATTTTTAAGAAGGTTATTAA  
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BuMi

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CAAAGTTAATGGGGATATGAGTTGGATTAATGTTTGAGCTAAAACCTGTTGATTTTTAAGAAGGTTAATAA  
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DS008

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CAAAGTTAATGGGGATATAAGTTAGATTAATGTTTGATCTATTACTGTTGATTTTTAAGAAGGTTATTAA  
ACAGGTATTGTTATAAATATACGAGAGGTCTAAATAATTAACCTGCTCTATAAAAATAGGTTGTATGTCTTAA  
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DS049

GTCAGATGCCTCCTGAAGCGGGATTACTGCCACTCTTGTGTTAGGTCCCTTGGAGATTAAGATCTCCAAGT  
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DS031

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BuMi2

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DS041

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CAAAGTTTAATGGGGATATAAGTTAGATTAATGTTTGATCTATTACTGTTGATTTTTAAGAAGGTTATTAA  
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BuMi 3

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CAAAGTTTAATGGGTATATTAGTTAGATTAATGTTTGATCTAGTATTGTTGATTTTTAAGAAGGTTATTAA  
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DS002

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DS025

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DS066

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DS063

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CAAAGTTTAATGGGGATATAAGTTAGATTAATGTTTGATCTATTACTGTTGATTTTTAAGAAGGTTATTAA  
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DS074

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DS067

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CAAAGTTTAAATGGGGATATAAGTTAGATTAATGTTTGATCTATTACTGTTGATTTTAAAGAAGGTTATTAA  
ACAGGTATTGTTATAAAATATACGAGAGGTCTAAATAATTAAGTCTCTATAAAATAGGTTGTATGTCTTAA  
ATCATTGATGATATAAGGAATATTA

## APPENDIX D

### COMBINED ND1 AND DLOOP MTDNA SEQUENCE DATA (1309 BASE PAIRS) ANALYZED AS PART OF THIS STUDY

BuMiAG75C

ACCCTTTCATAGAAGTTCAAATCTTCTCGTTAACTTTGAACCTATTTCTCATTATTTCTACACTTTGCTA  
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BuMiTC9C

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BuWo1C

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DS002C

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DS004C

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DS008C

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DS019C

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DS025C

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DS028C

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DS031C

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DS041C

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DS049C

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DS062C

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DS063C

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DS064C

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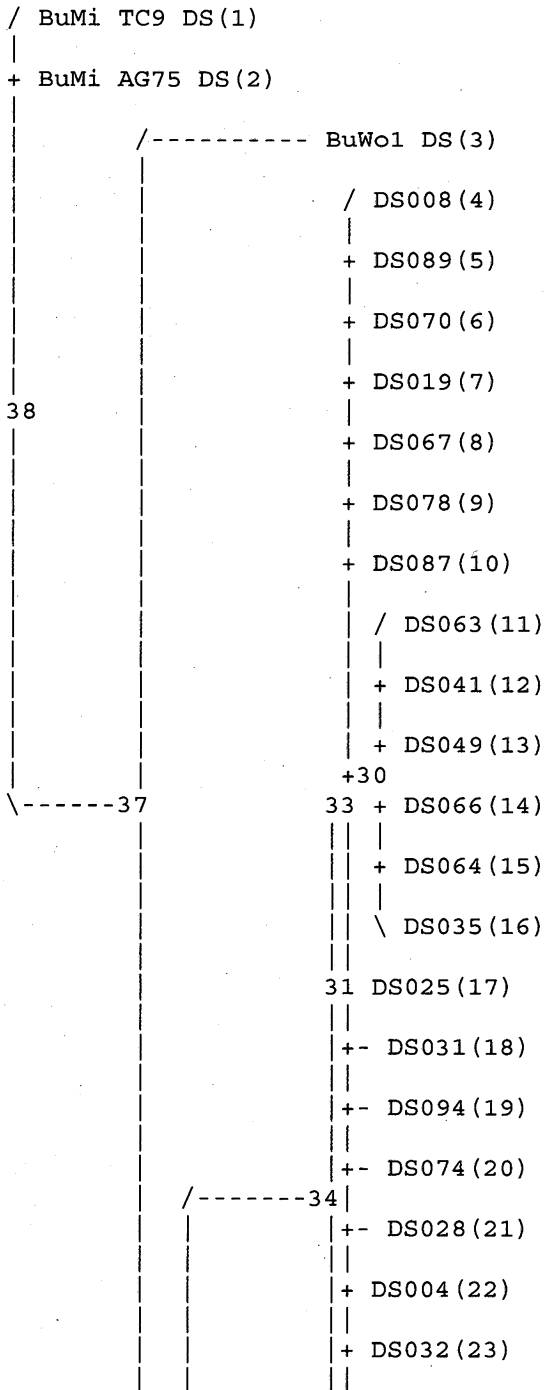
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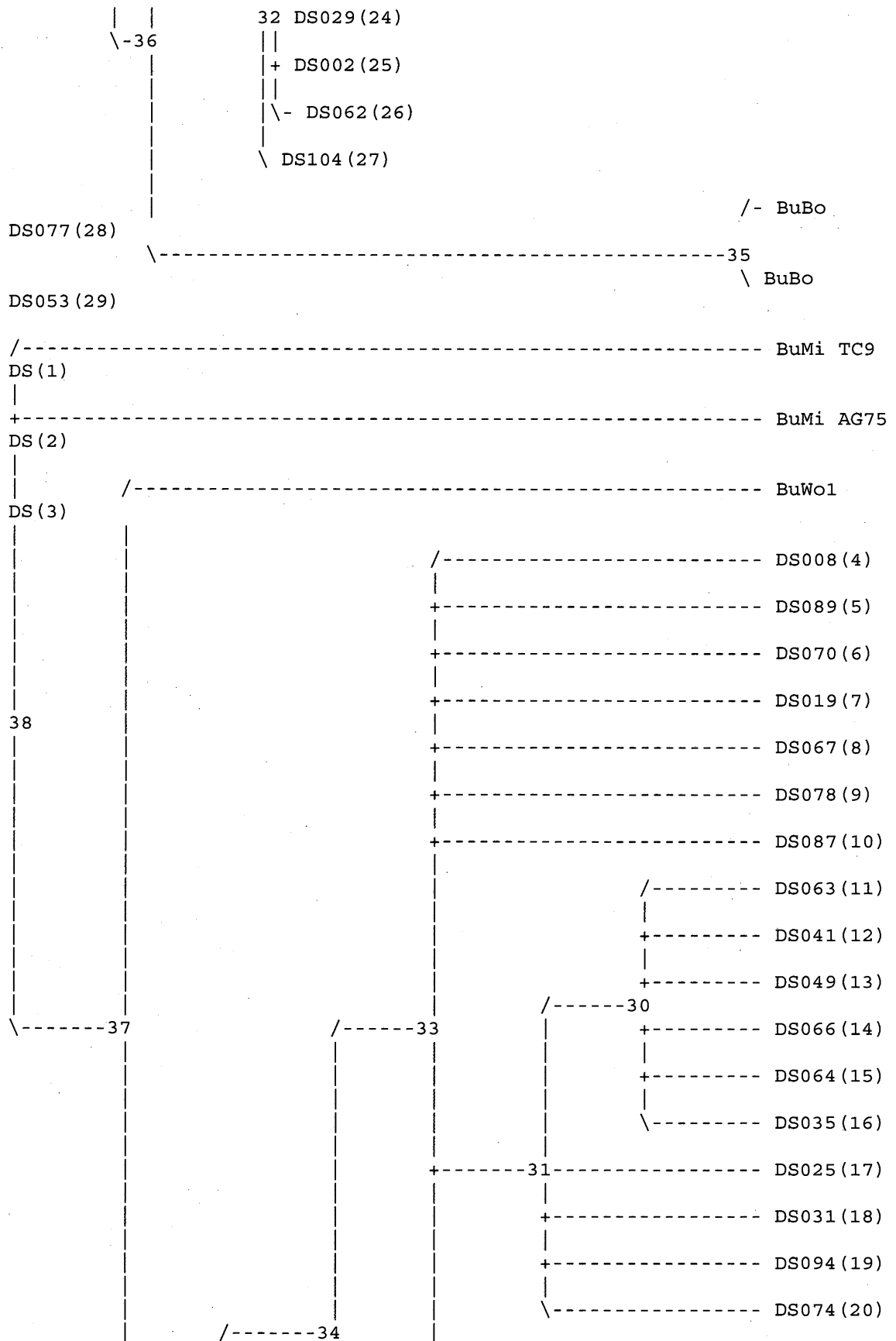
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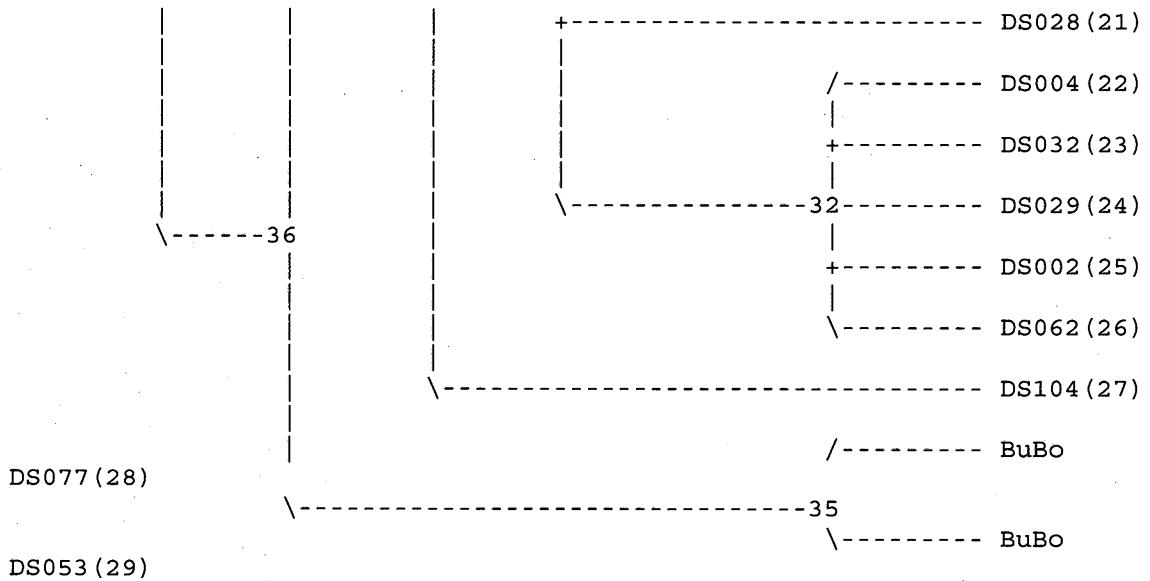
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APPENDIX E

ND1 APOMORPHY LIST







Apomorphy lists:

Branch	Character	Steps	CI	Change
node_38 --> BuMi TC9 DS	402	1	1.000	A ==> G
node_38 --> node_37	9	1	1.000	C ==> T
	135	1	1.000	C ==> T
	141	1	1.000	G ==> A
	189	1	1.000	T ==> C
	339	1	1.000	G ==> A
	342	1	1.000	T ==> C
	369	1	1.000	C ==> T
	456	1	1.000	G ==> A
	525	1	1.000	T ==> C
	633	1	1.000	T ==> C
	664	1	1.000	T ==> C
	690	1	1.000	C ==> T
	720	1	1.000	T ==> C
	783	1	1.000	A ==> C
	802	1	1.000	C ==> T
	838	1	1.000	T ==> C
node_37 --> BuWo1 DS	51	1	0.667	C --> T
	61	1	1.000	A ==> G
	156	1	1.000	T ==> C
	204	1	1.000	T ==> C
	258	1	1.000	T ==> C
	282	1	1.000	C ==> T
	306	1	1.000	C ==> T
	315	1	0.500	T --> C
	328	1	0.500	T --> C
	345	1	1.000	T --> C
	357	1	1.000	T ==> C
	361	1	0.500	C --> T
	366	1	1.000	C ==> T
	399	1	1.000	C ==> A
	471	1	1.000	T ==> C
	513	1	1.000	A ==> G

	573	1	0.500	C --> T
	576	1	0.500	C --> A
	594	1	0.500	G --> A
	648	1	1.000	C ==> A
	669	1	1.000	A ==> G
	762	1	1.000	T ==> A
node_37 --> node_36	253	1	1.000	T ==> C
	276	1	1.000	A ==> G
	477	1	1.000	C ==> T
	510	1	1.000	C ==> T
	663	1	1.000	A ==> G
	744	1	1.000	G ==> A
	763	1	0.500	A ==> G
node_36 --> node_34	61	1	1.000	A ==> T
	195	1	1.000	T --> C
	345	1	1.000	T --> A
	411	1	1.000	C ==> A
	414	1	1.000	A ==> C
	444	1	1.000	A --> T
	465	1	1.000	T ==> C
	474	1	1.000	A ==> G
	495	1	1.000	T ==> C
	585	1	1.000	A --> G
	591	1	1.000	C ==> T
	594	1	0.500	G --> A
	666	1	1.000	A ==> T
	771	1	1.000	T ==> C
	795	1	1.000	T --> C
	798	1	1.000	G ==> A
	804	1	1.000	A --> G
	843	1	1.000	T ==> C
	855	1	1.000	T ==> C
node_34 --> node_33	279	1	1.000	C ==> T
node_33 --> node_31	222	1	0.500	G ==> A
node_31 --> node_30	333	1	1.000	T ==> C
	571	1	1.000	A ==> G
	600	1	0.500	A ==> G
node_30 --> DS041	684	1	1.000	A ==> G
node_30 --> DS035	815	1	1.000	C ==> T
node_31 --> DS031	42	1	1.000	C ==> T
node_31 --> DS094	432	1	1.000	A ==> G
node_31 --> DS074	54	1	1.000	T ==> C
	250	1	1.000	A ==> G
node_33 --> DS028	49	1	0.500	C ==> T
	808	1	1.000	A ==> G
node_33 --> node_32	763	1	0.500	G ==> A
node_32 --> DS062	322	1	0.500	A ==> G
node_36 --> node_35	8	1	1.000	C ==> T
	44	1	1.000	T ==> C
	45	1	1.000	A ==> G
	49	1	0.500	C ==> T
	57	1	1.000	T ==> C
	69	1	1.000	C ==> T
	81	1	1.000	A ==> C
	109	1	1.000	C ==> T
	111	1	1.000	T ==> A
	117	1	1.000	A ==> G

153	1	1.000	A ==> G
156	1	1.000	T ==> G
192	1	1.000	C ==> T
195	1	1.000	T --> G
198	1	1.000	T ==> C
201	1	1.000	A ==> G
234	1	1.000	G ==> A
237	1	1.000	C ==> T
243	1	1.000	T ==> A
259	1	1.000	C ==> T
261	1	1.000	T ==> A
265	1	1.000	G ==> A
282	1	1.000	C ==> A
288	1	1.000	A ==> G
315	1	0.500	T --> C
318	1	1.000	A ==> G
321	1	1.000	T ==> C
322	1	0.500	A ==> G
324	1	1.000	T ==> C
325	1	1.000	C ==> T
328	1	0.500	T --> C
330	1	1.000	A ==> C
336	1	1.000	T ==> C
345	1	1.000	T --> G
351	1	1.000	A ==> T
354	1	1.000	A ==> G
361	1	0.500	C --> T
367	1	1.000	C ==> T
369	1	1.000	T ==> A
378	1	1.000	C ==> T
381	1	1.000	C ==> T
384	1	1.000	C ==> T
387	1	1.000	C ==> T
390	1	1.000	T ==> A
402	1	1.000	A ==> T
405	1	1.000	A ==> C
420	1	1.000	T ==> C
429	1	1.000	C ==> T
438	1	1.000	A ==> G
442	1	1.000	C ==> T
444	1	1.000	A --> G
459	1	1.000	A ==> G
481	1	1.000	T ==> C
483	1	1.000	A ==> G
498	1	1.000	A ==> T
528	1	1.000	T ==> C
540	1	1.000	C ==> T
543	1	1.000	T ==> C
546	1	1.000	T ==> C
549	1	1.000	T ==> C
559	1	1.000	T ==> A
565	1	1.000	C ==> T
567	1	1.000	T ==> A
573	1	0.500	C --> T
576	1	0.500	C --> A
579	1	1.000	T ==> C
582	1	1.000	A ==> G



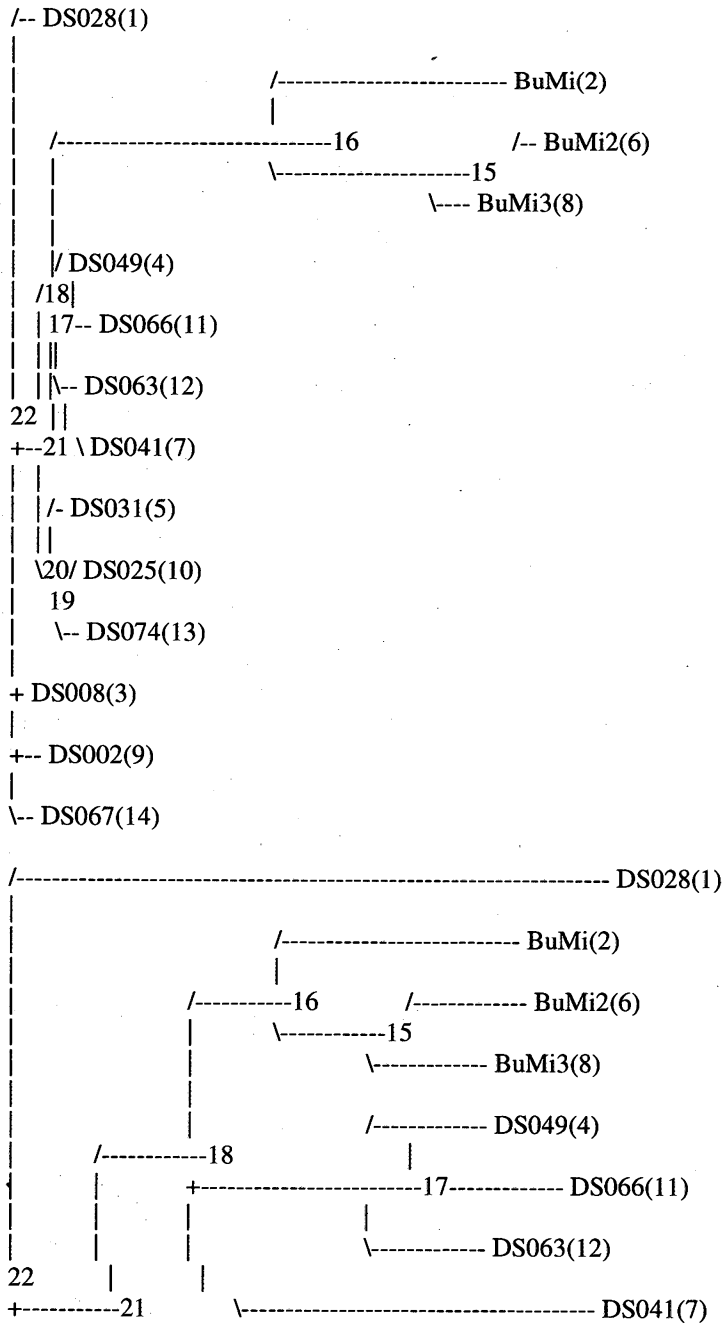
585	1	1.000	A --> C	
586	1	1.000	C ==> T	
597	1	1.000	A ==> G	
600	1	0.500	A ==> G	
624	1	1.000	T ==> A	
627	1	1.000	T ==> C	
636	1	1.000	A ==> G	
642	1	1.000	T ==> C	
645	1	1.000	C ==> T	
648	1	1.000	C ==> T	
657	1	1.000	G ==> A	
669	1	1.000	A ==> T	
693	1	1.000	A ==> T	
726	1	1.000	A ==> G	
750	1	1.000	C ==> T	
753	1	1.000	A ==> T	
759	1	1.000	T ==> C	
777	1	1.000	A ==> G	
793	1	1.000	T ==> C	
795	1	1.000	T --> A	
796	1	1.000	T ==> C	
804	1	1.000	A --> T	
807	1	1.000	A ==> T	
817	1	1.000	C ==> T	
825	1	1.000	T ==> A	
831	1	1.000	A ==> G	
835	1	1.000	A ==> G	
836	1	1.000	T ==> C	
837	1	1.000	T ==> C	
840	1	1.000	A ==> T	
849	1	1.000	T ==> A	
node_35 --> BuBo DS077	23	1	1.000	T ==> C
	51	1	0.667	C --> T
	222	1	0.500	G ==> A
node_35 --> BuBo DS053	51	1	0.667	C --> A

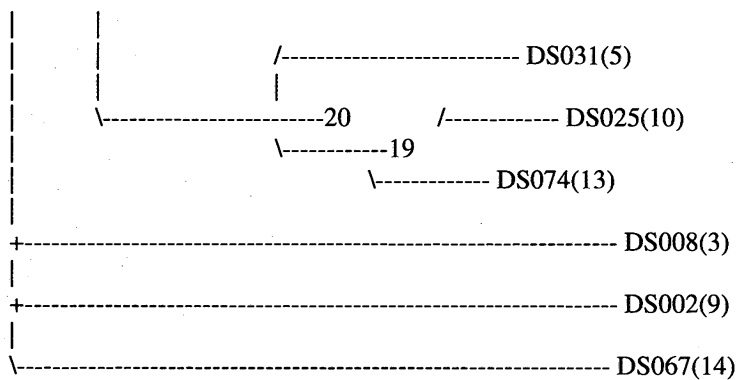
# APPENDIX F

## DLOOP APOMORPHY LIST

Dloop

Tree 1





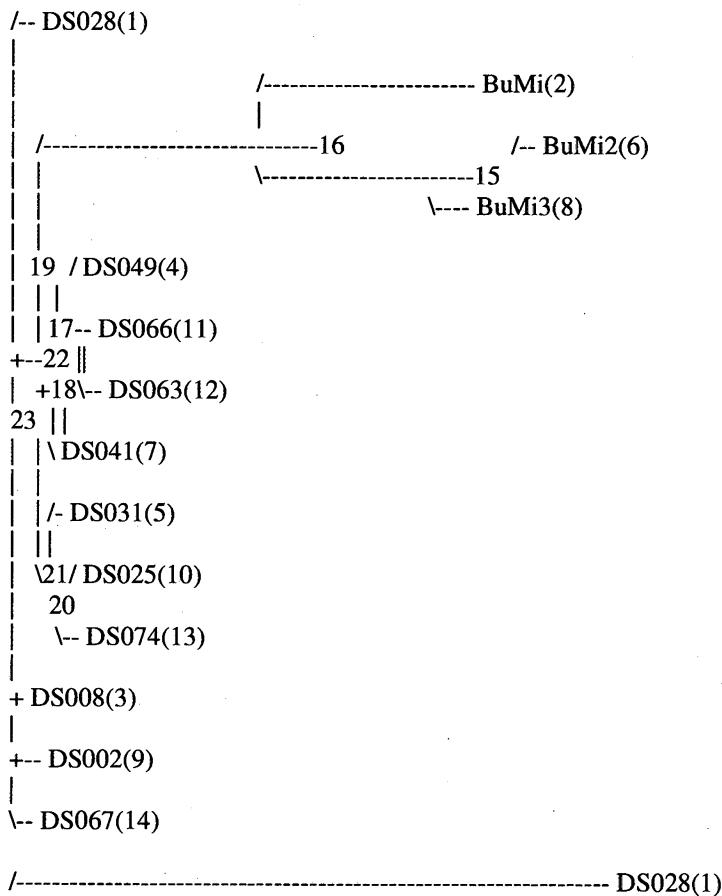
Tree 1

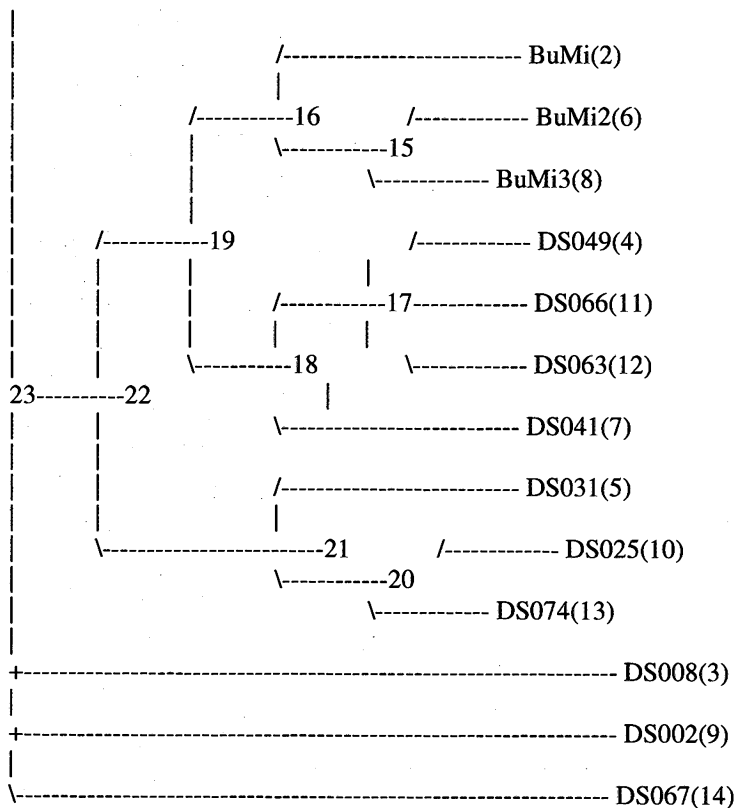
Apomorphy lists:

Branch	Character	Steps	CI	Change
node_22 --> DS028	297	1	1.000	G ==> A
node_22 --> node_21	239	1	1.000	G ==> A
	359	1	0.500	G ==> A
node_21 --> node_18	151	1	0.500	A ==> G
node_18 --> node_16	83	1	1.000	C ==> T
	85	1	1.000	G ==> T
	146	1	1.000	G ==> T
	165	1	1.000	C ==> T
	166	1	1.000	G ==> A
	169	1	1.000	T ==> A
	171	1	1.000	A ==> G
	172	1	1.000	A ==> G
	220	1	1.000	G ==> A
	228	1	1.000	A ==> T
	274	1	1.000	T ==> C
	360	1	1.000	G ==> T
	361	1	1.000	T ==> G
	377	1	1.000	C ==> T
	391	1	1.000	A ==> G
	397	1	1.000	C ==> T
	403	1	1.000	T ==> A
	413	1	1.000	G ==> A
node_16 --> BuMi	12	1	1.000	C ==> T
	30	1	1.000	C ==> T
	151	1	0.500	G ==> A
	163	1	1.000	G ==> A
	199	1	1.000	A ==> T
	203	1	1.000	T ==> A
	263	1	1.000	A ==> T
	266	1	1.000	A ==> T
	304	1	1.000	A --> G
	309	1	1.000	A ==> G
	323	1	1.000	T ==> G
	327	1	1.000	T --> A
	328	1	1.000	T ==> A
	352	1	1.000	T ==> A
node_16 --> node_15	117	1	1.000	A ==> G
	143	1	1.000	C ==> T

227	1	1.000	A ==> G
299	1	1.000	G ==> T
304	1	1.000	A --> T
327	1	1.000	T --> G
330	1	1.000	C ==> T
369	1	1.000	T ==> G
380	1	1.000	G ==> A
408	1	1.000	A ==> G
412	1	1.000	G ==> A
423	1	1.000	T ==> A
427	1	1.000	A ==> G
node_15 --> BuMi2	359	1	0.500 A ==> G
node_15 --> BuMi3	124	1	1.000 C ==> T
224	1	1.000	G ==> A
node_18 --> node_17	260	1	1.000 T ==> C
node_17 --> DS066	175	1	0.500 A ==> G
node_17 --> DS063	360	1	1.000 G ==> C
node_21 --> node_20	138	1	1.000 A ==> T
node_20 --> DS031	300	1	1.000 A ==> G
node_20 --> node_19	175	1	0.500 A ==> G
node_19 --> DS074	23	1	1.000 A ==> T
node_22 --> DS002	25	1	1.000 T ==> C
node_22 --> DS067	236	1	1.000 G ==> A

Tree 2





Tree 2

Apomorphy lists:

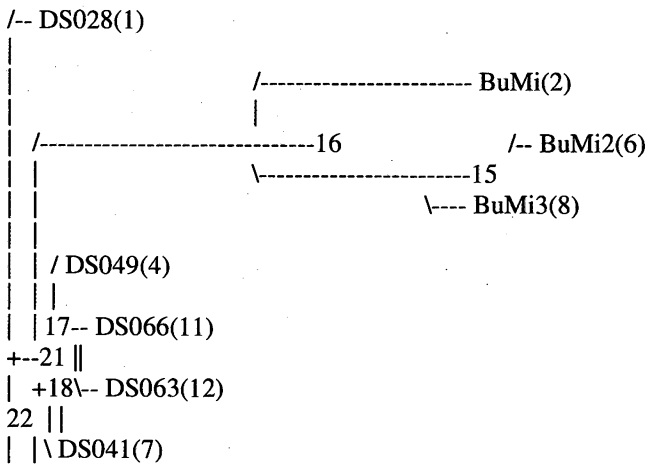
Branch	Character	Steps	CI	Change
node_23 --> DS028	297	1	1.000	G ==> A
node_23 --> node_22	239	1	1.000	G ==> A
	359	1	0.500	G ==> A
node_19 --> node_16	83	1	1.000	C ==> T
	85	1	1.000	G ==> T
	146	1	1.000	G ==> T
	165	1	1.000	C ==> T
	166	1	1.000	G ==> A
	169	1	1.000	T ==> A
	171	1	1.000	A ==> G
	172	1	1.000	A ==> G
	220	1	1.000	G ==> A
	228	1	1.000	A ==> T
	274	1	1.000	T ==> C
	360	1	1.000	G ==> T
	361	1	1.000	T ==> G
	377	1	1.000	C ==> T
	391	1	1.000	A ==> G
	397	1	1.000	C ==> T
	403	1	1.000	T ==> A
	413	1	1.000	G ==> A
node_16 --> BuMi	12	1	1.000	C ==> T
	30	1	1.000	C ==> T
	163	1	1.000	G ==> A

```

199      1 1.000 A ==> T
203      1 1.000 T ==> A
263      1 1.000 A ==> T
266      1 1.000 A ==> T
304      1 1.000 A --> G
309      1 1.000 A ==> G
323      1 1.000 T ==> G
327      1 1.000 T --> A
328      1 1.000 T ==> A
352      1 1.000 T ==> A
node_16 --> node_15 117      1 1.000 A ==> G
143      1 1.000 C ==> T
151      1 0.500 A --> G
227      1 1.000 A ==> G
299      1 1.000 G ==> T
304      1 1.000 A --> T
327      1 1.000 T --> G
330      1 1.000 C ==> T
369      1 1.000 T ==> G
380      1 1.000 G ==> A
408      1 1.000 A ==> G
412      1 1.000 G ==> A
423      1 1.000 T ==> A
427      1 1.000 A ==> G
node_15 --> BuMi2 359      1 0.500 A ==> G
node_15 --> BuMi3 124      1 1.000 C ==> T
224      1 1.000 G ==> A
node_19 --> node_18 151      1 0.500 A --> G
node_18 --> node_17 260      1 1.000 T ==> C
node_17 --> DS066 175      1 0.500 A ==> G
node_17 --> DS063 360      1 1.000 G ==> C
node_22 --> node_21 138      1 1.000 A ==> T
node_21 --> DS031 300      1 1.000 A ==> G
node_21 --> node_20 175      1 0.500 A ==> G
node_20 --> DS074 23       1 1.000 A ==> T
node_23 --> DS002 25       1 1.000 T ==> C
node_23 --> DS067 236      1 1.000 G ==> A

```

Tree 3





172	1	1.000	A ==> G
220	1	1.000	G ==> A
228	1	1.000	A ==> T
274	1	1.000	T ==> C
360	1	1.000	G ==> T
361	1	1.000	T ==> G
377	1	1.000	C ==> T
391	1	1.000	A ==> G
397	1	1.000	C ==> T
403	1	1.000	T ==> A
413	1	1.000	G ==> A
node_16 --> BuMi	12	1	1.000 C ==> T
30	1	1.000	C ==> T
163	1	1.000	G ==> A
199	1	1.000	A ==> T
203	1	1.000	T ==> A
263	1	1.000	A ==> T
266	1	1.000	A ==> T
304	1	1.000	A --> G
309	1	1.000	A ==> G
323	1	1.000	T ==> G
327	1	1.000	T --> A
328	1	1.000	T ==> A
352	1	1.000	T ==> A
node_16 --> node_15	117	1	1.000 A ==> G
143	1	1.000	C ==> T
151	1	0.500	A ==> G
227	1	1.000	A ==> G
299	1	1.000	G ==> T
304	1	1.000	A --> T
327	1	1.000	T --> G
330	1	1.000	C ==> T
369	1	1.000	T ==> G
380	1	1.000	G ==> A
408	1	1.000	A ==> G
412	1	1.000	G ==> A
423	1	1.000	T ==> A
427	1	1.000	A ==> G
node_15 --> BuMi2	359	1	0.500 A ==> G
node_15 --> BuMi3	124	1	1.000 C ==> T
224	1	1.000	G ==> A
node_21 --> node_18	151	1	0.500 A ==> G
node_18 --> node_17	260	1	1.000 T ==> C
node_17 --> DS066	175	1	0.500 A ==> G
node_17 --> DS063	360	1	1.000 G ==> C
node_21 --> node_20	138	1	1.000 A ==> T
node_20 --> DS031	300	1	1.000 A ==> G
node_20 --> node_19	175	1	0.500 A ==> G
node_19 --> DS074	23	1	1.000 A ==> T
node_22 --> DS002	25	1	1.000 T ==> C
node_22 --> DS067	236	1	1.000 G ==> A

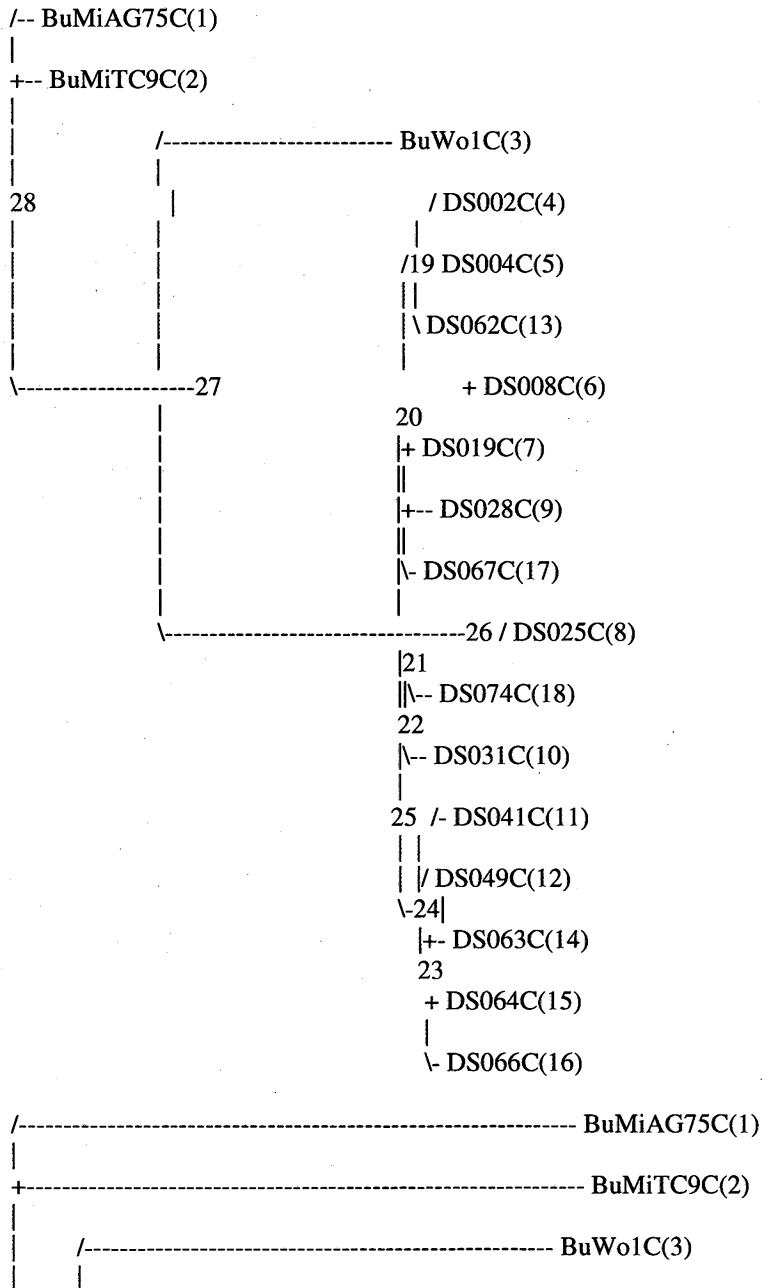


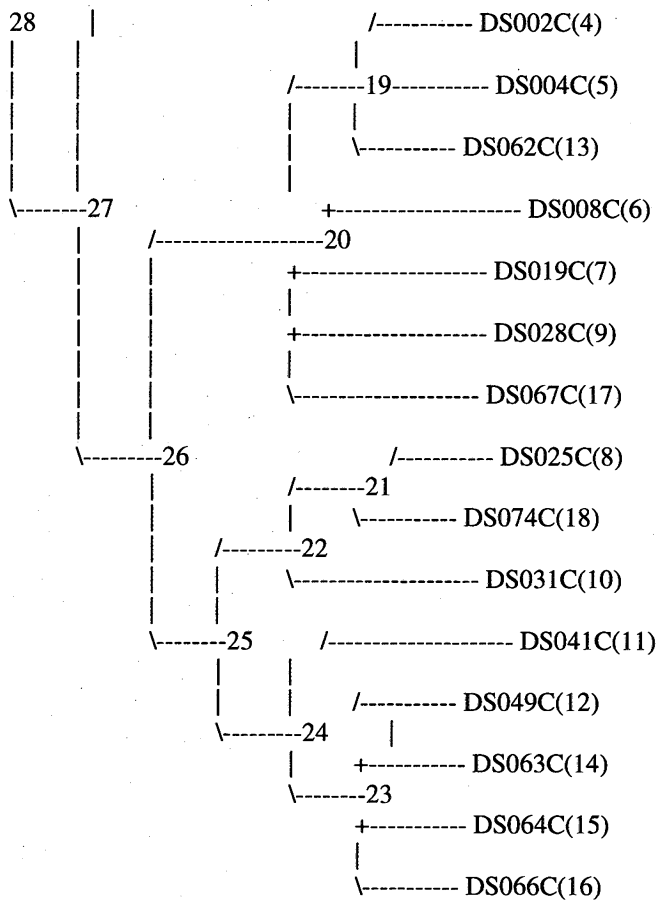
APPENDIX G

COMBINED ND1 AND DLOOP APOMORPHY LIST

Combined

Tree 1





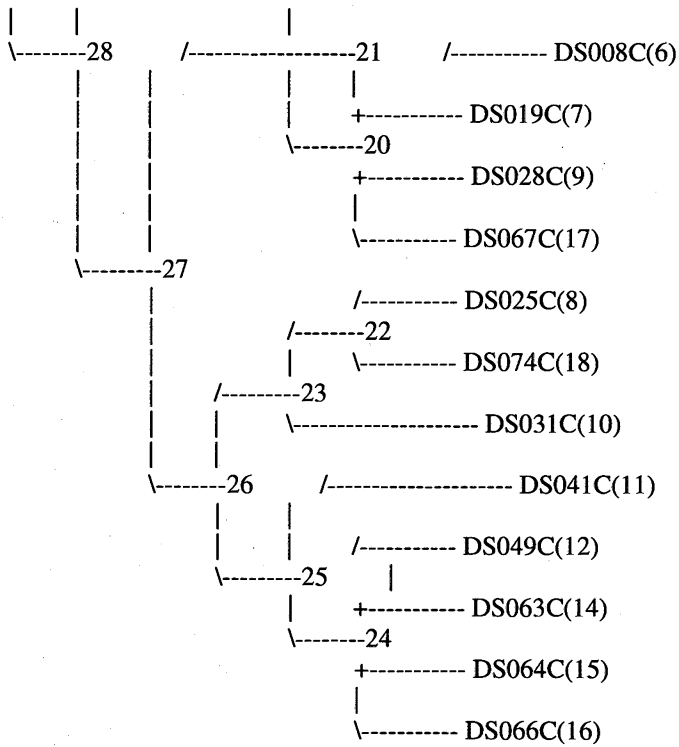
Tree 1  
Apomorphy lists:

Branch	Character	Steps	CI	Change
node_28 --> BuMiAG75C	982	1	1.000	C ==> T
	1082	1	1.000	G ==> A
node_28 --> BuMiTC9C	402	1	1.000	A ==> G
	1217	1	0.500	A ==> G
node_28 --> node_27	9	1	1.000	C ==> T
	135	1	1.000	C ==> T
	141	1	1.000	G ==> A
	189	1	1.000	T ==> C
	339	1	1.000	G ==> A
	342	1	1.000	T ==> C
	369	1	1.000	C ==> T
	456	1	1.000	G ==> A
	525	1	1.000	T ==> C
	594	1	1.000	G ==> A
	633	1	1.000	T ==> C
	664	1	1.000	T ==> C
	690	1	1.000	C ==> T
	720	1	1.000	T ==> C
	783	1	1.000	A ==> C
	802	1	1.000	C ==> T
	838	1	1.000	T ==> C

975	1	1.000	G ==> A
1001	1	1.000	T ==> C
1009	1	0.500	G ==> A
1085	1	1.000	G ==> A
1157	1	1.000	T ==> G
1188	1	1.000	T ==> C
1227	1	1.000	G ==> T
1238	1	1.000	A ==> G
1266	1	1.000	G ==> A
1270	1	1.000	A ==> G
1281	1	1.000	A ==> T
1285	1	1.000	G ==> A
node_27 --> BuWo1C	51	1	1.000 C ==> T
61	1	1.000	A --> G
156	1	1.000	T ==> C
204	1	1.000	T ==> C
258	1	1.000	T ==> C
282	1	1.000	C ==> T
306	1	1.000	C ==> T
315	1	1.000	T ==> C
328	1	1.000	T ==> C
345	1	1.000	T --> C
357	1	1.000	T ==> C
361	1	1.000	C ==> T
366	1	1.000	C ==> T
399	1	1.000	C ==> A
471	1	1.000	T ==> C
513	1	1.000	A ==> G
573	1	1.000	C ==> T
576	1	1.000	C ==> A
648	1	1.000	C ==> A
669	1	1.000	A ==> G
762	1	1.000	T ==> A
870	1	1.000	C ==> T
888	1	1.000	C ==> T
1021	1	1.000	G ==> A
1057	1	1.000	A ==> T
1061	1	1.000	T ==> A
1121	1	1.000	A ==> T
1124	1	1.000	A ==> T
1162	1	1.000	T --> G
1167	1	1.000	A ==> G
1181	1	1.000	T ==> G
1185	1	1.000	G --> A
1186	1	1.000	T ==> A
1210	1	1.000	T ==> A
node_27 --> node_26	61	1	1.000 A --> T
195	1	1.000	T ==> C
253	1	1.000	T ==> C
276	1	1.000	A ==> G
279	1	1.000	C ==> T
345	1	1.000	T --> A
411	1	1.000	C ==> A
414	1	1.000	A ==> C
444	1	1.000	A ==> T
465	1	1.000	T ==> C

474	1	1.000	A ==> G
477	1	1.000	C ==> T
495	1	1.000	T ==> C
510	1	1.000	C ==> T
585	1	1.000	A ==> G
591	1	1.000	C ==> T
663	1	1.000	A ==> G
666	1	1.000	A ==> T
744	1	1.000	G ==> A
763	1	0.500	A ==> G
771	1	1.000	T ==> C
795	1	1.000	T ==> C
798	1	1.000	G ==> A
804	1	1.000	A ==> G
843	1	1.000	T ==> C
855	1	1.000	T ==> C
941	1	1.000	T ==> C
943	1	1.000	T ==> G
1004	1	1.000	T ==> G
1023	1	1.000	T ==> C
1024	1	1.000	A ==> G
1027	1	1.000	A ==> T
1029	1	1.000	G ==> A
1030	1	1.000	G ==> A
1078	1	1.000	A ==> G
1086	1	1.000	T ==> A
1132	1	1.000	C ==> T
1162	1	1.000	T --> A
1185	1	1.000	G --> T
1218	1	1.000	T ==> G
1219	1	1.000	G ==> T
1235	1	1.000	T ==> C
1249	1	1.000	G ==> A
1255	1	1.000	T ==> C
1261	1	1.000	A ==> T
1271	1	1.000	A ==> G
node_26 --> node_20	1097	1	1.000 A ==> G
	1217	1	0.500 A ==> G
node_20 --> node_19	763	1	0.500 G ==> A
	883	1	1.000 T ==> C
node_19 --> DS062C	322	1	1.000 A ==> G
node_20 --> DS028C	49	1	1.000 C ==> T
	808	1	1.000 A ==> G
	1155	1	1.000 G ==> A
node_20 --> DS067C	1094	1	1.000 G ==> A
node_26 --> node_25	222	1	1.000 G ==> A
node_25 --> node_22	996	1	1.000 A ==> T
node_22 --> node_21	1033	1	0.500 A ==> G
node_21 --> DS074C	54	1	1.000 T ==> C
	250	1	1.000 A ==> G
	881	1	1.000 A ==> T
node_22 --> DS031C	42	1	1.000 C ==> T
	1158	1	1.000 A ==> G
node_25 --> node_24	333	1	1.000 T ==> C
	571	1	1.000 A ==> G
	600	1	1.000 A ==> G





Tree 2

Apomorphy lists:

Branch	Character	Steps	CI	Change
node_29 --> BuMiAG75C	982	1	1.000	C ==> T
	1082	1	1.000	G ==> A
node_29 --> BuMiTC9C	402	1	1.000	A ==> G
	1217	1	0.500	A ==> G
node_29 --> node_28	9	1	1.000	C ==> T
	135	1	1.000	C ==> T
	141	1	1.000	G ==> A
	189	1	1.000	T ==> C
	339	1	1.000	G ==> A
	342	1	1.000	T ==> C
	369	1	1.000	C ==> T
	456	1	1.000	G ==> A
	525	1	1.000	T ==> C
	594	1	1.000	G ==> A
	633	1	1.000	T ==> C
	664	1	1.000	T ==> C
	690	1	1.000	C ==> T
	720	1	1.000	T ==> C
	783	1	1.000	A ==> C
	802	1	1.000	C ==> T
	838	1	1.000	T ==> C
	975	1	1.000	G ==> A
	1001	1	1.000	T ==> C
	1009	1	0.500	G ==> A
	1085	1	1.000	G ==> A
	1157	1	1.000	T ==> G

1188	1	1.000	T ==> C
1227	1	1.000	G ==> T
1238	1	1.000	A ==> G
1266	1	1.000	G ==> A
1270	1	1.000	A ==> G
1281	1	1.000	A ==> T
1285	1	1.000	G ==> A
node_28 --> BuWolC	51	1	1.000 C ==> T
61	1	1.000	A --> G
156	1	1.000	T ==> C
204	1	1.000	T ==> C
258	1	1.000	T ==> C
282	1	1.000	C ==> T
306	1	1.000	C ==> T
315	1	1.000	T ==> C
328	1	1.000	T ==> C
345	1	1.000	T --> C
357	1	1.000	T ==> C
361	1	1.000	C ==> T
366	1	1.000	C ==> T
399	1	1.000	C ==> A
471	1	1.000	T ==> C
513	1	1.000	A ==> G
573	1	1.000	C ==> T
576	1	1.000	C ==> A
648	1	1.000	C ==> A
669	1	1.000	A ==> G
762	1	1.000	T ==> A
870	1	1.000	C ==> T
888	1	1.000	C ==> T
1021	1	1.000	G ==> A
1057	1	1.000	A ==> T
1061	1	1.000	T ==> A
1121	1	1.000	A ==> T
1124	1	1.000	A ==> T
1162	1	1.000	T --> G
1167	1	1.000	A ==> G
1181	1	1.000	T ==> G
1185	1	1.000	G --> A
1186	1	1.000	T ==> A
1210	1	1.000	T ==> A
node_28 --> node_27	61	1	1.000 A --> T
195	1	1.000	T ==> C
253	1	1.000	T ==> C
276	1	1.000	A ==> G
279	1	1.000	C ==> T
345	1	1.000	T --> A
411	1	1.000	C ==> A
414	1	1.000	A ==> C
444	1	1.000	A ==> T
465	1	1.000	T ==> C
474	1	1.000	A ==> G
477	1	1.000	C ==> T
495	1	1.000	T ==> C
510	1	1.000	C ==> T
585	1	1.000	A ==> G

591	1	1.000	C ==> T
663	1	1.000	A ==> G
666	1	1.000	A ==> T
744	1	1.000	G ==> A
771	1	1.000	T ==> C
795	1	1.000	T ==> C
798	1	1.000	G ==> A
804	1	1.000	A ==> G
843	1	1.000	T ==> C
855	1	1.000	T ==> C
941	1	1.000	T ==> C
943	1	1.000	T ==> G
1004	1	1.000	T ==> G
1023	1	1.000	T ==> C
1024	1	1.000	A ==> G
1027	1	1.000	A ==> T
1029	1	1.000	G ==> A
1030	1	1.000	G ==> A
1078	1	1.000	A ==> G
1086	1	1.000	T ==> A
1132	1	1.000	C ==> T
1162	1	1.000	T --> A
1185	1	1.000	G --> T
1218	1	1.000	T ==> G
1219	1	1.000	G ==> T
1235	1	1.000	T ==> C
1249	1	1.000	G ==> A
1255	1	1.000	T ==> C
1261	1	1.000	A ==> T
1271	1	1.000	A ==> G
node_27 --> node_21	1097	1	1.000 A ==> G
	1217	1	0.500 A ==> G
node_21 --> node_19	883	1	1.000 T ==> C
node_19 --> DS062C	322	1	1.000 A ==> G
node_21 --> node_20	763	1	0.500 A --> G
node_20 --> DS028C	49	1	1.000 C ==> T
	808	1	1.000 A ==> G
	1155	1	1.000 G ==> A
node_20 --> DS067C	1094	1	1.000 G ==> A
node_27 --> node_26	222	1	1.000 G ==> A
	763	1	0.500 A --> G
node_26 --> node_23	996	1	1.000 A ==> T
node_23 --> node_22	1033	1	0.500 A ==> G
node_22 --> DS074C	54	1	1.000 T ==> C
	250	1	1.000 A ==> G
	881	1	1.000 A ==> T
node_23 --> DS031C	42	1	1.000 C ==> T
	1158	1	1.000 A ==> G
node_26 --> node_25	333	1	1.000 T ==> C
	571	1	1.000 A ==> G
	600	1	1.000 A ==> G
	1009	1	0.500 A ==> G
node_25 --> DS041C	684	1	1.000 A ==> G
node_25 --> node_24	1118	1	1.000 T ==> C
node_24 --> DS063C	1218	1	1.000 G ==> C
node_24 --> DS066C	1033	1	0.500 A ==> G



## APPENDIX H

### *Bufo californicus* MATERIAL EXAMINED FROM BAJA CALIFORNIA, MÉXICO

Locality records of *Bufo californicus* in Baja California. Watersheds (**bold**) presented in map (Fig. 14) are listed in order from north to south. Asterisk (\*) denotes vouchers collected as part of this study. Museum abbreviations follow Leviton (1985): CAS (California Academy of Science), LACM (Los Angeles County Museum Natural History), MVZ (Museum Vertebrate Zoology), SDSNH (San Diego Natural History Museum), UABC (Universidad Autonoma de Baja California), USNM (United States National Museum). Non-standard abbreviations are as follows: CRM 2002 (Clark R. Mahrtd, 2002), HHW 1988 (Hartwell H. Welsh, 1988), PC (photo collection voucher), SDFN (San Diego Natural History Museum Field Number), UABC-T (Universidad Autonoma de Baja California, tissue collection).

MEXICO: Baja California: **Rio Las Palmas**: UABC 1510\*, UABC 1512\* (Canon El Alamo, Arroyo Las Palmas), UABC-T 376\* (Canon El Alamo, 5km upstream from Hwy 3); **Rio Guadalupe**: CRM 2002\* (Ejido la Mision, nr. Hwy 1 bridge), CRM PC uncatalogued (west of Ejido la Mision, Hwy 1 bridge), SDNHM 23507 (6 mi N San Faustino), SDNHM 56470 (2 km W of Agua Caliente), UABC 1507-1509\*, UABC 1532-1535\* (Arroyo Guadalupe, approx. 1.5 km E La Misión), UABC-T 368-373\* (Cañada Los Alisos, 4.5 km E La Misión), UABC-T 374-375\* (Cañon Agua Caliente, nr. Rancho Agua Caliente); **Rio San Carlos**: SDNHM 23456-23459 (Ojos Negros), SDFN 1176 (1 km N Laguna Hanson, Sierra Juarez), UABC 0885 (Cañon San Carlos,

Arroyo Maneadero, Ensenada [Santiago]); **Rio El Zorrillo**: UABC 1538-39\* (Arroyo El Zorrillo, nr. Las Animas, Hwy 1), UABC-T 377\* (Arroyo La Grulla, ca. 1 km downstream from the turn-off to Uruapan); **Rio Santo Tomas**: UABC-T 391-394\* (16 km E of Santo Tomas nr. Rancho Las Aguillas); **Rio San Vicente**: LACM 88055-88056 (0.3 mi N San Vicente on Hwy 1), UABC 1516-1518 (100 m S of Hwy 1 bridge), UABC-T 378-380\* (Arroyo San Vicente, 100 m N [upstream] of Hwy 1 bridge); **Rio San Rafael**: HHW 1988 (Arroyo de San Rafael, 6 mi E Mike's Sky Rancho), HHW 1988 (Arroyo de San Rafael, head), MVZ 140719 (2.6 mi E Mike's Sky Ranch, Sierra San Pedro Martir), SDFN 740 (nr. Rancho Los Mezcales), SDFN 1365\* (1 km SE Mike's Sky Ranch), SDFN 1368\* (Mike's Sky Ranch), UABC 1543\* (2 km E Rancho Los Mezcales), UABC-T 381-383\* (18 km E Punta Colonet), UABC 0282 (Arroyo San Rafael, Sierra San Pedro Martir); **Rio San Telmo**: SDNHM 47353 (15 mi E, 6 mi N Rancho Concepcion), UABC 1545\* (upstream nr. Ranch Meling), USNM 225285 (San Telmo, E of, 13.4 km SE of junction of Observatory and Valladares Roads); **Rio Santo Domingo**: HHW 1988 (Rancho Viejo), CAS 93419 (5.8 mi upstream from Hamilton Ranch), MVZ 9853, MVZ 9855 (Valladores, San Pedro Martir region), MVZ 9854, MVZ 9856-9857 (San Antonio Ranch, Santo Domingo River, San Pedro Martir region), MVZ 140715-140718, MVZ 147512 (Rancho San Antonio Sierra San Pedro Martir), MVZ 37242-37243 (Rio Santo Domingo at Hamilton Ranch), MVZ 140713, MVZ 140738 (SE end La Grulla Meadow, Sierra San Pedro Martir), MVZ 140714 (Mision San Pedro Martir, Sierra San Pedro Martir), MVZ 140739 (Santo Tomas, Sierra San Pedro Martir region) MVZ 145230, MVZ 150013-150029, MVZ 171767-171768, MVZ 175913, MVZ 175975 (3.7 mi by dirt road E Colonia Guerrero), SDNHM 5007 (above the [San

Antonio] falls), Santo Tomas, Sierra San Pedro Martir region), SDFN 1259, 1264, 1325\*, 1327\*, 1856 (nr. Rancho El Potrero, Arroyo El Potrero), UABC 0879, UABC 1222 (Arroyo San Antonio de Murillos, Rancho San Antonio, Sierra San Pedro Martir), UABC 1126-1133\* (nr. Colonia Vicente Guerrero, Arroyo Santo Domingo), UABC 1523\*, UABC 1528-1529\* (Arroyo Santo Domingo, nr. Rancho El Divisadero), UABC PC uncatalogued (Bocana de Santo Domingo); **Rio Santa Maria:** LACM PC1316 (Arroyo San Simón, ca. 16 km SE of San Quintin), UABC 1548\* (Arroyo San Simón, 3 km N upstream of Rancho José Maria Morelos y Pavón).