

Cryptic Neogene vicariance and Quaternary dispersal of the red-spotted toad (*Bufo punctatus*): insights on the evolution of North American warm desert biotas

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Abstract

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

Keywords: *Bufo punctatus*, mitochondrial DNA, nested clade, North American deserts, phylogeography, vicariance

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Introduction

In his biogeographical analysis of the herpetofauna of the Chihuahuan Desert, Morafka (1977) presented a general model for the evolution of North American desert biotas that promoted the importance of two major pre-Pleistocene vicariant events in the diversification of a widely distributed, ancestral desert biota. These vicariance events were the formation and uplift of the Colorado Plateau–Sierra Madre

Occidental (roughly the Continental Divide) separating the Chihuahuan Desert from the Sonoran and Mojave deserts, and the expansion of the Sea of Cortés, separating the peninsula of Baja California from the continental mainland. Morafka (1977) hypothesized that the closure of a last portal of desert habitat between the Chihuahuan and Sonoran deserts was largely caused by climatic cooling starting approximately 3 million years ago (Ma). Concurrently, landscape and climatic changes in mainland desert regions near the head of the Sea of Cortés resulted in the development of the modern biotic patterns in that region. While the details of his models have been modified or refined, particularly

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Table 1 Representative codistributed sister phylogroups providing evidence of (A) pre-Pleistocene Peninsular vs. western Continental deserts (Mojave and/or Sonoran desert) vicariance, or (B) pre-Pleistocene western versus eastern (Chihuahuan) Continental deserts vicariance. Distributions across multiple deserts are indicated for some taxa

Codistributed sister phylogroups by desert region		References
A	Peninsular desert	Western deserts
	<i>Chaetodipus rudinoris</i>	<i>C. baileyi</i> 7; 8
	<i>Centruroides exilicauda</i> (Peninsular clade)	<i>C. exilicauda</i> (Continental clade) 2
	<i>Lophocereus schottii</i> (Peninsular clade)	<i>L. schottii</i> (Continental clade) 3
	<i>Neotoma lepida</i> (Peninsular clade)	<i>N. lepida</i> + <i>N. devia</i> (Continental clade) 7
	<i>Peromyscus fraterculus</i> + <i>P. eva</i>	<i>P. eremicus</i> + <i>P. merriami</i> (+ Eastern Desert) 7; 9
	<i>Poliophtila californica</i>	<i>P. melanura</i> (+ Eastern Desert) 12
	<i>Sauromalus obesus</i> (Peninsular clade)	<i>S. obesus</i> (Continental clade) 4
B	Western deserts	Eastern desert
	<i>Ammospermophilus leucurus</i> (+ Peninsular Desert)	<i>A. interpres</i> 7; 14
	<i>Callipepla douglasii</i>	<i>C. squamata</i> 12; 13
	<i>Chaetodipus penicillatus</i>	<i>C. eremicus</i> 1; 8
	<i>Chaetodipus artus</i> + <i>C. goldmani</i>	<i>C. nelsoni</i> 8; 14
	<i>Kinosternon arizonense</i>	<i>K. durangoense</i> 10
	<i>Onychomys torridus</i>	<i>O. arenicola</i> 5; 6
	<i>Uta stansburiana</i> (+ Peninsular Desert)	<i>U. stansburiana stejnegeri</i> 11

1, Lee *et al.* 1996; 2, Gantenbein *et al.* 2001; 3, Nason *et al.* 2002; 4, Petren & Case 2002; 5, Riddle 1995; 6, Riddle & Honeycutt 1990; 7, Riddle *et al.* 2000a; 8, Riddle *et al.* 2000b; 9, Riddle *et al.* 2000c; 10, Serb *et al.* 2001; 11, Upton & Murphy 1997; 12, Zink & Blackwell 1998; 13, Zink *et al.* 2000; 14, unpublished data (Riddle & colleagues).

the model associated with development of the peninsular desert biota (e.g. Grismer 1994; Riddle *et al.* 2000a; Murphy & Aguirre-Léon 2002), Morafka (1977) articulated a new paradigm. This perspective countered previous scenarios that emphasized Pleistocene glacial–interglacial cycles as the dominant force in the evolution of North American desert biotas (e.g. Savage 1960; Findley 1969; Hubbard 1973).

Support for the hypothesis of pre-Pleistocene vicariance in the structuring of North American arid-adapted biotas has come from a greater understanding of the geological and climatic processes that formed the deserts and empirical observations of phylogeographic patterns (Hafner & Riddle in press; Riddle & Hafner in press). Numerous widespread species and species groups consist of two or more genealogical lineages that are sufficiently divergent to be consistent with postulated pre-Pleistocene vicariance. Many of these taxa have congruent phylogeographic patterns that are generally coincident with the boundaries of the Chihuahuan, Sonoran–Mojave, and/or peninsular deserts (Table 1).

Morphologically defined species with distributions spanning two or more of these desert regions have often been explained by, and taken as evidence for, late Pleistocene dispersal (Riddle & Hafner 1999). For example, Grismer (1994) stated that there is a lack of strong morphological difference between populations of the red-spotted toad, *Bufo punctatus* Baird & Girard, 1852, on the peninsula of Baja California and on the continental mainland. He saw this pattern as evidence that this widespread, arid-dwelling toad evolved on the mainland and dispersed onto the pen-

insula relatively recently (Pleistocene–Holocene), well after Pliocene vicariance had led to the evolution of other desert taxa unique to the peninsula. Under this interpretation, *B. punctatus* would provide evidence of biotic reticulation between the peninsular and Sonoran–Mojave deserts, obscuring the distinction between the biotas of these regions. This perspective was later rejected by Riddle *et al.* (2000a) when peninsular populations of *B. punctatus* were found to comprise a single monophyletic mitochondrial DNA (mtDNA) lineage divergent from lineages on the continental mainland. The observed phylogeographic distribution and deep level of sequence divergence were congruent with patterns evident within several other taxa, and were determined to be more consistent with postulated Pliocene vicariance than with more recent dispersal scenarios.

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

Bufo punctatus is broadly distributed across warm arid and arid-subtropical regions of southwestern North America (Fig. 1). It occurs throughout the Deming Plains (Cochise

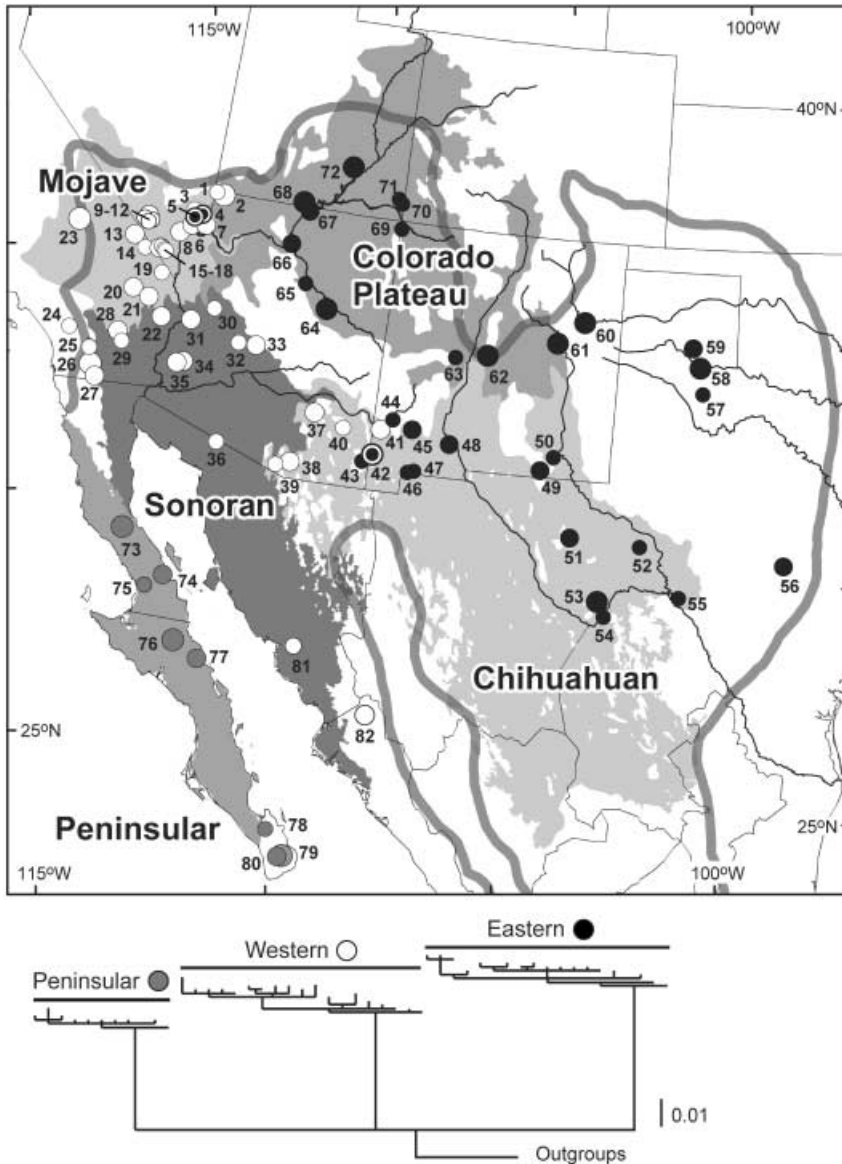


Fig. 1 Sample sites for *Bufo punctatus* in relationship to North American regional warm deserts and the Colorado Plateau (shaded areas; modified from ecoregions described by Ricketts *et al.* 1999). The heavy grey line indicates the approximate distributional limit of the species (after Stebbins 1985). Circle shadings indicate the major mtDNA clades identified in the maximum-likelihood tree (bottom of the figure) and in Fig. 2. Locations 4, 5, and 42 contain both Western and Eastern clade haplotypes. Circle size reflects sample size at each location (small circles = 1, mid-sized circles = 2–3, large circles = 4–5). Numbers identify sample localities listed in Appendix and referenced in Fig. 2. The maximum-likelihood tree is outgroup rooted (distances to outgroups not shown).

filter barrier) between the Sonoran and Chihuahuan deserts (Morafka 1977). This narrow desert corridor between the Sierra Madre Occidental and Colorado Plateau may have been ephemeral during the Pleistocene (Morafka 1977). During the last glacial maximum (Wisconsin glacial, 18 000 years ago; Ka), this region of the Chihuahuan Desert was a pinyon–juniper–oak (*Pinus*, *Juniperus*, and *Quercus*) woodland and apparently only transformed to arid-grassland and desert scrub vegetation in the middle Holocene, as recently as 4 Ka (Van Devender *et al.* 1984; Van Devender 1990). The lack of readily recognized morphological differentiation in populations of *B. punctatus* on either side of the Deming Plains suggests the possibility of late Quaternary dispersal between the Sonoran and Chihuahuan deserts (i.e. range expansion from one desert region to the other) or repeated contact between regional populations (i.e. migration). Either

process should have left a signature of reduced genetic divergence among the regional desert populations. Riddle *et al.* (2000a), however, revealed a deep east–west mtDNA divergence between continental populations of *B. punctatus*. Although this pattern implicated pre-Pleistocene divergence, samples were insufficient for further assessment.

Evidence from pollen records and packrat middens has documented substantial changes in the distributions of desert plant assemblages within North America during the latest glacial–interglacial cycle (Bentancourt *et al.* 1990; Thompson & Anderson 2000). Presumably, these biotic responses to climatic change reflected similar distributional responses during the multiple climatic oscillations of the late Quaternary (about the last 700 000 years; Webb & Bartlein 1992). The regional deserts that existed during interglacials, and may have reached their maximum extent during the

Holocene, were more limited in distribution during glacial periods. To the degree that niche requirements of *B. punctatus* have remained stable over time (e.g. niche conservatism; Wiens 2004), we predict that the distribution of this species would have tracked shifts in its habitat distribution through glacial cycles. Such distributional shifts need not erode phylogeographic structure produced by earlier episodes of isolation and divergence, if range shifts are constrained in spatial extent by abiotic or biotic factors (Riddle 1998). Indeed, elucidation of population responses to the most recent episode of climatically induced habitat changes might provide insight into the mechanisms that have maintained deeper-scale phylogeographic pattern in the face of multiple glacial–interglacial cycles.

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

Materials and methods

Samples

Samples of *Bufo punctatus* were field collected or acquired from university collections or other researchers (see Appendix). A total of 192 *B. punctatus* were sampled from 82 locations representing the majority of the species distribution (Fig. 1), except for portions of the range in the lower Sonoran and Chihuahuan deserts in Mexico. *Bufo debilis* and *Bufo retiformis* were selected as outgroups for phylogenetic analyses based on a hypothesis of phylogenetic relationships (Ferguson & Lowe 1969) and support from interspecific mtDNA analyses that consistently demonstrated some alliance between *B. punctatus* and a clade containing *B. debilis* and *B. retiformis* (Graybeal 1997).

Laboratory methods

Total genomic DNA was extracted from heart, muscle, liver, toe tissue, and in a few cases frozen haemolysate using standard phenol–chloroform extraction. The mitochondrial gene cytochrome *b* (*cyt b*) was selected for genetic analysis.

This gene has an evolutionary rate within bufonids that is useful for analyses within late Cenozoic time frames (Graybeal 1993, 1997). A portion of the *cyt b* gene was amplified by polymerase chain reaction (PCR) method using primers MVZ43 (Graybeal 1993) and a slightly modified version of MVZ16 (Moritz *et al.* 1992). Generally, PCR was performed in 50- μ L reactions using approximately 30–200 ng of total genomic DNA, 1.2 U AmpliTaq polymerase (PE Applied Biosystems, Inc.), and a final concentration of 3 mM of MgCl₂ in a standard reaction mix (50 mM KCl, 10 mM Tris-HCl, 0.001% gelatin, 0.5 μ M each primer, 0.2 mM each dNTP). Thermal cycling was accomplished using a 55 °C annealing temperature. PCR fragments were purified using GeneClean (II Kit, BIO 101, Inc.).

Fluorescence-based cycle sequencing was conducted on the purified PCR templates using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit chemistry (PE Applied Biosystems, Inc.) with unincorporated dye labels removed by ethanol precipitation (following manufacturer's protocol) or by Sephadex gel separation (e.g. Sambrook *et al.* 1989). Sequencing primers consisted of those used in PCR and two internal primers designed specifically for sequencing *B. punctatus* *cyt b* (Riddle *et al.* 2000a). Electrophoresis and visualization of sequences were completed on an ABI PRISM 310 Automated Sequencer (PE Applied Biosystems, Inc.).

Phylogenetic analyses

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

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

For ML analysis, the associated model parameters from MODELTEST were subsequently used in a heuristic search (random stepwise addition, 10 replications, 1 tree held at each step, tree-bisection–reconnection branch swapping, and collapsing zero length branches) as implemented in PAUP*

(version 4.0b10; Swofford 2000). Model parameters were then re-estimated from the resulting trees and applied in a subsequent heuristic search using the original ML trees as starting trees.

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

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

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

Nested clade analyses

We conducted NCA (Templeton *et al.* 1995; Templeton 1998, 2004) for each of the major clades. Haplotype networks (Smouse 1998; Posada & Crandall 2001) used a parsimony algorithm (Templeton *et al.* 1992) and 95% probability criterion for connections as implemented in the program tcs (version 1.6, Clement *et al.* 2000). Resulting networks were converted into a hierarchical nested design following the rules of Templeton *et al.* (1987) and Crandall (1996). Haplotype hierarchical position and geographical locations (latitude and longitude) were then incorporated into NCA using the program GEODIS (Posada *et al.* 2000). Null distributions for permutational contingency table tests comparisons

were generated from 10 000 random permutations and considered significant at $\alpha = 0.05$. NCA statistics (i.e. D_c , D_{nr} , I-T) were then jointly evaluated for their fit to expectations from various models of population structure and historical events (Templeton *et al.* 1995; Templeton 1998) using a reference key (Templeton 2004). Inferences were considered significant for only those clades in which the null distribution (i.e. a random association between haplotypes and geography) were rejected in the contingency table tests. Inferences of population expansion from NCA were corroborated by Fu's F_S test as implemented in ARLEQUIN. Fu (1997) has shown that F_S is a powerful test for detecting population growth, and in the absence of selection, strongly negative values are expected from population expansion. Critical values for the tests were considered significance at $P \leq 0.02$ (Schneider *et al.* 2000).

Results

Phylogenetic analyses

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

Under the estimated model parameters from MODELTEST, four equally likely ML trees were derived and the following model parameters were estimated from those trees (transitions/transversion ratio = 13.4471; base frequencies, A = 0.27610, C = 0.30567, G = 0.13774, T = 0.28049; α shape parameter = 0.13208). The second iteration analysis (rearrangements evaluated = 214 816) found four equally likely ML trees ($-\ln L = 2189.31148$). All four ML trees shared the same major topology in depicting three distinct clades within *B. punctatus*. The most conservative tree depicted the association between the major clades as a polytomy (Fig. 1). The other trees differed predominately in the order of branching among the major clades (with each of the major lineages being shown as branching earlier than the others in one of the three trees). Branch lengths associated with the patterns among major clades were diminutive.

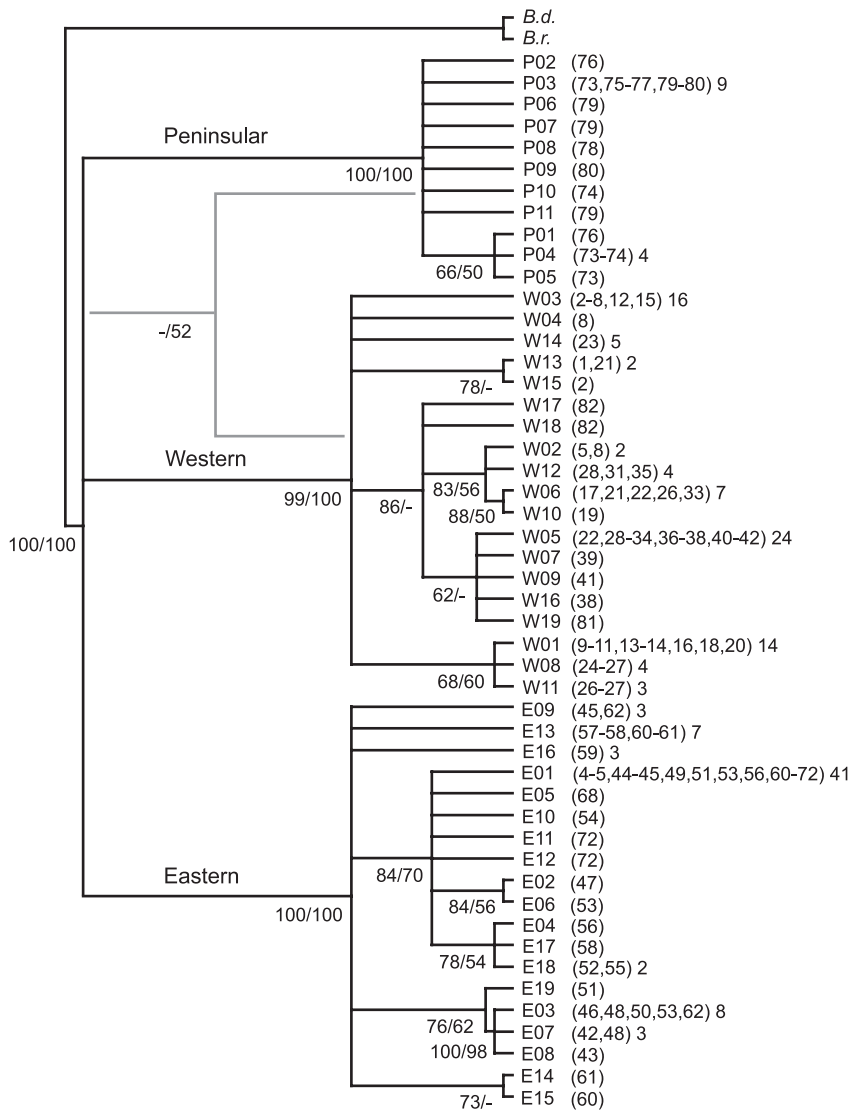


Fig. 2 Bayesian inference cladogram of *Bufo punctatus* haplotypes derived from 666 bp of *cyt b* sequence data. The three major clades are discussed in text and referenced in Fig. 1. There was weak support from maximum-parsimony analysis for the grouping of Peninsula and Western clades with a more basal branch leading to the Eastern clade (indicated by grey lines). Supports for clades (> 50%) are shown with numbers prior to forward-slash indicating Bayesian posterior probabilities (consensus of 30 000 sampled trees from three 1×10^6 generations runs) and numbers following the slash indicating maximum-parsimony bootstrap scores (1000 repetitions). The tree is outgroup-rooted on *Bufo debilis* and *Bufo retriformis*. Unique haplotypes are identified by letter and number designations. Numbers in parentheses following haplotype designations indicate sample locations referenced in Fig. 1. Numbers following parenthesis indicated sample sizes greater than one. Sample and locality descriptions are listed in the Appendix.

Tree reconstructions under BI converged on similar average likelihood values in all runs ($-\ln L = 2255.31, 2257.45, 2259.40$, respectively), with consensus trees (50% majority rule) revealing the same three major clades depicted in the ML analysis. Posterior probabilities (PP) revealed strong support for the major clades ($PP \geq 0.99$ per clade). As in the ML analysis, the BI consensus tree indicated a polytomy between the major clades with no support for any particular branching order (Fig. 2).

Unweighted MP resulted in 190 trees (score = 261), with the strict consensus depicting the same three major clades as in the other analyses (Fig. 2). Bootstrap support values (BS) for these clades under the MP criteria were high (BS = 100 per clade). The MP analysis indicated a particular branching order among the clades (Fig. 2); however, there was very little support (BS = 52) for the indicated topology.

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

The geographical distributions of haplotypes within the three major clades exhibited very little overlap. Two continental clades, generally eastern and western in distribution, and a clade concordant with the boundaries of the peninsular desert were identified. Henceforth, these three major clades are referred to as the Eastern, Western, and Peninsular, respectively (Fig. 2). Sequence divergences among these

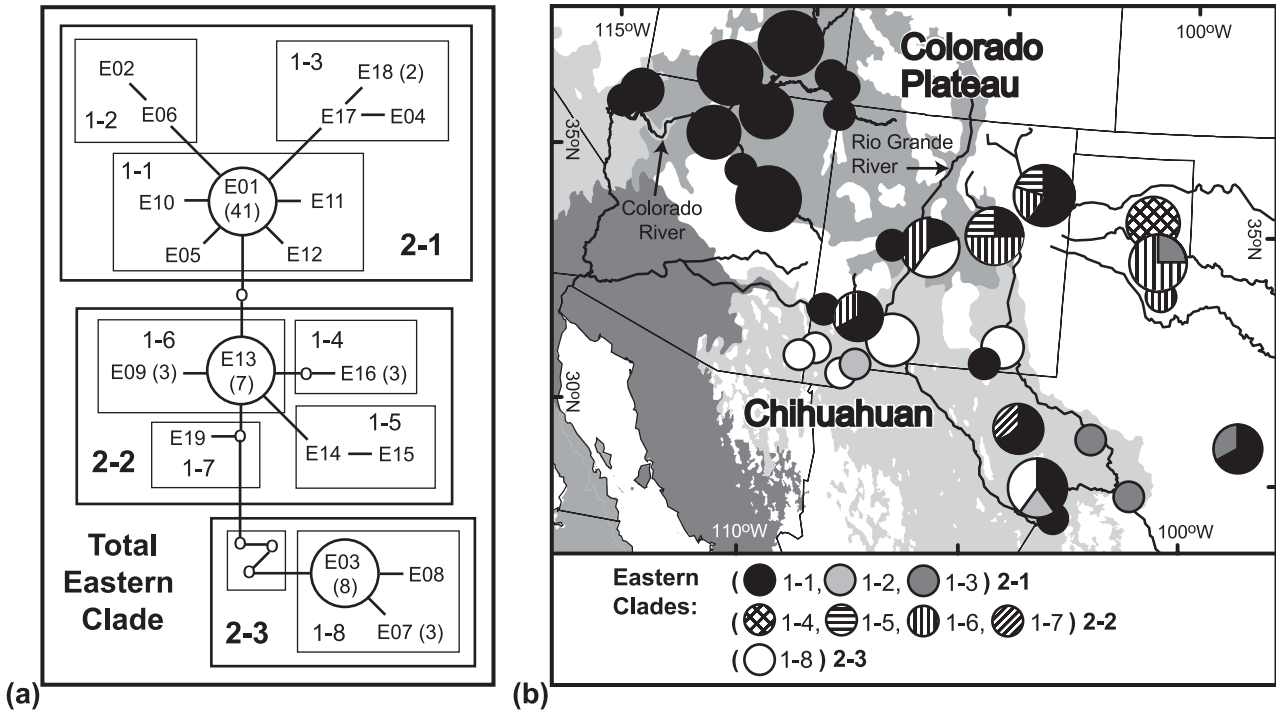


Fig. 3 (a) Nested haplotype network for mtDNA sequences of Eastern clade *Bufo punctatus* constructed under a criterion of statistical parsimony. Haplotypes are identified by letter and number designations. Numbers in parentheses following haplotype designations indicate sample sizes greater than one. Within the network, each line between haplotypes represents a mutational change. Zeros indicate unsampled haplotypes inferred from the data. Circles surround internal haplotypes with larger sample sizes. Boxes and line thickness indicate hierarchical nesting, with hierarchical clades designated by two-digit labels. (b) Distribution of salient lower-order clades from the nested clade analysis of Eastern clade *B. punctatus*. Pie graph size reflects sample size at each location progressing from smallest ($n = 1$) to largest ($n = 5$).

clades (adjusted for within clade variation) were all very similar (p -distances: Western–Eastern = 0.0669; Western–Peninsular = 0.0683; Eastern–Peninsular = 0.0670). This level of divergence was lower than that observed between the recognized sister taxa *Bufo debilis* and *Bufo retiformis* included in this study (= 0.0796). Sequence divergences within clades were about an order of magnitude lower (Western = 0.0084; Eastern = 0.0070; Peninsular = 0.0039).

Geographical distribution of major clades

The continental clades were geographically separated predominantly along the Rocky Mountains and Sierra Madre Occidental (roughly the Continental Divide). Haplotypes in the Eastern clade occurred throughout the Colorado Plateau and northern regions of the Chihuahuan Desert, and extended into the grassland regions of eastern New Mexico and western Texas. Haplotypes of the Western clade occurred throughout the Mojave and Sonoran deserts. Within the eastern Mojave Desert near the transition to the Colorado Plateau, Eastern and Western haplotypes were found in sympatry at sites near the confluence of the Colorado and Virgin rivers (now the Overton Arm of Lake

Mead; Fig. 1, locations 4 and 5). Within the region of contact between Sonoran and Chihuahuan deserts, Eastern and Western haplotypes occurred in sympatry within the Peloncillo Mountains (Fig. 1, location 42), a long recognized point of contact between these desert biotas (e.g. Dessauer *et al.* 1962; Findley 1969; Morafka 1977).

No sites of sympatry were located among haplotypes from Peninsular and Western clades, but our sampling within the border region of the peninsular and Sonoran deserts was insufficient to localize an area of contact, if one exists. Several peninsular desert taxa reach their northern limits in the Peninsular Mountain Ranges of southern California (Murphy 1983; Grismer 1994). The distribution of the Peninsular clade of *B. punctatus* does not appear to extend northward into this region where we found only haplotypes from the Western clade (Fig. 1, locations 25–27).

Nested clade analyses

Nested clade analysis of the Eastern clade was based on 19 haplotypes identified within 79 samples collected from 33 locations and the haplotype network and nesting design presented in Fig. 3a. Contingency table tests determined

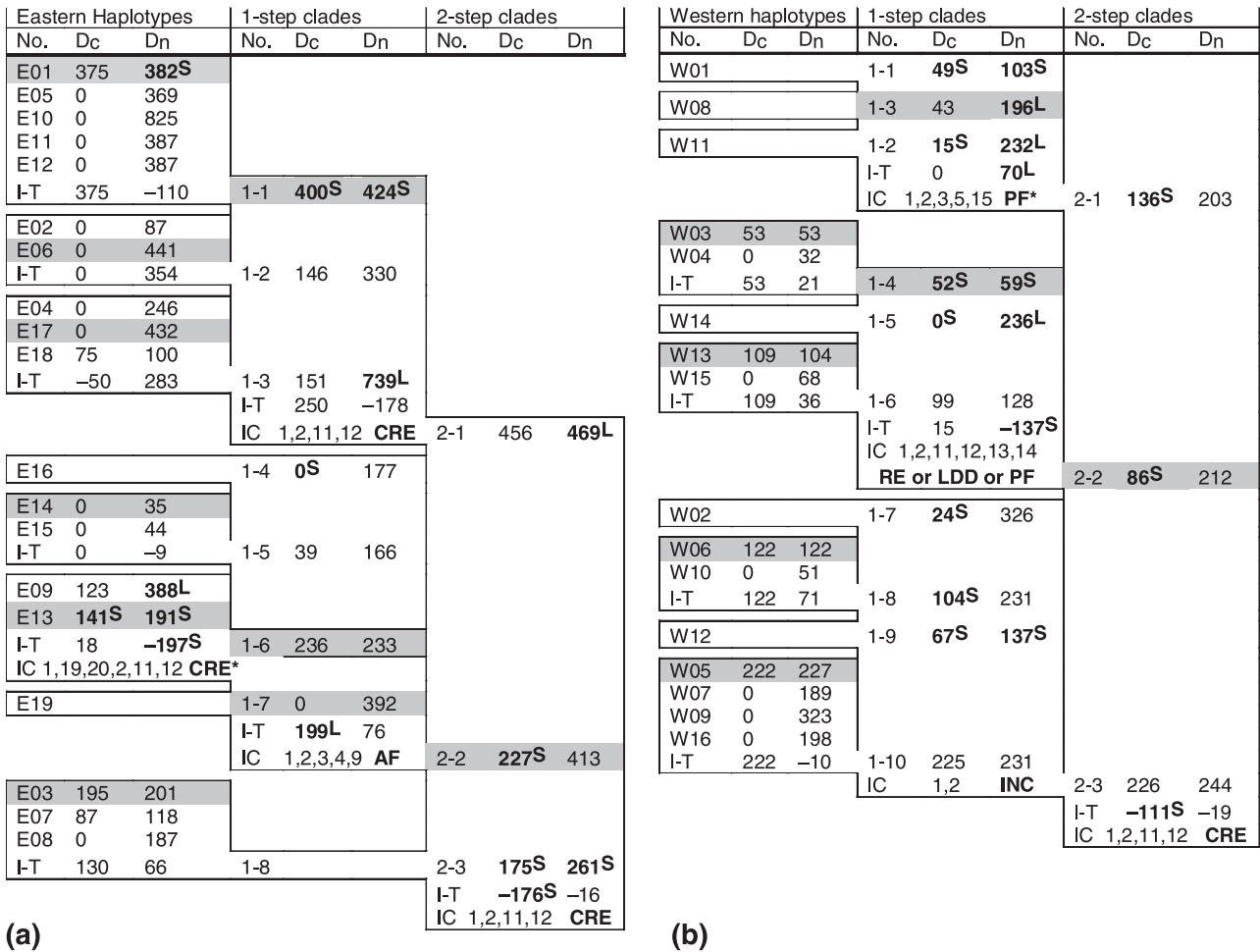


Fig. 4 Summary flow chart of nested clad analyses results for *Bufo punctatus* haplotypes from the (a) Eastern and (b) Western clades. Nesting level increases from left to right. Numbers indicate hierarchical clades. Clade distance (D_c), nested clade distance (D_n), and interior versus tip contrast (I-T) values are indicated with those showing significantly ($P \leq 0.05$) small or large values in bold and designated by a S or L, respectively. Shading indicates interior haplotypes or clades. Inference chain (IC) and interpretations of statistical results with abbreviations: CRE (contiguous range expansion), RE (range expansion), LDD (long-distance dispersal), PF (past fragmentation), and INC (inconclusive result). Asterisks indicate inferences for clades lacking support from initial contingency tests.

significant nonrandom association between sampling locations and Eastern clades 2-1, 2-2, and for the total Eastern cladogram (all P value ≤ 0.05). Interpretations of distance measures and relative haplotype/clade ages (i.e. interior versus tips) using the inference key revealed a significant signature of contiguous range expansion within the Chihuahuan Desert–Colorado Plateau region (Fig. 4a). The significantly negative F_s statistic value for the Eastern clade (Table 2) supported the inference that this population has undergone substantial growth. This range expansion was particularly conspicuous within Eastern clade 2-1 with haplotypes from Eastern clade 1-1 found throughout most of the region but comprising all samples from the Colorado Plateau west of the Rio Grande (Fig. 1, locations 4, 5, 63–72; Fig. 3b). In this region of the Colorado

Plateau, haplotypes were mostly identical (i.e. E01) with a few derived haplotypes that differed from the common haplotype by a single base pair (Fig. 3a). A significantly negative F_s statistic value, consistent with population expansion (Table 2), was derived from the samples in this region, whereas the samples from the Chihuahuan Desert (Fig. 1, locations 42–62) showed a nonsignificant F_s statistic value, indicating that the major population growth within the Eastern clade occurred on the Colorado Plateau. Allopatric fragmentation was implicated for clades within Eastern clade 2-2, but this signal should be interpreted with much caution since it is predominately derived by the significant geographical restriction of Eastern clade 1-4 which is represented by only three samples from a single location.

Table 2 Measures of haplotype and nucleotide diversity of *Bufo punctatus* mtDNA *cyt b* sequence data within major clades and specific geographic regions discussed in text. The northeast Sonoran Desert includes some areas identified in Fig. 1 as Chihuahuan Desert habitat

Clade or region	No. of samples (no. of haplotypes)	Haplotype diversity ± SD	Nucleotide diversity ± SD	Fu's F_S
Eastern Clade	79 (19)	0.7147 ± 0.0531	0.0045 ± 0.0026	-5.969 ($P = 0.02$)*
Western Colorado Plateau	29 (4)	0.1995 ± 0.0977	0.0003 ± 0.0004	-3.324 ($P < 0.01$)*
Chihuahuan Desert	50 (16)	0.8661 ± 0.0320	0.0060 ± 0.0034	-3.181 ($P = 0.13$)
Western Clade	91 (19)	0.8635 ± 0.0209	0.0067 ± 0.0037	-2.374 ($P = 0.24$)
Mojave Desert	49 (11)	0.8053 ± 0.0369	0.0061 ± 0.0034	0.186 ($P = 0.58$)
NE Sonoran Desert south of Gila River	15 (4)	0.3714 ± 0.1532	0.0006 ± 0.0007	-2.369 ($P < 0.01$)*
Peninsular Clade	22 (11)	0.8182 ± 0.0740	0.0033 ± 0.0021	-5.114 ($P < 0.01$)*

*Significant values.

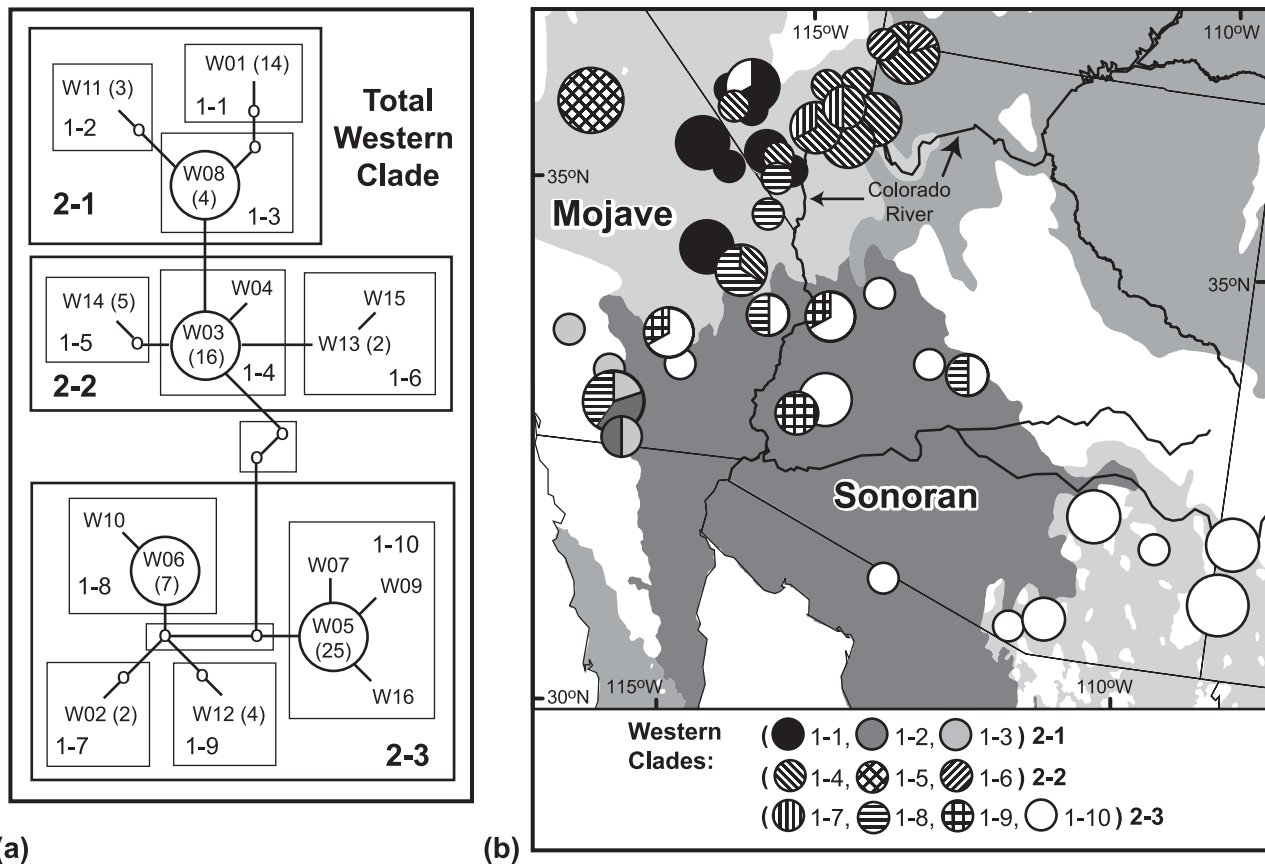


Fig. 5 (a) Nested haplotype network for mtDNA sequences of Western clade *Bufo punctatus* constructed under a criterion of statistical parsimony. (b) Distribution of salient lower-order clades from the nested clade analysis of Western clade *B. punctatus*. See Fig. 3 legend for figure explanations.

The haplotype network and nesting design of the Western clade NCA (Fig. 5a) were derived from 16 haplotypes identified within 88 samples from 42 locations. We excluded the three samples from the southern Sonoran Desert because of the large geographical distance separating them from the more densely sampled populations in the northern Sonoran and Mojave deserts. Contingency table tests determined

significant nonrandom association between sampling locations and Western clades 2-2, 2-3, and the total Western cladogram (all P value ≤ 0.001). The only conclusive inference from this NCA, however, was a signal of contiguous range expansion at the total clade level (Fig. 4b). The interior status of Western clade 2-2, necessary for inference at the total clade level, can be weakly inferred from the

phylogenetic analyses. All haplotypes within this clade appear on trees to have diverged earlier than haplotypes within the other Western clades (Fig. 2), thus indicating that clade 2-2 may well contain the oldest haplotypes. Although there is a tendency for haplotypes of high frequency to be near the root of a genealogy (Castelloe & Templeton 1994), the most common haplotype in this analysis (W05), as well as the most common haplotype in the Eastern clade NCA (E01), both appear to have resulted from participation in range expansions. The high frequencies of these haplotypes in the data sets may be artefacts of the expansion process and our sampling.

An inference of population expansion for the Western clade was not supported by Fu's F_S test, although the test statistic was strongly negative (Table 2). Population growth within the northeastern Sonoran Desert, south of the Gila River (Fig. 1, locations 36–42), was supported by a significantly negative F_S statistic value (Table 2). The lack of diversity in this region appears to have been a major factor in the inference of contiguous range expansion inferred from the NCA at the total Western clade level.

Within Western clade 2-1, a signature of past fragmentation was implicated in the geographical separation between Western clade 1-1 (haplotype W08), found in populations within the eastern Mojave Desert of California and Nevada, and Western clades 1-2 and 1-3 (haplotypes W01 and W11, respectively) in populations within the Peninsular Ranges of southern California (Fig. 5b). Contingency table analysis, however, failed to reject the null hypothesis of random association between geographical location and haplotypes within these clades.

The NCA of the Peninsular clade was based on 11 haplotypes identified within 22 samples from eight locations (Fig. 6a, b). No patterns were supported in the contingency table tests of these data (all P value > 0.05; nested clade statistics not shown), but a strongly negative F_S statistic value (Table 2) indicated the possibility of population growth for the Peninsular clade. There also appeared to be a north–south trend in haplotype structure (Fig. 6b). Further sampling, however, is needed to assess these patterns.

Discussion

Pre-Pleistocene vicariance

The patterns of genealogical relationships suggest a near simultaneous divergence among the major clades of *Bufo punctatus*. Sequence divergences among the three major clades were almost identical, and phylogenetic analyses revealed no significant support for any particular branching order among these clades (Fig. 2). Haplotypes from these clades showed little overlap in their geographical distributions (Fig. 1). We propose that this phylogeographic pattern was likely initiated by two late Neogene vicariant

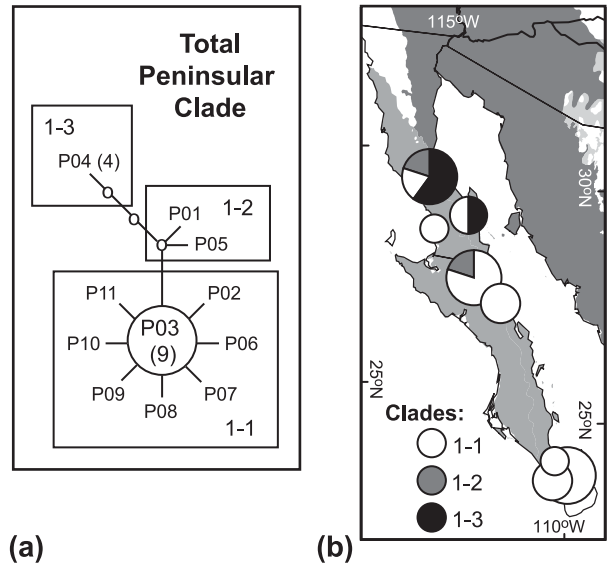


Fig. 6 (a) Nested haplotype network for mtDNA sequences of Peninsula clade *Bufo punctatus* constructed under a criterion of statistical parsimony. (b) Distribution of salient lower-order clades from the nested clade analysis of Peninsular clade *B. punctatus*. See Fig. 3 legend for figure explanations.

events — the early stages in the development of the peninsula of Baja California, and the uplifting of the Colorado Plateau, Mexican Plateau, and the Sierra Madre Occidental.

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

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

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

Pleistocene–Holocene patterns

Secondary contact of continental clades within the eastern Mojave Desert is likely a recent occurrence (late Pleistocene–Holocene). Very low haplotype and nucleotide diversity throughout the Colorado Plateau west of the Rio Grande (Fig. 3; western Colorado Plateau in Table 2) is consistent with an interpretation that *B. punctatus* colonized this area recently and rapidly. The NCA inference of contiguous range

expansion for haplotypes within this region supports this interpretation, as does the signal of population growth derived from Fu's F_S test. Reconstructions of Pleistocene macrohabitats within the southwest (Lomolino *et al.* 1989; Bentancourt 1990; Thompson *et al.* 1993; Thompson & Anderson 2000) indicate that arid vegetation assemblages, currently inhabited by *B. punctatus*, did not persist on most of the Colorado Plateau during the latest glacial period (Wisconsin). The Grand Canyon of the Colorado River forms a low-elevation corridor extending from the west into the higher-elevation Colorado Plateau. Even lower elevations within the canyon were mostly occupied at that time by a pluvial desert scrub comprised principally of species from the Great Basin, which typically dominate in areas of greater seasonality (Cole 1990).

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

In contrast, the Mojave Desert contains low-elevation areas that appear to have mitigated colder climatic conditions during recent glacial periods. Death Valley and the Amargosa River drainage in the northern Mojave Desert both appear to have retained desert scrub vegetation during the Wisconsin, but this assemblage contained numerous species common to colder regions (Spaulding 1990). The area of the lower Colorado River, extending northward into southern Nevada, appears to have maintained more desert-like conditions (Betancourt *et al.* 1990; Thompson & Anderson 2000). Haplotype and nucleotide diversity for *B. punctatus* are much higher in the Mojave Desert (Fig. 1, locations 1–21, 28) than on the western Colorado Plateau (Table 2) likely reflecting Pleistocene persistence of populations in the Mojave Desert.

Bradford *et al.* (2003) suggested that the Amargosa River and the lower Colorado River were the sources for post-Pleistocene expansions of *B. punctatus* into the surrounding areas. There is some support for this perspective in the form of east–west structuring of haplotypes between populations on the lower Colorado River in Nevada (clade 2-2; Fig. 5)

and populations in mountains further to the west (clade 1-1; Fig. 5). There is also evidence of past fragmentation between clades centred in the Mojave Desert (clade 1-1; Fig. 5) and those in the extreme northwestern edge of the Sonoran Desert (clades 1-2, 1-3; Fig. 5), which could be attributed to Pleistocene refugia patterns. None of these patterns, however, have statistical support from initial permutational contingency table tests in the NCA.

We interpret the pattern of low haplotype and nucleotide diversity on the Colorado Plateau as provisional support for rapid post-Pleistocene range expansion from populations in the northern Chihuahuan Desert. The most likely scenario is that as the climate warmed, toads within the Rio Grande rift valley, or expanding northward up the valley, jumped the continental divide into the San Juan or Little Colorado River systems. Toads then quickly expanded throughout the Colorado Plateau along the major river corridors. This range expansion is consistent with a leading edge, or pioneer model (Hewitt 1993) of long-distance dispersants establishing rapidly expanding populations of low genetic diversity within newly colonized areas (reviewed in Hewitt 1996, 1999). The spread of Eastern haplotypes towards the Mojave Desert may have moved quickly along the southwestward-flowing Colorado River, facilitated by passive dispersal of adults and larvae. Expansion of Eastern haplotypes into the Mojave Desert may now be limited as immigrants are entering regions occupied by established local populations (Hewitt 1993). Eastward dispersal of Western haplotypes upriver into the Grand Canyon was likely hindered by river flow and topography.

Maintaining vicariant structure in the face of Pleistocene climate oscillation

The current contact between major continental clades of *B. punctatus* in at least two localities begs the question: Why has deep phylogeographic structure not been eroded during the multiple climatic oscillations during the Pleistocene period of the last 2 Myr, or more specifically, during the more extreme glacial–interglacial climatic cycles (Webb & Bartlein 1992) of the last 700 000 years? Seemingly, if current climatic conditions exemplify previous interglacial climates, contact between clades of *B. punctatus* would have occurred multiple times through the past million years. We could account for the lack of empirical evidence for regional introgression of haplotypes between clades by two scenarios.

Perhaps arid habitats were less extensive during previous interglacials. If true, then the clades of *B. punctatus* may not have actually come into contact during the Pleistocene and the current contact may be unique. The expansion of Eastern clade haplotypes through the Colorado Plateau and into the eastern Mojave Desert appears to be a Holocene event. The apparent recent expansion of the Western clade into the northeastern Sonoran Desert and northwestern fringe

of the Chihuahuan Desert may potentially be explained by recent development of favourable desert conditions within this region. Evidence from packrat midden data (Van Devender *et al.* 1984) indicates that the development of arid conditions in the northern Chihuahuan Desert following the Wisconsin glacial did not occur until the mid to late Holocene (*c.* 4 Ka). Unless climatic conditions were similarly developed during previous interglacials, aridization of areas peripheral to the core deserts may not have occurred. Under conditions less climatically or ecologically favourable, *B. punctatus* may not have been able to overcome barriers between desert regions during previous interglacials. A detailed evaluation of the distribution of arid habitats in these regions across multiple interglacial cycles would provide insight into this possibility, but we know of no palaeoecological records currently available of sufficient resolution.

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

Bufo punctatus as a member of the warm deserts biota

A profound change in our understanding of the biogeography of North American warm deserts began with the notion that widespread taxa might have been isolated by tectonically driven landscape transformations (e.g. Morafka 1977; Murphy 1983). These alternatives to scenarios dominated by Pleistocene climatic responses were not tractable prior to the advent of phylogeographic approaches to assessing the relationships of distributional and phylogenetic patterns to geology and palaeoclimate. We recognize

that isolation and divergence between regional desert biotas is likely the result of multiple events over several time frames, including the Pleistocene, but debate continues over the importance of late Neogene events to current North American biotic diversification and structure (e.g. Johnson & Cicero 2004). While our interpretations about *B. punctatus* are based on a single mtDNA gene, the available data indicate that this species has three divergent maternal lineages resulting from early vicariant events. The three-way split (polytomy) between the peninsular and continental clades implies a near-simultaneous set of vicariant events. Peninsular populations of *B. punctatus* appear to have been separated from the mainland by either a Pliocene northern gulf vicariance or a latest Miocene–early Pliocene southern gulf vicariance. Populations in the Sonoran and Mojave deserts were likely separated from those in the Chihuahuan Desert by the uplifting of the Colorado and Mexican plateaus and the Sierra Madre Occidental. These inferences are supported by the observations that where major clades have dispersed across historical barriers and are now found in sympatry, the patterns are ones of secondary contact resulting from recent population expansions. Furthermore, the deep splits between maternal lineages of *B. punctatus* among the regional deserts are generally congruent with phylogenetic subdivisions within an array of codistributed warm desert taxa (see Riddle & Hafner in press) that implicated late Neogene vicariant events as causal factors.

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This research is part of Jef Jaeger's PhD dissertation. Brett Riddle's interest in this work centers on its application to understanding the biogeography and evolution of North American aridlands biotas. David Bradford initiated this research with his studies on the ecology and population structure of *Bufo punctatus*.

Appendix Descriptions of sample locations, haloptypes, and sample identifications, by country, state, county, and map number (referenced in Fig. 1). Latitude and longitude (negative number) follow in parentheses, after location descriptions. Sample identification numbers (LVT) follow after haplotype identifications. Sequences are referenced in GenBank under accession numbers: AY010121–AY010166 and DQ085629–DQ085776.

United States: Arizona: Cochise County: 43-Paradise Cemetery, Chiricahua Mountains (31.923, 109.205), E08 (LVT4432); Coconino County: 65-Roden Spring, SW. of Roden Crater (35.416, 111.288), E01 (LVT6051); 66-Little Colorado River, at confl. with Colorado River (36.192, 111.801), E01 (LVT4480, 4483, 4486); 67-Page Golf Course, Page (36.920, 111.470), E01 (LVT4417-4419); Graham County: 40-SR266, 7 mi. SW. of jct. with US191, Pinaleno Mountains (32.559, 109.782), W05 (LVT6072); La Paz County: 31-Bill Williams River National Wildlife Refuge (34.267, 114.033), W05 (LVT5565-5566), W12 (LVT5567); Maricopa County: 32-Wickenburg (33.975, 112.733), W05 (LVT4445); Mojave County: 1-Mormon Wells, Beaver Dam Wash (36.975, 113.985) W13 (LVT5570); 2-Cedar Break, Virgin River Gorge (36.949, 113.792), W03 (LVT5616-5619), W15 (LVT5620); 30-Kaiser Spring Wash, 10 mi. S. of Wikieup (34.586, 113.495) W05 (LVT5621); Navajo County: 64-Clear Creek near McHood Park Lake (34.968, 110.644), E01 (LVT5622-5626); Penal County: 37-Buzzard Roost Spring, Galiuro Mountains (32.786, 110.547), W05 (LVT3573-3575); Pima County: 39-Arivaca Road, 2.2 mi. NW. of Arivaca (31.595, 111.316), W07 (LVT4446); Santa Cruz County: 38-Mt. Hopkins Road, Santa Rita Mountains (31.693, 110.957), W05 (LVT6070), W16 (LVT6071); Yavapai County: 33-Humbug Creek, N. of Lake Pleasant (33.975, 112.291), W05 (LVT4577), W06 (LVT4576); Yuma County: 34-High Tank 8, Kofa Mountains (33.379, 114.031), W05 (LVT5583-5585); 35-Palm Road, Kofa Mountains (33.337, 114.147), W12 (LVT6060, 6062); California: Inyo County: 23-Great Falls Basin, Argus Range (35.855, 117.381), W14 (LVT5592-5596); Riverside County: 24-Rawson Canyon (33.617, 117.025), W08 (LVT4596); 28-Cottonwood Spring, Eagle Mountains (33.738, 115.810), W05 (LVT5589, 5591), W12 (LVT5590); 29-Canyon Spring, Orocoopia Mountains (33.546, 115.655), W05 (LVT5588); San Bernardino County: 13-Horse Thief Springs, Kingston Range (35.773, 115.886), W01 (LVT3478-3480); 14-Colosseum Gorge Spring, Clark Mountain (35.554, 115.554), W01 (LVT4256); 19-Fort Piute, Piute Range (35.112, 114.997), W10 (LVT4459); 20-Vernandyles Spring, Marble Mountains (34.687, 115.642), W01 (LVT3507-3508, 4407); 21-Dripping Spring, Old Women Mountains (34.560, 115.209), W06 (LVT5598, 5599), W13 (LVT5600); 22-Horn Spring, Turtle Mountains (34.208, 114.788), W05 (LVT5587), W06 (LVT5586); San Diego County: 25-Borrego Palm Canyon, Anza Borrego Desert State Park (33.278, 116.428), W08 (LVT4448); 26-Augua Caliente Springs, Anza Borrego Desert State Park (32.948, 116.305), W06 (LVT4474, 4476), W08 (LVT4473), W11 (LVT4475, 4477); 27-Dos Cabezas Spring, Anza Borrego Desert State Park (32.715, 116.143), W08 (LVT4478), W11 (LVT4479); Nevada: Clark County: 3-Magnesite Wash, S. of Overton (36.501, 114.471), W03 (LVT2762); 4-Red Bluff Spring, Gold Butte area (36.464, 114.256), E01 (LVT2825-2826), W03 (LVT2824); 5-Surplus Spring, E. of Muddy Mountains (36.377, 114.445), E01 (LVT2777), W02 (LVT2776), W03 (LVT2778); 6-Cottonwood Spring, Black Mountains (36.204, 114.476), W03 (LVT4147-4149); 7-Connolly Spring, Gold Butte area (36.246, 114.108), W03 (LVT2900-2902); 8-Pupfish Refugium Spring, Black Canyon (36.009, 114.747), W02 (LVT2959), W03 (LVT2958), W04 (LVT2960); 9-Kiup Spring, Spring Mountains (36.163, 115.722), W01 (LVT4311); 10-La Madre Spring, Spring Mountains (36.187, 115.506), W01 (LVT3022-3023), W05 (LVT3024); 11-Pine Creek, Spring Mountains (36.122, 115.470), W01 (LVT2666); 12-Lost Cabin Spring, Spring Mountains (36.085, 115.650), W03 (LVT4236); 15-McCullough Spring, McCullough Range (35.649, 115.128), W03 (LVT3251); 16-Railroad Spring, McCullough Range (35.604, 115.198), W01 (LVT4287, 4291); 17-Highland Spring, Highland Range (35.597, 115.058), W06 (LVT3290); 18-Cow Spring, Highland Range (35.569, 115.014), W01 (LVT3313); New Mexico: Dona Ana County: 48-Corralitos Ranch Road, 19.4 mi. NW. of jct. with I10 (32.500, 107.100), E03 (LVT6073), E07 (LVT6074-6075); Eddy County: 49-SR137, 12.3 mi. S. of US285 (32.453, 104.483), E01 (LVT4436); 50-SR137, 12.6 mi. N. of TX State Line (32.158, 104.792), E03 (LVT4435, 4437); Grant County: 44-Evan's Lake (32.868, 108.580) E01 (LVT4449); 45-Martin Canyon, 1.5 mi. N. & 4.2 mi. E. of Hurley (32.717, 108.075), E01 (LVT4453, 4455), E09 (LVT4454); Guadalupe County: 61-SR91, 0.6–3.6 road mi. N. of Puetu de Luna (34.835, 104.619), E01 (LVT5629), E13 (LVT5627, 6054), E14 (LVT5628); Hidalgo County: 41-Box Canyon, SE. of Verden Bridge (32.628, 108.857), W05 (LVT4451-4452), W09 (LVT4450); 42-Granite Gap, Peloncillo Mountains (32.089, 108.974), E07 (LVT4426), W05 (LVT4427-4430); Luna County: 46-SR9, 26.2 mi. W. of Columbus (31.813, 108.048), E03 (LVT3416); 47-SR9, 19.2 mi. W. of Columbus (31.848, 107.944), E02 (LVT3414); San Juan County: 69-US64, 4.4 mi. E. of AZ state line (36.828, 109.001), E01 (LVT4441); San Miguel County: 60-SR104, between Cochas and Tucumcari, 12 mi. N. of county line (35.315, 103.972), E01 (LVT5631, 5634–5635), E13 (LVT5632), E15 (LVT5633); Socorro County: 63-Carbon Spring, NE. of Magdalena (34.321, 107.195), E01 (LVT4401); Torrance County: 62-US60, 2.5 mi. E. of Socorro County Line (34.439, 106.380), E01 (LVT4438), E03 (LVT4439-4440), E09 (LVT5641-5642); Texas: Brewster County: 53-Road from Panther Jct. to Persimmon Gap, Big Bend National Park (29.517, 103.123), E01 (LVT4412, 4415), E03 (LVT4414, 4416), E06 (LVT4413); 54-Rio Grande Village, Big Bend National Park (29.192, 102.950), E10 (LVT4595); Gillespie County: 56-On loop road, 2.35 mi. N. of Willow City (30.448, 98.655), E01 (LVT4404, 4406), E04 (LVT4405); Hall County: 58-SR70, 9.8 mi. N. of Turkey (34.528, 100.929), E13 (LVT6056-6067, 6059), E17 (LVT6058); Jeff Davis County: 51-FM1832, 10.6 mi. W. of SR17 (30.808, 103.910), E01 (LVT6076, 6078), E19 (LVT6077); Motley County: 57-SR70, 2.1 mi. S. of Matador (33.982, 100.829), E13 (LVT6055); Pecos County: 52-FM2886, 14.5 mi. S. of I10 (30.708, 102.193), E18 (LVT6069); Randall County: 59-Rim of Palo Duro Canyon (34.933, 101.140), E16 (LVT5636-5638); Val Verde County: 55-Comstock, Jct. US90 and SR163 (29.688, 101.173), E18 (LVT6068); Utah: Garfield County: 72-Hog Spring, SE. of jct. SR95 and SR276 (37.969, 110.511), E01 (LVT5607-5609), E11 (LVT5606), E12 (LVT5610); Kane County: 68-Wahweap State Fish Hatchery, Big Water (37.092, 111.667), E01 (LVT4422-4425), E05 (LVT4411); San Juan County: 70-Visitor centre, Hovenweep National Monument (37.390, 109.082), E01 (LVT6053); 71-Hovenweep Road, 0.2 mi. W. of state line (37.423, 109.048), E01 (LVT6052); Mexico: Baja California: 73-Catavina (29.733, 114.717), P03 (LVT1789), P04 (LVT1785, 1787–1788), P05 (LVT1786); 74-Bahia de Los Angeles (29.019, 113.800), P04 (LVT4468), P10 (LVT4469); 75-Rosorito (southern city) (28.516, 114.030), P03 (LVT4470); Baja California Sur: 76-San Francisco de la Sierra (27.583, 113.033), P01 (LVT1780), P02 (LVT1781), P03 (LVT1782-1784); 77-Santa Agueda 27.259, 112.350, P03 (LVT4471-4472); 78-San Pedro (23.790, 110.130), P08 (LVT4465); 79-Augua Caliente (23.442, 109.803), P03 (LVT4460), P06 (LVT4462), P07 (LVT4463), P11 (LVT4461); 80-Boca de La Sierra (23.388, 109.816), P03 (LVT4467), P09 (LVT4466); Sinaloa: 82-Hwy, 32 N. of Hiox (26.833, 108.370), W17 (LVT6358), W18 (LVT6359); Sonora: 36-Sonoita (Soroita) (31.850, 112.850), W05 (LVT4464); 81-Between Hornos and San Nicolas 27.776, 109.537, W19 (LVT6360).