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Molecular Systematics of Short-Horned Lizards: Biogeography and Taxonomy of a Widespread Species Complex

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Abstract.—We surveyed mitochondrial DNA (mtDNA) sequence variation in short-horned lizards (Phrynosoma douglasi) from throughout western North America and used these data to estimate an intraspecific phylogeny and to assess biogeographic scenarios underlying the geographic structure of lineages in this species. We sequenced 783 base pairs from 38 populations of P. douglasi and three putative outgroups (P. ditmarsi, P. orbiculare, P. platyrhinos). We detected high levels of nucleotide variation among populations and a spatial distribution of mtDNA lineages compatible with major geographic regions. The phylogenetic hypotheses best supported by the data suggest that P. douglasi, as currently described, is paraphyletic with respect to P. ditmarsi. Populations of P. douglasi from the Pacific Northwest (ID, CA, OR, WA) form a monophyletic group that is sister to the subsequent radiation of P. ditmarsi and other P. douglasi clades. These results suggest that diversifications within this widespread species are fairly old. We focused on the genetic structure of populations of P. douglasi from a geographic perspective and interpreted the intraspecific phylogeny in light of geologic and climatic changes in western North America during the last 20 million years. The generally high levels of genetic variation found in these population comparisons are in accord with high levels of morphological variation in this species group; however, only in the Pacific Northwest region is there spatial congruence between these phylogenetic results and subspecific ranges based on previous morphological studies. We compared the evolutionary units delineated in this study with previously described subspecies of P. douglasi and evaluated the support (from morphology and mtDNA) for each population lineage in the phylogeny and the implications for the taxonomy of this group. [Biogeography; cytchrome b; geographic variation; mitochondrial DNA; ND4; paraphyly; Phrynosoma ditmarsi; Phrynosoma douglasi; phylogenetics; phylogeography; population structure.]

Considerable interest has centered around the degree to which phylogeographic patterns of plant and animal taxa are related to historical changes in the environment (Brooks, 1985; Vrba, 1985; Cracraft, 1988; Riddle, 1995). Addressing this issue at a molecular level requires an understanding of the sequence of diversification among and within lineages and its concordance with environmental shifts that may have occurred at the same scale (Riddle, 1995). Because of the complexity of historical changes in the environment and the biotic responses to those changes, establishing concordance between lineage diversification and environmental change has not always been possible (Cronin and Schneider, 1990; Brown, 1995). In many cases, sequential diversifications in species with deep histories have been difficult to evaluate because more recent events may mask genetic evidence of older divergences. Thus, most studies of lineage diversification have emphasized recent divergence histories in a number of taxa and their concordance with recent environmental changes such as late Quaternary glacial cycles (Van Devender et al., 1987; Betancourt et al., 1990; Hewitt, 1993). In North America, however, some of the most dramatic changes in biome distribution and connectivity occurred 3–28 million years ago (MYA), primarily in response to regional climatic change associated with mountain building and changes in ocean currents (Axelrod, 1958, 1979, 1985; Barnosky, 1984; Leopold and Denton, 1987). With the molecular assessment of lineage

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differentiation within widespread taxa at a regional scale, genetic evidence of these environmental changes has been found (da Silva and Patton, 1993; Riddle, 1996).

A widespread taxon, the short-horned lizard (*Phrynosoma douglasi*), was chosen for study in an attempt to infer population genetic structure and phylogeny, explore the geographic context of these phylogenetic relationships, and infer the relationship between divergences in this group and historical environmental change in western North America. Short-horned lizards are a suitable subject for phylogeographic studies for three main reasons. First, previous phylogenetic studies within the genus *Phrynosoma* (Presch, 1969; Montanucci, 1987; Reeder, unpubl. data) and the fossil record (Robinson and Van Devender, 1973) indicate that *P. douglasi* has a long history (dating to the mid-Miocene), offering the opportunity to investigate lineage differentiation in response to both deep and more recent historical environmental changes. Second, this species has an extensive geographic distribution, ranging from southern Canada to central Mexico (Fig. 1), making it possible to assess population differentiation over a large part of western North America. Finally, *P. douglasi* is characterized by high levels of morphological and life-history variation among populations (Reeve, 1952; Nussbaum et al., 1983; Stebbins, 1985; Zamudio, 1996). These levels of differentiation are indicated by the recognition of six subspecies (Reeve, 1952) based on external morphological characters. Although the
taxonomic validity of these groups has been questioned (Nussbaum et al., 1983), this diversity in morphology suggests that regional substructuring is likely in this species.

The purpose of this study was twofold. First, we wanted to describe the phylogeographic pattern of a widespread species with a long history and to determine the presence and distribution of evolutionary lineages within this group. To do that, we used mitochondrial DNA (mtDNA) sequences to reconstruct a population-level gene phylogeny of Phrynosoma douglasi. Detailed phylogeographic studies of species with continental distributions are rare, yet these species offer a unique opportunity to examine processes of population differentiation over a heterogeneous landscape. Our results support previous findings that modern biota worldwide have deeper histories than previously considered (Patton et al., 1981; da Silva and Patton, 1993; Riddle, 1996). We focused on the genetic structure of populations of P. douglasi from a geographic perspective and interpreted our intraspecific phylogenetic hypothesis in light of geologic and climatic changes in western North America during the last 20 million years. Second, we used this hypothesis to illustrate how monophyletic population assemblages can be used for recognizing evolutionary units and species boundaries, to the extent that the data resolve relationships among populations of widespread species. Proponents of lineage-based species concepts argue that species boundaries should be defined by monophyly, such that the historical pattern underlying each evolutionary unit is recovered (e.g., Donoghue, 1985; Cracraft, 1987; Baum and Shaw, 1995). We compared the evolutionary units delineated in our study with previously described subspecies of P. douglasi (based on morphological variation) and evaluated the support (from morphology and mtDNA) for each population lineage in our proposed phylogeny and the implications of our findings for the taxonomy of this group.

**Materials and Methods**

**Population Sampling**

Short-horned lizards were collected from throughout their range during the summers of 1990–1993, or tissues were obtained from private and institutional tissue collections. The analysis included multiple populations from five of the six subspecies of *P. douglasi* (Fig. 1); we were unable to obtain samples of the Mexican subspecies *P. d. brachycercum*. We obtained mtDNA sequences from 64 individuals from 38 localities throughout the western United States (Fig. 1; Table 1) and from at least 1 individual from each of three outgroups (*P. ditmarsi*, *P. orbiculare*, *P. platyrhinos*) identified in previous phylogenetic work within the genus *Phrynosoma* (Montanucci, 1987; Reeder, unpubl. data). All lizards collected for this study were prepared as vouchers and deposited in collections (see the Appendix).

**Laboratory Protocols**

Total cellular DNA was isolated from frozen tissue samples by standard proteinase K extraction followed by phenol/chloroform purifications (Maniatis et al., 1982). Two segments of the mitochondrial genome were amplified via the polymerase chain reaction (PCR) (Saiki et al., 1988) using two pairs of primers (Table 2). The sequenced regions correspond to 345 bases of the ND4 gene and 438 bases of the cytochrome b gene (Fig. 2). Amplification conditions for cytochrome b consisted of 30 thermal cycles of 1-min denaturation at 94°C, 1-min annealing at 45°C, and 2-min extension at 72°C, followed by a 5-min extension at 72°C. Amplification of the ND4 fragment also consisted of 30 thermal cycles of 1-min denaturation at 93°C, 30-sec annealing at 56°C, and 2-min extension at 72°C, followed by a 5-min extension at 72°C. In every PCR reaction, one primer was biotinylated at the 5’ end. Single-stranded template for sequencing was obtained directly from the amplified product by use of streptavidin-coated magnetic beads (Dynal) that anneal to the biotinylated primer. The bead/DNA solution was
Table 1. Unique *Phrynosoma* composite mtDNA haplotypes, sample sizes, geographic locality, and subspecies included in this study.

<table>
<thead>
<tr>
<th>Lineage</th>
<th>Locality</th>
<th>n</th>
<th>Subspecies or species</th>
<th>Lineage</th>
<th>Locality</th>
<th>n</th>
<th>Subspecies or species</th>
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<td>hernandezii</td>
<td>51</td>
<td>Sonora, Mexico</td>
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<td>P. dimarsi</td>
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<td>1</td>
<td>hernandezii</td>
<td>52</td>
<td>Sonora, Mexico</td>
<td>1</td>
<td>P. dimarsi</td>
</tr>
<tr>
<td>26</td>
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<td>hernandezii</td>
<td>53</td>
<td>Nuevo Leon, Mexico</td>
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<td>P. orbiculare</td>
</tr>
<tr>
<td>27</td>
<td>Iron Co., UT</td>
<td>1</td>
<td>hernandezii</td>
<td>54</td>
<td>Kern Co., CA</td>
<td>1</td>
<td>P. platyrhinos</td>
</tr>
</tbody>
</table>

used directly in dideoxy chain-termination sequencing (Sanger et al., 1977) with Sequenase version 2.0 (U.S. Biochemicals) and 35S-labeled dATP.

Data Analyses

Sequences were read from one strand and aligned by eye to each other and to the published sequences of *Xenopus* (Roe et al., 1985). All sequences have been deposited with the EMBL/GenBank Data Libraries under accession numbers U71457-U71597. Pairwise sequence comparisons to determine the distribution and amount of variation and the degree of saturation by codon position were performed using MEGA 1.01 (Kumar et al., 1993). Levels of phylogenetic structure in the data were

Table 2. Oligonucleotide primers used for amplification and sequencing of *Phrynosoma* in this study. Primers are listed from 5' to 3' end. The position of each primer is given relative to the published sequence of *Xenopus* mtDNA (Roe et al., 1985).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position</th>
<th>Sequence</th>
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</thead>
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<tr>
<td>MVZ49a</td>
<td>16268-16291</td>
<td>AA TCT CAT CCA TTA ATT AAA ATT A</td>
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<tr>
<td>MVZ14b</td>
<td>17415-17439</td>
<td>GTC TIG TAA (G/A)CC (G/A/T)(A/G)A GAT GAA GAC C</td>
</tr>
<tr>
<td>ND4c</td>
<td>12900-12941</td>
<td>CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA GC</td>
</tr>
<tr>
<td>LEUc</td>
<td>13831-13857</td>
<td>AC CAC GTG TAG GTT CAT TTT CAT TAC</td>
</tr>
<tr>
<td>ND4intd</td>
<td>13095-13115</td>
<td>CGT CAA ACA GAT CTA AAA TCA</td>
</tr>
</tbody>
</table>

*a* Source: C. Schneider (pers. comm.).


*c* Source: Arévalo et al., 1994.

*d* Source: this study.
evaluated by examining tree length distributions and calculating the skewness statistic (g1) from 10^6 randomly generated trees (Hillis, 1991). Probability of phylogenetic structure was assessed using the values provided by Hillis and Huelsenbeck (1992). Phylogenetic analyses were performed using aligned sequences for all gene regions combined (total 783 nucleotides). We used only unique mtDNA lineages in the phylogenetic reconstruction, thus the final data set, including outgroup species, is composed of 47 unique mtDNA haplotypes (Table 1).

To assess levels of saturation of base substitutions at each codon position, we plotted percent sequence divergences (based on two substitution classes: transitions and transversions) against Tamura–Nei estimates of relative divergence for the same substitution classes (modified from Moritz et al., 1992; Villablanca, 1993). The Tamura–Nei divergences are analogous to the uncorrected percent divergences; however, they take into account deviations from equal base compositions and differences in substitution rates among bases. Nonisometric plots indicate increasing saturation of substitutions for transitions or transversions at each codon position. Heuristic analysis of saturation levels in six different substitution categories (Fig. 3) suggested that first and third codon position transitions might be saturated. For this reason, we explored the effect of two schemes that downweight these classes: (1) differentially weighting transitions and transversions and (2) weighting each codon position in reverse proportion to the observed rates of substitutions at each site.

Two methods for phylogenetic reconstruction were used, maximum likelihood (ML) (Felsenstein, 1981, 1993) and parsimony (Swofford, 1993), in combination with various character weighting schemes. Each base position was treated as an unordered character with four alternative states. In both analyses, trees were rooted using outgroup comparisons (Watrous and Wheeler, 1981), with the most distant species (*P. platyrhinos*) as the only designated outgroup. Sequence of taxon entry in both parsimony and ML heuristic analyses can bias species position in the resulting tree (Maddison, 1991; Swofford et al., 1996); thus, we used repeated randomized taxon input orders for all analyses.

We reconstructed and evaluated ML trees using the DNaML program in PHYLIP 3.5 (Felsenstein, 1993) or fastDNAm1 1.1 (Olsen et al., 1994). Three weighting schemes were used in the ML analyses: (1) equal weighting for all characters, (2) differential weighting of transitions/transversions, and (3) codon position weighting. In the first scheme, all substitutions were weighted equally, regardless of codon position or substitution type. In the second scheme, transitions (ts) were downweighted relative to transversions (tv) by a factor of 5 (ts:tv = 1:5) or 10 (ts:tv = 1:10). This range of transition/transversion ratios was chosen because it encompasses the observed transversion bias in the data. For the codon-position weighting scheme, nucleotides in each gene fragment were
grouped into domains corresponding to first, second, and third codon positions. The observed rates of variability at each codon position were calculated as a percentage of total positions in each domain that showed at least one substitution among the ingroup taxa sequenced (Table 3). Thus, this estimate of substitution rates at different codon positions is independent of tree topology and simply reflects the proportion of sites in each domain that vary. These rates were standardized by dividing all values by the smallest rate across all codon positions and gene fragments, and the resulting proportional rates were then used in the "categories" option of fastDNAML. This scheme effectively downweights all third position changes (those with the highest proportional rates) relative to first and second position substitutions. Alternative topologies were tested for significance at the 95% level using the Kishino–Hasegawa test for ML (Hasegawa and Kishino, 1989) implemented by DNAML and fastDNAML.

Maximum parsimony phylogenies were estimated using PAUP 3.1.1 (Swofford, 1993). Because of the large number of mtDNA lineages in the analysis, we used the heuristic search option in PAUP. Random addition sequences increase the effectiveness of heuristic searches because they decrease the likelihood that a search will find suboptimal trees in "tree islands" other than those containing the most-parsimonious trees (Hendy et al., 1988; Maddison, 1991). Parsimony analyses were performed with 100 replicate searches with random addition of taxa, TBR branch swapping, zero-length branches collapsed to yield polytomies, and the steepest descent option not in effect. As with the ML analysis, we searched for most-parsimonious trees using three weighting schemes: (1) equal weighting for all codon positions and substitution types, (2) transitions and transversions weighted separately (using step matrices of 1:5 and 1:10), and (3) the inverse of the observed rates of change at each codon position calculated previously for the ML analyses. The inverse of the proportional rates of change at each codon position were multiplied by 10 and rounded to the nearest integer, and these inte-
TABLE 3. Rates of base substitutions at each codon position for the three Phrynopsoma gene fragments sequenced for this study. Rates of substitution at each codon position were calculated as the proportion of these positions that vary among all ingroup taxa. The proportional rates were used to differentially weight changes in each domain in ML analyses. The inverses of these rates, multiplied by 10 and rounded to the nearest integer, were used as weights in the parsimony analyses.

<table>
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<th>Gene segment</th>
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<th>2</th>
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<td></td>
</tr>
<tr>
<td>No. changes</td>
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<tr>
<td>No. bases sequenced</td>
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<td>68</td>
<td>68</td>
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<tr>
<td>Rate</td>
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</table>

Phylogenetic Relationships

Maximum likelihood.—For each of the weighting schemes (equal weighting, ts:tv = 1:5, ts:tv = 1:10, codon position weighting), we performed 10 independent ML analyses to guard against biases resulting from taxon input order. Equal weighting at all positions yielded one ML tree (ln L = -4812.62) (Fig. 4a). An identical tree was obtained when transitions were down-weighted relative to transversions by a ratio of 1:5 (ln L = -4644.66). Multiple ML runs using a ts:tv ratio of 1:10 resulted in two trees that differ primarily in the placement of one taxon: P. ditmarsi (ln L = -4659.08 and -4659.00; Fig. 5). Weighting by codon position resulted in one tree (ln L = -4535.44; Fig. 4b) similar to that obtained with the 1:10 weighting scheme. A log-likelihood comparison test (Hasegawa and Kishino, 1989) did not differentiate statistically among the four trees obtained in all analyses. The "best" tree is the one obtained both by equal weighting and by using a ts:tv ratio of 1:5 (Fig. 4a), although it is not significantly more likely than the others when compared under the assumptions of equal weighting (tree 5a: ln L =

RESULTS

Sequence Variation

We aligned 783 base pairs (coding for 261 amino acids); of these, 285 were variable and 212 were phylogenetically informative. Alignment was straightforward because no insertions or deletions were found in the genes sequenced. Levels of sequence divergence (uncorrected) between outgroup and ingroup lineages range from 11.7% (between P. orbicularis and lineage 26, UT) to 16.5% (between P. platyrhinus and lineage 15, AZ). Percent sequence divergence among ingroup taxa (including P. ditmarsi) ranges from 0.1% (between lineages 17 and 18, two nearby populations from UT) to 11.9% (between P. ditmarsi and lineage 2, CA). A transition bias is evident in these data. The average transition/transversion ratio across all pairwise sequence comparisons in our data set is 6.15. This level of transition bias is within the range of biases previously reported for other vertebrates (Reeder, 1995) and serves as a basis for the transition/transversion weighting ratios we used in phylogenetic reconstruction. The tree length distribution for 10^6 randomly generated trees was significantly skewed to the left (g_1 = -0.59, P < 0.01), suggesting phylogenetic signal in the data (Hillis and Huelsenbeck, 1992).
-4699.88, SD = 5.47, P > 0.05; tree 5b: ln L = -4700.22, SD = 9.00, P > 0.05; tree 4b: ln L = -4699.47, SD = 8.68, P > 0.05). Thus, all four trees are equally likely working hypotheses for the evolution of this group (Figs. 4, 5).

The main features of the ML trees are (1) paraphyly of the species *P. douglasi* as a whole with respect to *P. ditmarsi*; (2) a geographic division of *P. douglasi* populations into three main clades: a monophyletic group of populations in the Pacific Northwest (PNW, including ID, WA, OR, and CA), populations inhabiting mostly the Great Basin and Colorado Plateau region (GB/CP), and a large clade including the remaining populations of *P. douglasi* south and east of the Rocky Mountains (SER); (3) basal position of the PNW clade relative to *P. ditmarsi* and remaining lineages of *P. douglasi*; and (4) some geographic separation between the GB/CP and SER clades. The four trees are similar and differ primarily in the placement of one important taxon, *P. ditmarsi*. Although both topologies result in a paraphyletic *P. douglasi*, it is uncertain whether *P. ditmarsi* is most closely related to the populations in the GB/CP clade (as in Figs. 4b, 5b) or whether it is the sister taxon to the two internal clades, GB/CP and SER (Figs. 4a, 5a). Using log-likelihood tests (Hasegawa and Kishino, 1989) under different models of evolution, we compared the four ML trees with user-defined trees that constrained the monophyly of *P. douglasi* populations.
(Table 4). In all cases, the paraphyletic ML trees obtained from the original analyses were more likely than the hypothetical monophyletic tree. In three cases, this difference was significant, as judged by the likelihood tests; the only times significant differences were not found between the paraphyletic and monophyletic trees were for the topologies that place *P. ditmarsi* as sister taxon to the GB/CP clade.

**Parsimony.**—In most parsimony analyses, shortest trees were found by all addition sequence replicates, suggesting there is a high probability that we have found the island with the overall shortest tree. The only exception to this was the analysis that included weighting by codon position, in which the shortest trees were found by 75% of the replicates. Phylogenetic analysis of all 783 nucleotide positions weighted equally resulted in 10 most-parsimonious trees 658 steps in length (consistency index [CI] = 0.522, retention index [RI] = 0.788). A strict consensus of these 10 trees (Fig. 6a) shows the same major groupings among the populations of *P. douglasii* and shows the paraphyly of this taxon indicated by the ML analysis, with *P. ditmarsi* placed as sister taxon to the two internal clades of *P. douglasii* populations. Weighting by codon position in parsimony yields similar results (Fig. 6b). This weighting scheme resulted in 18 equally parsimonious trees, 1,110 steps in length (CI = 0.574,
Table 4. Comparison of ML trees (from Figs. 4, 5) with hypothetical trees constrained to represent monophyletic *Phrynosoma douglasi* under different models of evolution. The log likelihood (ln L) and standard deviation (SD) of the original paraphyletic ML trees and hypothetical trees constrained to monophyly were calculated and compared using a likelihood ratio test. Asterisks represent significant differences between the original and constrained trees.

<table>
<thead>
<tr>
<th>Evolutionary model</th>
<th>Tree</th>
<th>Topology</th>
<th>ln L</th>
<th>SD</th>
<th>P &lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ts:tv = 1:1</td>
<td>4a</td>
<td>paraphyletic</td>
<td>-4812.62</td>
<td>8.46</td>
<td>**</td>
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<td></td>
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<tr>
<td>Ts:tv = 1:5</td>
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<td>paraphyletic</td>
<td>-4644.66</td>
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<td></td>
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</tr>
<tr>
<td>Ts:tv = 1:10</td>
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<td>-4659.00</td>
<td>11.47</td>
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<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Ts:tv = 1:10</td>
<td>5b</td>
<td>paraphyletic</td>
<td>-4659.08</td>
<td>9.04</td>
<td>**</td>
</tr>
<tr>
<td></td>
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<td>-4678.68</td>
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<tr>
<td>Codon weights</td>
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<td>-4535.44</td>
<td>8.98</td>
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<td></td>
<td></td>
<td>monophyletic</td>
<td>-4551.04</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Ts:tv = transition: transversion ratio.*

RI = 0.818). The strict consensus of the codon-weighted trees uncovers the GB/CP and PNW clades; however, the topology differs from all other ML and parsimony reconstructions in that the SER clade is not unambiguously monophyletic. Three populations in southern Arizona are basal to the SER and GB/CP clades, possibly resulting in a paraphyletic SER clade. However, despite this discrepancy, the support for these differences is low, as reflected by very low bootstrap values along branches supporting this paraphyly (Fig. 6b).

Differentially weighting transitions and transversions in parsimony does not significantly alter tree topology. A 1:5 ts:tv weighting scheme resulted in 10 equally parsimonious trees, 1,086 steps in length (CI = 0.510, RI = 0.778). As in the ML analysis, parsimony reconstruction with transitions and transversions weighted 1:5 was also unable to unambiguously resolve the branching order of *P. ditmarsi* in relation to the GB/CP and SER clades; thus, the strict consensus for these trees reflects the GB/CP clade, SER clade, and *P. ditmarsi* as a trichotomy (Fig. 7a). Using a 1:10 ts:tv ratio yielded congruent results; this weighting scheme yielded five minimum-length trees, 1,611 steps in length (CI = 0.516, RI = 0.783). The three clades of *P. douglasi* populations are resolved in this topology, and *P. ditmarsi* is unambiguously placed as sister taxon to the GB/CP clade (Fig. 7b).

As with the ML analyses, the parsimony analyses yielded four different tree topologies. The parsimony trees differ primarily in the position of *P. ditmarsi* relative to the GB/CP and SER clades and in the position of some populations of the SER clade, resulting in a paraphyletic SER clade under one weighting scheme. Despite these differences, the general arrangement of populations in well-supported clades remains constant. In fact, the lengths of all consensus trees under equal weighting are very similar. The consensus for trees obtained under equal weighting is 667 steps long, compared with 683, 675, and 676 steps for the consensus trees obtained with weighted characters. A comparison of all four trees using Templeton's test (Templeton, 1983) indicates that the 'best' tree is the one obtained by equal weighting of all substitutions (Fig. 6a), although the four topologies cannot be distinguished statistically based on our data (ts:tv = 1:5, SD = 4.475, P > 0.05; ts:tv = 1:10, SD = 5.295, P > 0.05; codon position weighting; SD = 6.004, P > 0.05). We compared the shortest tree obtained in the parsimony analyses (Fig. 6a) with a hypothetical tree that constrains the monophyly of *P. douglasi* populations. A Templeton test indicated significant differences between the original (and
Figure 6. Strict consensus trees for Phrynosoma douglassi, P. ditmarsi, and outgroup mtDNA lineages. Numbers above each branch represent bootstrap values derived from 100 replicates. (a) Consensus of 10 most-parsimonious trees obtained with equal weighting of all substitutions. (b) Consensus tree of 18 most-parsimonious trees obtained with codon position weighting.
Figure 7. Strict consensus trees for *Phrynosoma douglasi*, *P. ditmarsi*, and outgroup mtDNA lineages. Numbers above each branch represent bootstrap values derived from 100 replicates. (a) Consensus of 10 most-parsimonious trees obtained with a 1:5 transition:transversion weighting scheme. (b) Consensus tree of five most-parsimonious trees obtained with a 1:10 transition:transversion weighting scheme.
FIGURE 8. Simplified interpretation of *Phrynosoma douglasii* phylogeography, illustrating the geographic distribution of mtDNA haplotypes in each clade (PNW, SER, GB/CP, *P. ditmarsi*). Every monophyletic group of localities in the unweighted maximum likelihood analysis (Fig. 4a) is circled to illustrate the degree of local differentiation and regional integrity of haplotypes throughout the range of this species. Populations 28 (NM) and 40 (CO) are included in the GB/CP clade but have not been united with the western populations in that clade for ease of illustration. A simplified phylogenetic hypothesis indicates the relationship among all clades of *P. douglasii* and *P. ditmarsi* supported by our data. Letters on the tree indicate divergence events discussed in the text.

shorter) paraphyletic tree topology (658 steps in length) and a tree in which all *P. douglasii* populations are constrained to be monophyletic (668 steps in length, SD = 3.164, *P* < 0.05).

**DISCUSSION**

**Phylogeographic Pattern and Historical Biogeography**

The mtDNA lineages of *P. douglasii* exhibit clear geographic structuring. All tree topologies in our analyses recover an indisputably monophyletic Pacific Northwest (PNW) clade. This basal clade is well supported in all analyses, as indicated by branch lengths in ML and bootstrap values of ≥99% in parsimony. Remaining populations included in this study form a second strongly supported group (internal to or at the same hierarchical level as *P. ditmarsi*) composed of two clades that approximately follow geographic lines (GB/CP, SER). Figure 8 illustrates the geographic distribution of mtDNA haplotypes and the degree of geographic localization; for each of the major clades in our trees (PNW, SER, GB/CP, *P. ditmarsi*), all monophyletic assemblages of localities represented in the ML tree (Fig. 4a) are successively circled (for clarity, populations 28 and 40 were not included with the western GB/CP populations, although they belong to that clade). The distribution of *P. douglasii* haplotypes throughout most of the species’ range is characterized by high diversity and in general high regional integrity. The northeastern section of this species’ range (within the SER clade) is an exception to this pat-
tern and is characterized by very low haplotype diversity and the predominance of few haplotypes over large geographic areas.

The clades identified in our analyses are not geographically continuous in two cases, both involving populations of the GB/CP and SER clades. Two lineages basal to the GB/CP clade (28NM and 40CO) are found within the range of the SER clade. This "leakage" of haplotypes between two phylogeographic regions might reflect an area of complex intergradation of haplotypes in this region, and our sampling intervals may be too large to offer resolution at this level. A more complete sampling across this part of the range might shed some light on more detailed patterns of mtDNA haplotype distributions in this region. Another possible explanation is that this discontinuity is the result of a historical split of populations in a previously widespread GB/CP clade; perhaps haplotypes similar to those represented in the GB/CP clade once were widespread in the southwestern United States, and the two population groups (east and west) were separated when a southern lineage (SER) expanded northward and came to occupy regions in the central and northern Rocky Mountains. This sequence of events would be consistent with the topologies proposed here; however, the mechanism underlying the separation of GB/CP populations is currently unknown.

The genus *Phrynosoma* diverged from other phrynosomatid lizards during the late Oligocene–early Miocene (Montanucci, 1987; Etheridge and de Queiroz, 1988), and during the early to mid-Miocene the ancestral *Phrynosoma* differentiated into the lineages represented today (Presch, 1969; Montanucci, 1987). Species of the short-horned lizard clade, including *P. douglasi* and its allies, are currently primarily associated with mesic habitats (woodlands and forests) and are usually found at higher elevations throughout their range. Judging from habitat preferences and geographic distributions of modern species (Montanucci, 1987), the evolution of this lineage was probably associated with developing mesic woodlands in the upland environments of North America during the early Miocene (Axelrod, 1975; Wing, 1987). The fossil record for *P. douglasi*, although fragmentary, extends back to the mid-Miocene. Although *P. douglasi* and its close relatives are well represented in Plio-Pleistocene and more recent deposits (Holman, 1970, 1977, 1995; Rickart, 1977; Estes, 1983; Mead and Bell, 1994), records dating back to the early history of the species are rare. The oldest fossil tentatively assigned to *P. douglasi* was recovered from the mid-Miocene Split Rock Formation, Wyoming (42°N latitude; Robinson and Van Deven- der, 1973). This discovery suggests that short-horned lizards, or ancestors to this lineage, were already present in western North America during the mid-Miocene (17–20 MYA) and that this lineage probably originated prior to that time. Thus, the evolutionary history of the genus *Phryno- soma* and the fossil record for this species in particular lead us to consider the mid-Miocene as the minimum date for the evolution and differentiation of this species.

Geological and paleocological studies of the western United States indicate that the Miocene was a time of unstable climatic conditions (Potts and Behrensmeyer, 1992). Beginning in the late Oligocene, the mountain ranges of the western North American Cordillera were uplifted in close succession (King, 1958; McKee, 1972; Barnosky and Labar, 1989; Riddiman et al., 1989; Burbank and Barnosky, 1990) and served as strong regional modifiers of climate. These geological changes resulted in the aridification of newly formed rain shadows and ultimately in the regional differentiation of the West into distinct vegetational provinces by the late Miocene (Axelrod, 1985, 1992, 1995; Leopold and Denton, 1987; Axelrod and Schorn, 1994). These changes have been proposed as important factors in the differentiation and even extinction of other organisms (Webb, 1983; Riddle, 1995). The three main clades within our proposed phylogeny are associated with vegetational provinces that resulted from the physiographic changes in
North America during the Miocene. The three main divergences in our proposed phylogeny could be related to the climatic and physiographic changes that occurred during the Miocene: the basal divergence of PNW populations from populations in other parts of the range (A), the separation between the GB/CP and SER clades (B), and the formation of a relatively undifferentiated clade in the northern sections of the SER clade (C) (Fig. 8).

The isolation of the PNW lineage of \textit{P. douglasi} (divergence A, Fig. 8) is the deepest divergence in our proposed phylogeny and may have occurred simultaneously with a well-documented vegetational shift in the Miocene. Paleoeocological data suggest that aridification of most of the West began gradually during early and mid-Miocene followed by a rapid late-Miocene expansion of regional deserts, grasslands, and steppe communities (Axelrod, 1983, 1985). By the late Miocene, forested environments in the Great Plains were replaced by extensive grassland environments (Leopold and Denton, 1987), and the Sierra Nevada–Cascade orogeny brought increasing aridity to the intermountain region west of the Rocky Mountains (Wolfe, 1985; Leopold and Denton, 1987; Axelrod, 1992; Potts and Behrensmeier, 1992; Axelrod and Schorn, 1994). In stark contrast to this drying process throughout most of western North America, biotic change was much more gradual in areas northwest of the Rocky Mountains from the Continental Divide throughout the Columbia Plateau (Leopold and Denton, 1987; Axelrod, 1992; Axelrod and Schorn, 1994). Thus, during most of the Miocene (from 18 to 4.5 MYA) the area presently occupied by the PNW clade maintained a more typical ancestral flora of predominantly mesic vegetation; widespread grasslands and steppe developed in this region approximately 10 million years later than in comparable areas east of the Rocky Mountains (Leopold and Denton, 1987) and in the Great Basin (Axelrod, 1992).

The differences between the Pacific Northwest and the other regions probably influenced the differentiation of the PNW clade of \textit{P. douglasi}. As a consequence of these ecological shifts, southern populations may have been forced into more restricted ranges at the foothills and in the mountains of the southwest, while populations in the Pacific Northwest remained in the more mesic environments that persisted in that region. These changes could result in strong, discontinuous divergent selection on separate populations. These regional changes would have necessitated the evolution of specific ecological, physiological, and behavioral attributes in response to different environments. Comparisons among modern individuals from the Pacific Northwest and localities further south support this notion; short-horned lizards from the Pacific Northwest generally exhibit marked differences in behaviors, especially those associated with predator avoidance, when compared with individuals from other parts of the range (Zamudio, pers. obs.).

The second divergence is between the GB/CP and the SER clades (divergence B, Fig. 8). Populations included in the GB/CP clade are mostly restricted to the intermountain region and may have become isolated from southern populations by the uplifting of the Colorado Plateau and by the regional development of vegetational and elevational differences approximately 8–5 MYA. Fossil floras from the Sonoran and Great Basin deserts indicate the presence of forested regions during the Miocene and gradual replacement with individual xerophytic communities in each of these provinces (Axelrod, 1979, 1983). The western deserts are topographically complex, and given their regional differences in paleoclimate and vegetation, opportunities for restricted gene flow and differences in selective environments for the GB/CP populations and those further south in the Sonoran Desert have existed since the late Cenozoic (Shreve, 1942; Axelrod, 1983).

The widespread clade of \textit{P. douglasi} to the east of the Rocky Mountains is also of interest because population structure in this region offers information on more recent events in western North America. The
northernmost extent of this clade occupies
an area that was repeatedly covered by ice
sheets during the Pleistocene glacial cycles
(Potts and Behrensmeyer, 1992). Approx-
mately 18,000–22,000 years ago, during the
last glacial maximum, ice sheets covered
the northern part of the range occupied by
the SER clade, including at least the six
northernmost populations included in this
study. The lack of significant genetic struc-
ture in populations east of the northern
Rocky Mountains suggests that this area
was recolonized after the glacial retreat by
individuals from populations south of the
periglacial regions (event C, Fig. 8); thus,
the expansion of *P. douglasi* northeast of the
Rocky Mountains is relatively recent com-
pared with divergences of the main lin-
eages within this group. The continuity of
common haplotypes over a wide geo-
graphic area in the northeastern extent of
the range may also be maintained by gene
flow in the present or very recent past.

The reasons for the differentiation of *P.
ditmarsi* from the other populations of *P.
douglasi* are unclear. *Phrynosoma ditmarsi*
has been recorded only in the Sierra Man-
zanal, Sierra Baviacona, and the Rio Yaqui
drainage (Lowe et al., 1971; Lowe and
Howard, 1975; Perrill, 1983) in northeastern
Sonora. Apparently, the two "species" are
almost parapatric, with the nearest
populations of both approximately 19 km
apart. *Phrynosoma ditmarsi* inhabits rocky
outcrops associated with Madrean ever-
green woodland and open short-tree forest
(Lowe et al., 1971; Lowe and Howard,
1975). Evidence from captive-bred animals
suggests that *P. ditmarsi* differs in repro-
ductive timing from the Mexican popula-
tions of *P. douglasi* (Montanucci, 1989),
breeding early in the fall and presumably
storing sperm or arresting embryonic de-
velopment over the winter. In contrast, *P.
douglasi* breeds in the spring throughout its
range (Goldberg, 1971; Howard, 1974;
Montanucci, 1989; Zamudio, 1996). It is un-
known whether fall breeding is the pattern
in natural populations of *P. ditmarsi*, but if
so, this difference may act as a behavioral
reproductive isolating mechanism between
the two groups.

**Phylogenetic Structure and Taxonomic
Congruence**

Lineage-based species concepts in their
many forms are an important improve-
ment over other species models because
they attempt to incorporate historical pat-
terns of evolution into classification and
taxonomy (e.g., Donoghue, 1985; Cracraft,
1987; Baum and Shaw, 1995; Olmstead,
1995). Although the philosophical frame-
work underlying different lineage-based
species concepts has been developed and
argued extensively, the practical applica-
tion of lineage-based concepts awaits de-
tailed case studies. Whereas many system-
aticists now agree with the general
philosophy of recognizing species as evolu-
tionary lineages, which are uncovered or
diagnosed through studies of phylogenetic
systematics, few efforts to indentify line-
eage-based species have been published.
Practical recognition of lineage-based spe-
cies has been criticized because of prob-
lems that can arise in separating the his-
tory of a lineage from the history of the
characters used to identify that lineage
(Avise and Ball, 1990). This criticism has
been aimed especially at studies that rely
exclusively on monophyly in gene trees
and fixation of cryptic molecular charac-
ters to delimit species, resulting in ever-
finer and often impractical diagnoses of
species (Olmstead, 1995). In addition, in
some cases of recent differentiation, mole-
cular markers may offer conflicting views of
relationships at the population or species
level, making strict adherence to lineage-
based concepts difficult at best (Patton and
Smith, 1994).

The appropriateness of delimiting spe-
cies by monophyly of character trees is an
empirical issue and will depend on the to-
tal sum of information about lineage dif-
ferentiation available for the group of in-
terest and the resolution offered by those
data. Our proposed phylogeny for the
short-horned lizard clade is an example of
the application of the lineage-based species
concept and has implications for taxonomy
in this group. First, we addressed the issue
of resolution in our gene tree and com-
pared the evolutionary units delineated using molecular characters with units previously determined using morphology. We then used the concordant support from these independent sources as a basis for delimiting species boundaries.

Our phylogenetic analysis of mtDNA sequences generally does not mirror the current specific and subspecific taxonomy within this group. First, all our trees placed *P. ditmarsi* within *P. douglasi*, contradicting their current taxonomy. Montanucci (1987) proposed a phylogeny for *Phrynosoma* in which *P. douglasi* and *P. ditmarsi* are not closely related, based on morphological characters. However, Presch (1969) concluded that *P. ditmarsi* is a highly localized derivative of *P. douglasi*, and more recent work based on molecular data (Reeder, unpubl. data) also suggests that these two taxa are closely related. Evidence from our study of a *P. douglasi*–*P. ditmarsi* complex corroborates this second view of relationships within the genus *Phrynosoma*.

Beyond the issue of *P. douglasi* paraphyly, the mtDNA sequences further resolve lineages in our proposed intraspecific phylogeny, reflecting the evolutionary history of geographic units within this group. Our trees indicate that populations in the Pacific Northwest form an unambiguous and well-supported monophyletic group that corresponds closely to the previously named subspecies *P. d. douglasi*. In addition, there are two clades that are restricted to the Great Basin/Colorado Plateau and the areas south and east of the Rocky Mountains. Certain ambiguities exist in our gene trees, notably the affiliation of *P. ditmarsi* relative to the SER and GB/CP clades and the monophyly of the SER clade (uncovered by codon weighting in parsimony). Thus, a conservative assessment of our analyses results in two large and distinct clades of *P. douglasi* populations: the PNW clade (basal to all other lineages within this group) and a clade containing both the GB/CP and SER populations. This last clade may or may not be paraphyletic (relative to *P. ditmarsi*) and thus is reflected as a polytomy in the generalized proposed phylogeny in Figure 8.

The high levels of geographic variation in morphology in this species have long been recognized, and subspecific taxonomic units have been determined based on many of these geographically variant characters (Reeve, 1952). Nonetheless, no phylogenetic hypothesis has been proposed for the various morphological subspecies within *P. douglasi*. Therefore, we used the previously described morphological subspecies as evolutionary units for comparison with our gene tree. Superimposing subspecific designations on populations included in our proposed phylogeny (Fig. 9) makes clear the lack of concordance between evolutionary history and taxonomy in this group. Only the populations in the PNW clade form a monophyletic subspecies (*P. d. douglasi*); all other subspecies are poly- or paraphyletic. We compared the original tree with a hypothetical tree in which all subspecies are monophyletic; in both ML and parsimony, the original trees were significantly better than the trees with constrained monophyletic subspecies (Templeton test: constrained tree length = 796, SD = 18.01, *P* < 0.05; log likelihood test: constrained tree ln L = −5397.55, SD = 71.96, *P* < 0.05). Thus, our molecular tree is concordant with the morphological characters that separate *P. d. douglasi* from the remaining subspecies in this group.

Many of the morphological characters used in the original descriptions of the subspecies, such as background coloration, color of the dorsal spots, and body size (Reeve, 1952), are relatively plastic even within populations. However, several characters distinguish all populations of *P. d. douglasi* (the PNW clade) from neighboring populations. For example, the occipital and temporal spines (the "horns") are extremely reduced in the PNW populations, such that these lizards have only small inconspicuous tubercles projecting vertically from the head. In addition, the dorsal scales are of irregular size and distribution; the largest dorsal scales are keeled and set in a rosette of smaller keeled scales. In all populations outside of the PNW clade, the enlarged keeled dorsal scales are arranged in six to eight longi-
tudinal rows, and the rosette arrangement of scales is absent or poorly developed (Reeve, 1952). Although we did not sample the Mexican subspecies *P. d. brachy cercum*, this subspecies shares morphological characters with *P. d. hernandezi*; thus, we assume that the Mexican subspecies is affiliated with the two internal clades (SER and GB/CP) in our proposed phylogeny, although its exact position is currently unknown. A second lineage that is genetically and morphologically diagnosable is *P. ditmarsi*, described from two specimens from Sonora, Mexico (Stejneger, 1906). Al-
though morphological studies indicate a close affinity between *P. douglassi* and *P. ditmarsi* (Reeve, 1952; Presch, 1969), the two taxa are distinguished by numerous external features, and no intermediates between them are known (Reeve, 1952; Montanucci, 1987); thus, the validity of *P. ditmarsi* has never been questioned.

At least two evolutionary lineages are included in *P. douglassi* (sensu lato), based on the topology of the gene tree in our study and concordance with previously described morphological differentiation. The two segments of *P. douglassi* represent divergent lineages with no known contact zone, and one segment (the combined GB/CP and SER clades) may be more closely related to *P. ditmarsi* than it is to the other segment (the PNW clade). Based on all available information for this complex, we propose a taxonomic reorganization of this polytypic species. The name *P. douglassi* should be assigned to the PNW clade, with type locality of “Columbia River drainage” (Bell, 1829). For other populations outside the PNW region, the next oldest available name is *P. hernandezii*, with a type locality of “Sonora, Mexico” (Girard, 1858; Reeve, 1952; following Ellis and Henderson [1915] as first examiners and with spelling according to Hammerson and Smith [1991]).

Thus, we propose that three species be recognized for this group: *Phrynosoma douglassi*, *P. hernandezii*, and *P. ditmarsi* (Fig. 9). This new arrangement is conservative and recognizes basal and well-supported clades within our proposed phylogeny, all of which are morphologically distinct and evidently allopatric (Reeve, 1952; Lowe et al., 1971). This proposed rearrangement emphasizes the morphological and genetic distinctiveness of the PNW clade populations and of *P. ditmarsi*. Furthermore, areas of uncertainty in our proposed phylogeny (such as the possible paraphyly of the SER clade and the exact position of *P. ditmarsi*) are recognized as potential evolutionary signals that can be examined in more detail for further understanding of relationships within that clade. In this framework, species are units to be “discovered” as we develop working hypotheses of relationships among populations (Olmstead, 1995); further phylogenetic studies of morphological differentiation and additional molecular evidence may further refine divisions at the specific and subspecific level. The new taxonomy of this complex recognizes as species the evolutionary units uncovered by our current study. Distinguishing and recognizing these units is an important step toward understanding the patterns and processes of speciation within this group of lizards.

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APPENDIX