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PHYLOGEOGRAPHIC PATTERNS IN MITOCHONDRIAL DNA OF THE DESERT TORTOISE (*XEROBATES AGASSIZI*), AND EVOLUTIONARY RELATIONSHIPS AMONG THE NORTH AMERICAN GOPHER TORTOISES

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Abstract.—Restriction-fragment polymorphisms in mitochondrial DNA (mtDNA) were used to evaluate population-genetic structure in the desert tortoise *Xerobates agassizi* and to clarify evolutionary affinities among species of the gopher tortoise complex. Fourteen informative endonucleases were employed to assay mtDNAs from 56 *X. agassizi* representing 22 locations throughout the species' range. The mtDNA genotypes observed were readily partitioned into three major phylogenetic assemblages, each with striking geographic orientation. Overall, the *X. agassizi* mtDNA genotypes typify a common phylogeographic pattern, in which broad genetic uniformity of populations is interrupted by geographic features that presumably have functioned as dispersal barriers. The geologic history of the Colorado River area, which includes extensive marine incursions, may account for the marked mtDNA divergence between eastern and western *X. agassizi* assemblages.

In mtDNA comparisons among the four species of the gopher tortoise complex, both UPGMA and Wagner parsimony analysis strongly support the recognition of two distinct species groups previously suggested by traditional systematic approaches. Furthermore, the mtDNA data identify the eastern *X. agassizi* assemblage as the probable inceptive lineage of *X. berlandieri.* Results from both intra- and interspecific comparisons illustrate how clues to historical events may be present in the geographic structure of mtDNA phylogenies.

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The extended pedigree for any species constitutes its intraspecific phylogeny. For a species with limited historical dispersal and gene flow, branches in such a phylogeny are likely to have a geographic orientation. A new discipline concerned with these issues, and which draws on principles of both population genetics and phylogenetic systematics, has recently been termed "intraspecific phylogeography" (Avise et al., 1987). Methods of mitochondrial DNA (mtDNA) assay, which have provided most of the available data on "gene trees" (Avise et al., 1987; Nei, 1987) at the intraspecific level, were central to the development of this discipline. In general, mtDNA variability within species is extensive, and the qualitative mtDNA genotypes identifiable by restriction-enzyme assay lend themselves to phylogenetic assembly. It is not unusual for the resulting assemblages to display some degree of geographic structure; hence, the term "phylogeography."

Avise et al. (1987) outlined four categories of phylogeographic outcomes that could arise, in theory: I) discontinuities in a gene phylogeny, accompanied by spatial population separation; II) discontinuities in a gene phylogeny without spatial separation; III) phylogenetic continuity, spatial population separation; and IV) phylogenetic continuity, lack of spatial separation. Category I, in which populations within a species are characterized by phylogenetic differentiation with evident geographic orientation, has been the most commonly encountered situation (Avise et al., 1987). One explanation for such patterns involves historical, extrinsic (i.e., zoogeographic) barriers to genetic exchange, such that genic pedigrees across populations become recognizable branches on an intraspecific tree.

This study describes striking geographic structure in the mtDNA phylogeny of the desert tortoise, *Xerobates agassizi,* a species inhabiting desert and subtropical scrublands of the American Southwest (Fig. 1). Utilizing recent geologic findings, we implicate historical barriers to gene flow that appear to account, both geochronically and physiographically, for the observed phylogenetic discontinuities in mtDNA divergence. We also examine mtDNA from *Xerobates berlandieri, Gopherus flavomarginatus,* and *G. polyphemus,* which together with *X. agassizi* form a species complex

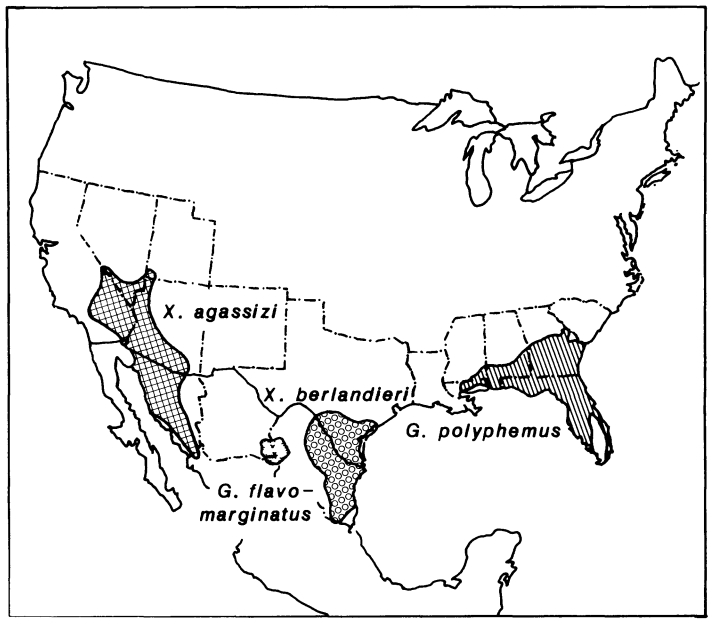

FIG. 1. Geographic ranges of the four extant species of gopher tortoises.

commonly known as the gopher tortoises. Our results complement and extend previous morphological and fossil-based interpretations concerning species formation and evolutionary history in the group. This survey represents the first extensive compilation of mtDNA data in turtles of any kind.

The Gopher Tortoises—Systematic Background

The gopher tortoises represent a distinctly North American lineage of testudinid turtles characterized by structural specialization for digging and burrowing. Until recently, the four extant species and a number of fossil forms were assembled under a single generic heading, *Gopherus,* though two complexes were apparent osteologically (Auffenberg, 1976). Employing additional morphological and extensive paleontological evidence, Bramble (1982) provided a case for generic separation of the two species complexes. He argued (Bramble, 1982 p. 864) that distinctions between the *agassizi* group (*G. agassizi* and *G. berlandieri*) and the *polyphemus* group (*G. flavomarginatus* and *G. polyphemus*) "generally exceed those that distinguish many (perhaps most) other genera of land tortoises." Furthermore, a fairly complete fossil record indicates that the two lineages were distinguishable by the Middle Miocene and have been largely allopatric since that time, though with shifting geographic distributions. The current ranges are shown in Figure 1. Thus, Bramble (1982) erected a new genus, *Scaptochelys,* for the structurally generalized *agassizi* group, while retaining *Gopherus* for the more fossorial *polyphemus* group. Bour and Dubois (1984) followed with a nomenclatural revision, noting that *Xerobates* Agassiz 1857 had priority over *Scaptochelys* Bramble 1982 as a valid published name. Honoring both revisions, we use the taxon name *Xerobates* in this study.

MATERIALS AND METHODS

Fifty-six *X. agassizi* collected from 22 localities throughout the species' range (Table 1, Fig. 2) were transported live to the laboratory in Aiken, SC. Two live *X. berlandieri* from near San Antonio, TX, were provided by F. Rose, and three *G. polyphemus* were collected from Calhoun, Dougherty, and Emanuel Counties, GA. J. Flannagan shipped frozen tissue samples from two juvenile *G. flavomarginatus* following autopsies at the Houston Zoo Hospital. Preparation and purification of mtDNA from heart and liver were conducted according to

TABLE 1. Collection sites and sample sizes for *Xerobates agassizi.*

Site code	Locality	State	*N*
C1	Fremont Valley, San Bernardino Co.	CA	4
C2	Superior Valley, San Bernardino Co.	CA	4
C3	Kramer Junction, San Bernardino Co.	CA	4
C4	Chuckwalla Mountains, Riverside Co.	CA	3
C5	Chemehuevi Valley, San Bernardino Co.	CA	4
C6	Arrowhead Junction, San Bernardino Co.	CA	4
C7	Ivanpah Valley, San Bernardino Co.	CA	4
N1	Piute Valley, Clark Co.	NV	1
N2	Good Springs, Clark Co.	NV	1
N3	Coyote Springs, Lincoln Co.	NV	1
N4	Mormon Mountains, Lincoln Co.	NV	1
N5	Gold Butte, Clark Co.	NV	2
U1	Hurricane, Washington Co.	UT	1
U2	Paradise Canyon, Washington Co.	UT	5
A1	Virgin Mountains, Mohave Co.	AZ	3
A2	Hulalapai Mountains, Mohave Co.	AZ	1
A3	Harquahala Mountains, Maricopa Co.	AZ	1
A4	South Mountain, Maricopa Co.	AZ	3
A5	Picacho Mountains, Pinal Co.	AZ	1
A6	Red Rock, Pima Co.	AZ	2
S1	Mazatan	SO	3
S2	Alamos	SO	3

Lansman et al. (1981), with two minor modifications. First, the concentration of EDTA in homogenization buffers (MSB-EDTA-Ca^{++}, and MSB-EDTA) was increased to 100 mM, thereby reducing DNAase activity and enhancing mtDNA yields. Second, cesium chloride concentration was increased to near saturation (a refractive index of 1.395–1.396), facilitating the separation of nuclear and mitochondrial DNA fractions, which tend to band closely in tortoise preparations during gradient centrifugation.

Seventeen restriction endonucleases were selected for the assay: three with pentanucleotide recognition sites (*Ava* I, *Ava* II, *Hinc* II) and 14 with hexanucleotide recognition sites (*Bam*H I, *Bcl* I, *Bgl* I, *Bgl* II, *Cla* I, *Eco*R I, *Hin*d III, *Kpn* I, *Nde* I, *Pst* I, *Pvu* II, *Sac* I, *Stu* I, and *Xba* I). Restriction digests were conducted overnight under conditions recommended by the enzyme suppliers (Bethesda Research Laboratories and New England Biolabs). Digestion fragments were end-labeled with the appropriate α-^{32}P-labeled nucleotide(s), separated by electrophoresis through 0.6–1.5% agarose gels, and revealed by autoradiography. Fragment sizes were compared against a 1-kb ladder standard available from Bethesda Research Laboratories. Estimates of mtDNA genome size were made from digestion profiles involving multiple fragments in the 0.5–8.0 kb range.

Within *X. agassizi,* each of the restriction profiles produced by a given endonuclease was identified and assigned an upper-case letter code. Following convention, the common profile was designated "C," profiles differing by a single restriction-site alteration (gain or loss) were encoded "B" or "D," and profiles involving two or more site changes received nonadjacent letter assignments. Compiling this information for all enzymes, we derived an array of composite codes, each reflecting a distinct mtDNA genotype (clone). Individual tortoises were then categorized according to the composite code identifying their particular mtDNA genotype. Due to the number of multiple site changes observed across species, no attempts were made to code the other tortoise taxa relative to *X. agassizi.*

Estimates of nucleotide sequence divergence (p) between *X. agassizi* clones and among tortoise species were calculated by the fragment approach of Nei and Li (1979). From the resulting distance matrix, a phenogram relating the various genotypes was constructed by UPGMA clustering (Sneath and Sokal, 1973), using the average linkage algorithm of the BMDP statistical package (Dixon, 1981). A data matrix encoding presence-absence status of each restriction

fragment in each mtDNA genotype was also used to generate a Wagner parsimony network by the METRO algorithm in the PHYLIP phylogenetic package distributed by J. Felsenstein. A bootstrap approach, BOOTM of the PHYLIP package, was employed to place confidence limits on the branches generated by the Wagner network (Felsenstein, 1985). The procedure comprised 200 separate runs: 100 replicates for each of two input orders of taxa. Although the statistical validity of bootstrapping is compromised by the lack of complete independence of character-state changes in fragment data, resulting confidence values can nonetheless be viewed as relative measures in support of observed network branches (see Bermingham and Avise, 1986).

Results

Three endonucleases, *Bam*H I, *Kpn* I, and *Sac* I, failed to produce more than one cut in any of the mtDNAs assayed and will not be considered further. The remaining 14 enzymes collectively accounted for 35 different digestion profiles involving 84 restriction fragments in *X. agassizi* and for 68 profiles involving 147 fragments overall. Profiles for at least four endonucleases were particularly amenable to genome-size estimation for each species. Averaging these estimates, we calculated the mtDNA genomes of both *X. agassizi* and *X. berlandieri* to be approximately 16.4 kb in length. Estimates for *Gopherus* mtDNA were somewhat smaller, approximately 15.8 kb; however, some small fragments (less than about 0.4 kb) may not have been detected. These values, which to our knowledge represent the first estimates of mtDNA genome size reported for turtles, fall near the lower end of values documented for other vertebrate species (Brown, 1985). Some mtDNA size polymorphism (<500 base pairs) appeared to be present in *X. agassizi,* but differences were neither pronounced nor readily scored relative to the size variation documented in certain other lower vertebrates (Densmore et al., 1985; Bermingham et al., 1986).

Xerobates agassizi

Five different mtDNA clones were resolved among the 56 *X. agassizi* assayed (Table 2). The small number of clones observed, coupled with the fact that they were largely interconvertible by a series of single restriction-site changes, permitted a hand-drawn phylogenetic network to be constructed by the parsimony approach of Avise et al. (1979). The network consists of three major genetic assemblages, each with a distinct geographic distribution (Figs. 2, 3).

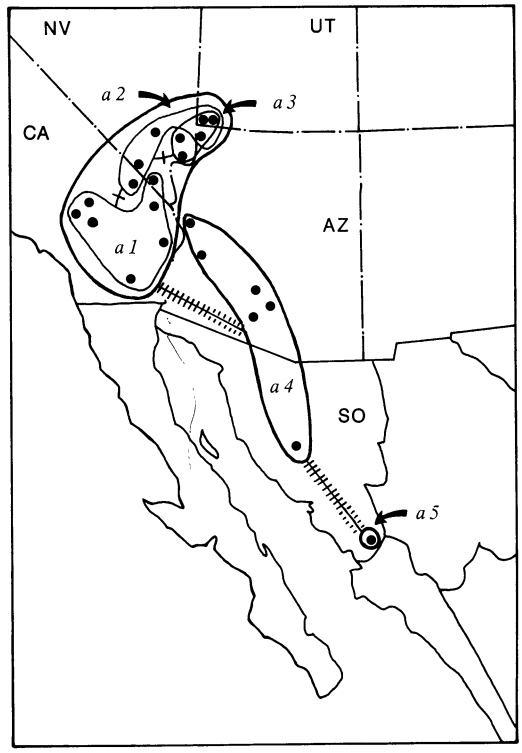

FIG. 2. The mtDNA phylogeny for *Xerobates agassizi* superimposed over the geographical sources of collections. The solid circles represent collection localities. Distribution of the five distinct mtDNA clones (a1–a5) are encircled, and branches interconnect related genotypes. The solid lines crossing branches represent the numbers of observed restriction endonucleases producing gel profile changes; the solid and dashed lines collectively represent the numbers of restriction-site changes responsible for these profile differences.

The first assemblage consists of three closely related clones, a1–a3, confined to and fixed in sampled populations north and west of the Colorado River. The most common genotype, a1, is distributed throughout the Colorado and Mojave deserts in California and extends into southern Nevada along the Piute Valley. Clones a2 and a3 are

TABLE 2. The mtDNA genotypes observed in *X. agassizi*. Letters of the composite genotypes refer to restriction-fragment patterns for the following endonucleases (in order, from left to right): *Ava* I, *Ava* II, *Bcl* I, *Bgl* I, *Bgl* II, *Cla* I, *Eco*R I, *Hinc* II, *Hind* III, *Nde* I, *Pst* I, *Pvu* II, *Stu* I, and *Xba* I. Collection sites are coded as in Table 1.

Clonal designation	Composite genotype	Number of tortoises	Collection site
a1	CCCCCCCCCCCCCC	25	C1–C6, N1
a2	CCCCCCCCCCCCCB	13	C7, N2–N3, U1–U2, A1
a3	CCCCCCCCCBCCCB	5	N4–N5, U2, A1
a4	ECHCBBDDBACCBD	11	A2–A6, S1
a5	FDFCCABBBACBDC	3	S2

restricted to the northeastern Mojave and are geographically coincident with the northern limit of the species' range. Within this area, clone a2 appears to be fairly widespread, ranging from Ivanpah Valley, California, eastward through Nevada, the Arizona strip (a narrow section northwest of the Grand Canyon), and into southern Utah. Clone a3 appears to be more limited in distribution, being represented by five individuals from four locales (A1, N4, N5 and U2) in the extreme northeastern portion of the Mojave.

A second assemblage, represented by the single clone a4, occurs in all assayed tortoises from west central and southern Arizona (samples from southwestern Arizona were sought but were not obtained). This "eastern" genotype is distinguished from mtDNA genotypes west of the Colorado River by a minimum of 17 restriction-site changes involving ten of the 14 informative enzymes. Furthermore, a4 is remarkably widespread, ranging from the Hulalapai Mountains site in west central Arizona to central Sonora, a linear distance of about 800 km. In southern Sonora, however, tortoises are characterized by another highly distinct genotype (a5) that differs from other clones at a level of divergence approaching that which distinguishes eastern and western assemblages. Thus, the southern clone, a5, constitutes a third major mtDNA assemblage.

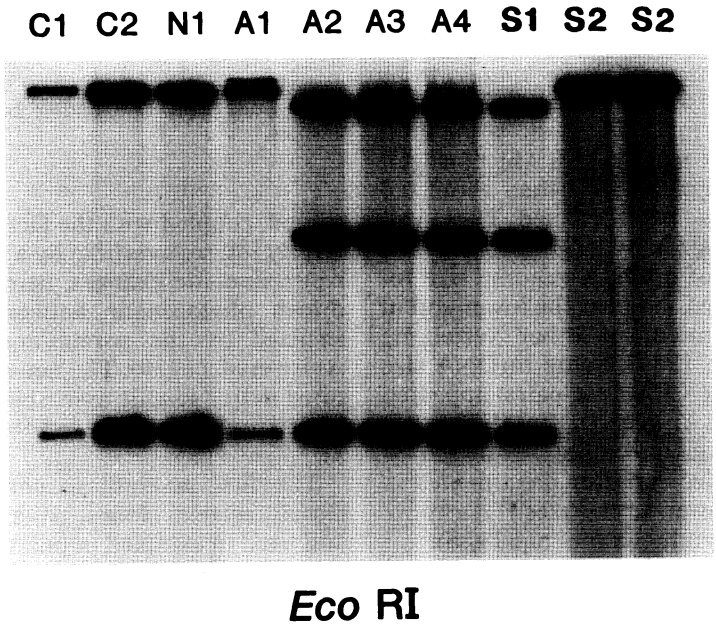

FIG. 3. Representative *Eco*R I digests of mtDNA from *Xerobates agassizi*. The three distinct profile patterns, from left to right, characterize the western, eastern, and southern assemblages observed in Figure 2. Site codes above each lane identify localities for the selected individuals.

TABLE 3. Estimates of base substitutions per nucleotide (p) among mtDNAs of the North American gopher tortoises. a1–a5 = clones observed for *X. agassizi*; ber = *X. berlandieri*; poly = *G. polyphemus*; and flavo = *G. flavomarginatus*.

	mtDNA							
mtDNA	a1	a2	a3	a4	a5	ber	poly	flavo
a1	0.000							
a2	0.002	0.000						
a3	0.004	0.002	0.000					
a4	0.055	0.056	0.051	0.000				
a5	0.050	0.055	0.050	0.042	0.000			
ber	0.068	0.069	0.063	0.048	0.071	0.000		
poly	0.135	0.136	0.135	0.101	0.100	0.105	0.000	
flavo	0.110	0.111	0.110	0.088	0.104	0.111	0.045	0.000

Within the western assemblage (a1–a3), estimates of numbers of base substitutions per nucleotide (p) were quite low (less than 0.005; Table 3). The three western genotypes are, in fact, distinguished by only 1–2 mutation steps: a1 differs from a2 by the loss of an *Xba* I restriction site, whereas a3 exhibits a second change involving an *Nde* I site gain. In contrast, clonal comparisons across the major genetic assemblages reflected substantial mtDNA differentiation, with levels of p ranging from 0.042 to 0.056 (Table 3). Although these estimates fall within the known range of genetic distances for other intraspecific comparisons, they are certainly among the largest values reported (see Avise et al., 1987).

In light of the high divergence levels (and the concordant profile changes observed for a large enzyme contingent), it is important to consider whether the apparent genetic "breaks" in *X. agassizi* mtDNA might conceivably be attributable to singular molecular genetic events, such as large-scale deletions/additions or genome rearrangements involving multiple restriction sites. If this were indeed the case, then resulting divergence values could mistakenly be interpreted to indicate long-term mtDNA lineage separation in *X. agassizi*. However, we can largely dismiss this possibility by relating several lines of argument put forth in Saunders et al. (1986) to our study: 1) the minor size variation observed in *X. agassizi* mtDNA was evident within each genetic assemblage and did not distinguish the various groups; 2) two enzymes (*Bgl* I and *Pst* I) showed identical multifragment pattern profiles in all individuals, providing evidence that gene arrangement is conserved in the area of the mtDNA molecule encompassing these sites; 3) whereas some pattern profiles differed by a single restriction-site change, others required at least two site differences (Table 2); 4) some changes involved single site losses in one assemblage relative to another (e.g., western to eastern: *Ava* I, *Bgl* II, *Cla* I, *Pvu* II), whereas others involved single site gains in the same direction of comparison (*Eco*R I, *Xba* I); and 5) not all profile changes were perfectly concordant with major breaks (e.g., certain restriction enzymes distinguishing eastern from southern assemblages had identical patterns for eastern and western samples, and vice versa [Table 2]). Overall then, it is most likely that many apparently independent mutation events account for the high mtDNA divergence levels observed among the phylogeographic assemblages of *X. agassizi*.

Interspecific Comparisons

Gopherus and *Xerobates* form highly distinct clusters in the UPGMA phenogram (Fig. 4), with a mean sequence divergence (base substitutions per nucleotide) of $\bar{p} = 0.112$. This branch dichotomy is also evident in the Wagner network topology (Fig. 4), in which generic separation is supported at the 100% confidence level by the bootstrap procedure. Within *X. agassizi*, the genetic distinctiveness of the western assemblage (a1–a3) is emphasized in both the UPGMA and Wagner trees, and the validity of this grouping is supported at the 100% confidence level by bootstrapping. Differences arise between the two tree-building methods in their placement of *X. berlandieri* relative to the *X. agassizi* clones. Pheneti-

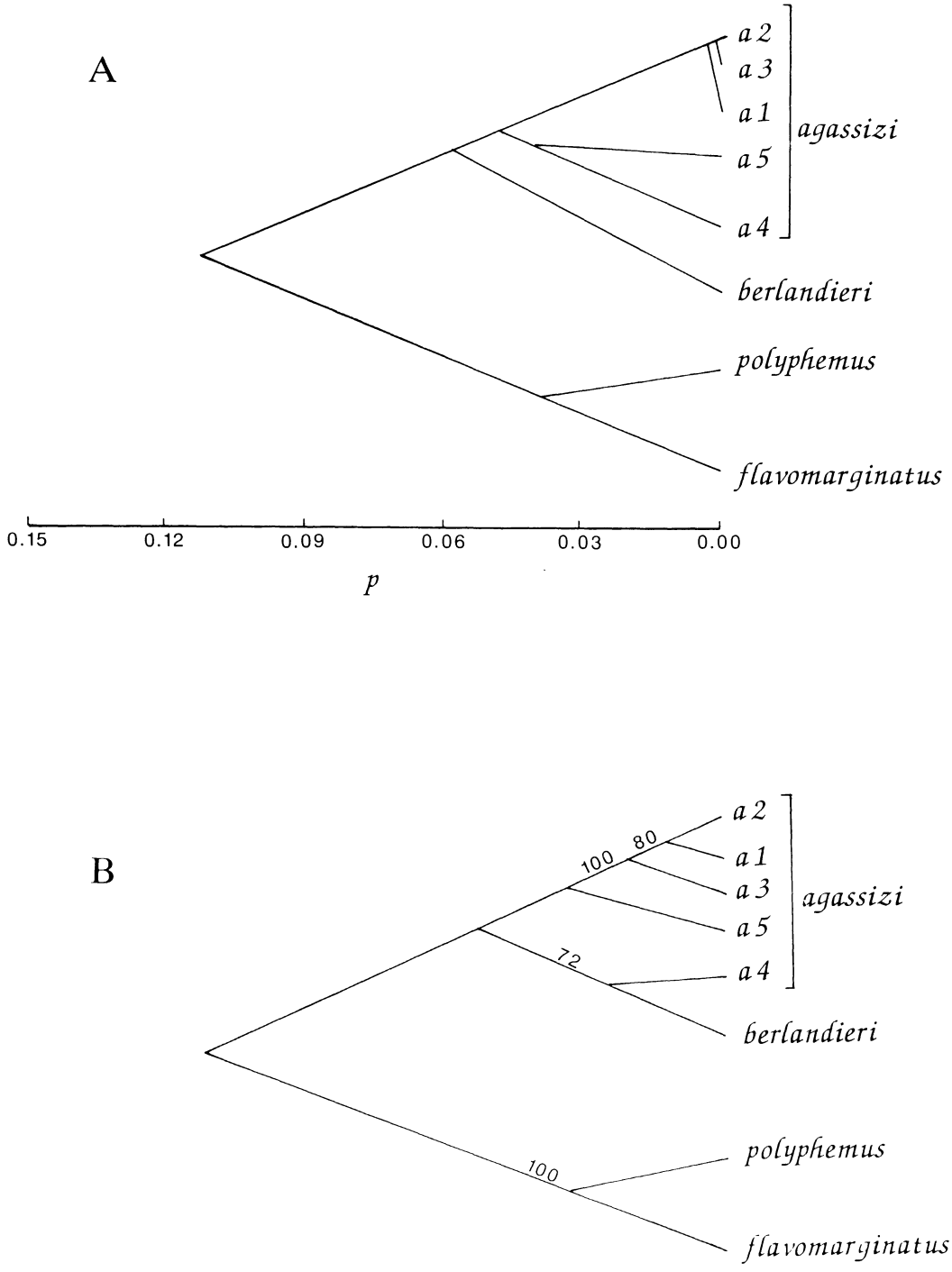

FIG. 4. A) Phenogram of mtDNA relationships within and among tortoise species generated by UPGMA analysis of genetic distance (*p*) estimates. Genotypes in *X. agassizi* are abbreviated as in Tables 2 and 3. B) Wagner parsimony network generated from the presence-absence mtDNA-fragment matrix and arbitrarily rooted along the branch separating *Gopherus* and *Xerobates*. Numbers along network branches indicate the percentage of times that a group was distinguished in the bootstrap analysis (only values greater than 70% are shown).

cally, *X. berlandieri* joins with a cluster containing all five *X. agassizi* genotypes, whereas in the Wagner network, *X. berlandieri* and a4 (the eastern assemblage) form a separate cluster, which in turn joins the branch containing the remaining four *X. agassizi* clones.

Discussion

Intraspecific Phylogeography of Xerobates agassizi

What could account for the pronounced genetic divergence observed between the eastern and western assemblages of *X. agassizi*? The geographic position of the genetic break implicates the general Colorado River area as a possible historical impediment to gene flow. Physiographic constraints are evident in the steep canyon areas of the upper Colorado, though it is difficult to view today's lower Colorado as a serious barrier to tortoise dispersal. To appreciate fully the role the lower Colorado River region may have played in shaping mtDNA phylogeny in *X. agassizi,* it is necessary to consider the middle and late Cenozoic history of this drainage within the surrounding "Basin and Range Province" (Lucchitta, 1972) of southern California and western Arizona.

Recent stratigraphic interpretations and compilation of regional geology indicate that, prior to the Miocene, the area now occupied by the Colorado Plateau was topographically lower than the Basin and Range Province lying to the south and west (Lucchitta, 1979). As a result, middle-Cenozoic drainages flowed north and northeast onto the plateau. During the Miocene, extensive volcanism and tectonism in the Basin and Range terrain enhanced structural separation from the Colorado Plateau, resulting in major drainage changes and diversions. Evidence suggests that the initiation of southwestward drainage of an early Colorado River on the plateau occurred during the middle Pliocene (Young and Brennan, 1974).

Within the Basin and Range region, two incursions of marine waters were important in the formation of the lower Colorado. The first, which extended just north of Yuma, Arizona, resulted from Miocene tectonism and presumably represented an early phase of opening of the Gulf of California (Lucchitta, 1979). Later, a far more extensive marine transgression, produced by downwarping, reached the Lake Mohave area, some 200 km north of the first incursion. Deposits from this inundation, known as the Bouse Formation (Metzger, 1968), indicate a shallow, relatively broad (40–50 km) embayment of brackish-to-marine water inhabited by foraminifers, mollusks, ostracods, charophytes, and barnacles (Smith, 1970). Some researchers have independently viewed the Bouse embayment as a Pliocene event (Metzger, 1968; Smith, 1970; Lucchitta, 1972, 1979). Although age assignments range over 3–8 million years, a K-Ar date of 5.47 ± 0.20 MY, determined from Bouse tuff near Yuma, Arizona, is considered the most reliable of the reported estimates (Shafiqullah et al., 1980). It is not altogether clear, however, whether the embayment had transgressed its full extent at that time. Varied uplifting along portions of the lower Colorado region and the adjacent Colorado Plateau later resulted in the retreat of the Bouse sea and the formation of the modern Colorado River.

In several vertebrate groups, mtDNA has been estimated to accumulate genetic differences at an initial rate of about 2% sequence divergence per million years (Brown et al., 1979; Wilson et al., 1985; Shields and Wilson, 1987). If this rate also holds for tortoises, then time of divergence between the eastern and western assemblages of *X. agassizi* dates to the Middle or Late Pliocene (about 2–3 MY B.P.). Thus, these two major lineages appear to be descended from a common ancestor that lived roughly within the broad time span corresponding to the marine transgression responsible for the Bouse Formation. Following the retreat of the Bouse sea, stream-flow rates of the lower Colorado might have continued to inhibit tortoise dispersal until very recent times (when drainage intervention practices of humans potentially diminished the barrier).

Other studies addressing intraspecific variation in *X. agassizi* offer independent support for the Colorado's role as a major zoogeographic barrier. Based on a survey of allozymes encoded by about 20 loci, Jennings (1985) identified two major groups that correspond roughly with populations inhabiting 1) the Mojave Desert and 2) the

Sonoran Desert and the Sinaloan Thornscrub. No fixed allelic differences were detected between samples, but phenograms generated from genetic distance values (Nei's *D*) displayed clusters generally distinguishing these geographically disparate sites. Overall, Jennings' data seem to associate reduced nuclear gene flow with the Colorado River region. Genetic patterns suggesting similar zoogeographic constraints for the Colorado River area have been documented for other vertebrate species (Avise et al., 1974; Patton and Yang, 1977).

An east-west break was also evident in a recent morphometric survey involving some 600 *X. agassizi* from U.S. localities (Weinstein and Berry, 1987). Discriminant analysis of eight shell characters readily separated Arizona tortoises from California-Nevada animals. Interestingly, tortoises from the Beaver Dam Slope area of Utah and northern Arizona emerged as an equally distinct morphometric group. This geographic region corresponds closely with the distribution of mtDNA clone a3.

Nonetheless, there may remain some difficulty with the hypothesis invoking Pliocene events as initiators of population divergence in *X. agassizi.* Fossil *Xerobates* from Pliocene beds, though represented by fragmentary material, are not readily assigned to *X. agassizi,* whereas fossils that have been identified as *X. agassizi* have been restricted to Pleistocene deposits (D. M. Bramble, pers. comm.). Though some morphological differentiation is apparent between eastern and western assemblages (Weinstein and Berry, 1987), perhaps more noteworthy is the degree of morphological stasis that accompanies the observed mtDNA divergence levels among *X. agassizi* genotypes. Thus another possibility is that the genetic differences between eastern and western *X. agassizi* populations arose over more recent evolutionary time scales. With respect to mtDNA, this would imply either an unusually rapid rate of mtDNA divergence or geographic sorting of mtDNA genotypes from a recent, highly polymorphic ancestral population. Theoretically, the latter possibility is the more feasible explanation, but it receives little support from the assayed populations of *X. agassizi,* each of which exhibited very limited sequence diversity. In any event, the genetic distinctions between eastern and western forms of *X. agassizi* suggest a significant level of population differentiation that should probably be recognized in management programs for this protected species.

Precise identification of the barrier(s) that conceivably separated eastern and southern mtDNA assemblages is hampered by both the distance between Sonoran locales (Fig. 2) and the numerous physiographic features encompassed by these sites. Two of Sonora's largest drainages, the Rio Yaqui and the Rio Mayo, lie between locales S1 (Mazatan) and S2 (Alamos). The influence of these rivers, their respective geologic histories, and the deeply dissected topography of southeastern Sonora could all effectively account for mtDNA differentiation between assemblages. In addition, the Alamos region is characterized by a distinct phytogeographic component, the Sinaloan Deciduous Forest (Gentry, 1982), in which tortoise habitat usage differs altogether from other parts of the species' range. Collections from additional sites, particularly those between the Rio Yaqui and Rio Mayo, would be necessary to delimit the eastern and southern assemblages. Until such a survey is undertaken, possible barriers that may have shaped these assemblages must remain loosely defined.

Interspecific Evolutionary Relationships

The qualitative pattern of the mtDNA phylogeny estimated for gopher tortoises (Fig. 4) provides strong support for the suggested recognition of *Gopherus* and *Xerobates* as distinct, natural taxa (Bramble, 1971, 1982; Auffenberg, 1976). The genera form highly divergent ($\bar{p} = 0.112$) mtDNA groupings recognized in both the UPGMA phenogram and the Wagner network.

If sequence divergence is again calibrated at 2% per million years, then the mtDNA data suggest that these two genera may have last shared a common female ancestor only as early as the Late Miocene or Early Pliocene, approximately 5–6 million years ago. This estimate is somewhat later than the early Middle Miocene separation proposed by Bramble (1971, 1982). He argued that the appearance of widespread sandy sedi-

ments in the Great Plains region during Early Miocene times was critical to the origin of *Gopherus,* a fossorial specialist of friable soils. Numerous fossil tortoises from the Middle and Late Miocene possessed, to varying degrees, the structurally derived features that characterize the genus (Bramble, 1971, 1982).

The discrepancy between the molecular and paleontological estimates for times of generic separation may be due, in part, to the nature of our mtDNA approach, i.e., analysis of fragment data. In cases where the divergence between nucleotide sequences greatly exceeds $\bar{p} \simeq 0.05$, the fragment method tends to underestimate sequence divergence relative to restriction-site mapping (Ferris et al., 1983; Shields and Wilson, 1987). Thus, our estimate for *Gopherus-Xerobates* lineage separation ($\bar{p} = 0.112$) may be low. Another possibility may be that the rate of mtDNA evolution in turtles, measured in absolute time units, is somewhat decelerated relative to that for other vertebrates previously assayed. This possibility would also be consistent with the rather low estimate of mtDNA divergence time ($\simeq$2–3 MY) for the eastern and western assemblages of *X. agassizi,* compared to geological age (K-Ar) of the Bouse formation (5.5 MY). Gopher tortoises have longer generation times (15–20 years) than do some (but not all) of the vertebrates from which mtDNA clock calibrations have been derived (Shields and Wilson, 1987). Thus, if genetic divergence in mtDNA accumulates as a function of number of generations, rate of divergence in absolute time would appear low. With current status of knowledge, further discussion along these lines becomes highly speculative.

An especially interesting aspect of the interspecific comparisons concerns the qualitative pattern of mtDNA differentiation among the assemblages of *X. agassizi* relative to *X. berlandieri.* Conflicting topologies arise between the phenogram and Wagner network; in the latter, the eastern *X. agassizi* assemblage and *X. berlandieri* form a separate matriarchal "clade" (Fig. 4).

Recent theoretical studies of mtDNA lineage sorting across speciation events have demonstrated that, under certain plausible demographic conditions, populations of one species may sometimes contain mtDNA genotypes more closely related to those of a different species than to other conspecifics (Tajima, 1983; Neigel and Avise, 1986). This is especially likely when a peripheral isolate (B) speciates from a geographically widespread but structured ancestor (A). Then the mtDNA (or other gene) lineages in A that were geographically adjacent to B, and from which B arose, would likely be phylogenetically closer to B than to other members of A. The separation of *X. berlandieri* from *X. agassizi* may represent an empirical example of this kind of phenomenon.

Xerobates berlandieri has traditionally been viewed as a peripheral isolate of *X. agassizi* or pre-*agassizi* stock following an eastward expansion of *Xerobates,* presumably in the Middle or Late Pleistocene (Bramble, 1971). However, the particular dispersal route is subject to question. Fossil evidence indicates that northern and southern dispersal routes are equally plausible; Pleistocene materials from New Mexico and western Texas have been assigned to *X. agassizi* (Van Devender et al., 1976; Moodie and Van Devender, 1979), and material from Pleistocene sediments at Aguascalientes, Mexico, are classified as *X. berlandieri* (= *X. auffenbergi*) (Mooser, 1972). In addition, the presence of a small Pleistocene tortoise from coastal Sonora that resembles *X. berlandieri* in its strongly ridged carapace (D. M. Bramble, pers. comm.) suggests access to a central corridor across the Mexican Plateau.

The Wagner network suggests that the matriarchal ancestry of *X. berlandieri* is more readily derived from the eastern assemblage of *X. agassizi* than from either southern or western assemblages. This relationship is corroborated by estimates of nucleotide sequence divergence, for which the *X. berlandieri*-eastern assemblage comparison ($p = 0.048$) is lower than comparisons with either southern ($p = 0.071$) or western ($\bar{p} = 0.067$) forms. Thus, our data indicate that *X. berlandieri* probably originated from *Xerobates* stock inhabiting the north central region of Sonora; a southern dispersal through Sinaloa seems less plausible. This interpretation must be qualified, however, by the fact that our sample of *X.*

berlandieri represents a single locality from a fairly widespread geographic range; we do not know the extent of mtDNA variation in this species, nor the degree to which it might influence the *X. berlandieri*-eastern assemblage relationship.

It does appear unlikely that the Late Pleistocene *X. agassizi* fossils of New Mexico and Texas represent the inceptive lineage, given the level of mtDNA divergence between *X. berlandieri* and the eastern assemblage ($P = 0.048$). Rather, an earlier *Xerobates* expansion in the Late Pliocene, possibly through New Mexico or Chihuahua, would more effectively account for the observed genetic distance. Such a Late Pliocene separation is temporally more consistent with the appearance of a distinct *X. berlandieri*-like form, *X. auffenbergi* (viewed as *X. berlandieri* by Bramble [1982]), in Early Pleistocene sediments from Aguascalientes.

The mtDNA relationship between *X. agassizi* and *X. berlandieri* is not the first reported instance of an apparent discrepancy between species affiliation and mtDNA phylogeny due to the phylogenetic sorting of lineages across speciation events. Avise et al. (1983) discussed a similar case in the *Peromyscus maniculatus* complex of mice, in which the mtDNA genotypes of *P. polionotus* branch from a node lying well within a larger network of *P. maniculatus* clones. In a strict phylogenetic context, *P. maniculatus* is "paraphyletic" with respect to *P. polionotus* in matriarchal ancestry (Avise et al., 1983). A discordance of this nature should not be viewed with consternation as a complication in phylogeny reconstruction. Rather, it is a biologically real phenomenon and an expected logical consequence for gene lineages evolving within population and organismal trees.

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Literature Cited

Auffenberg, W. 1976. The genus *Gopherus* (Testudinidae): Osteology and relationships of extant species. Bull. Florida State Mus. 18:121–251.

Avise, J. C., J. Arnold, R. M. Ball, E. Bermingham, T. Lamb, J. E. Neigel, C. A. Reeb, and N. C. Saunders. 1987. Intraspecific phylogeography: The mitochondrial DNA bridge between population genetics and systematics. Ann. Rev. Ecol. Syst. 18:489–522.

Avise, J. C., C. Giblin-Davidson, J. Laerm, J. C. Patton, and R. A. Lansman. 1979. Mitochondrial DNA clones and matriarchal phylogeny within and among geographic populations of the pocket gopher, *Geomys pinetis*. Proc. Nat. Acad. Sci. USA 76:6694–6698.

Avise, J. C., J. F. Shapira, S. W. Daniel, C. F. Aquadro, and R. A. Lansman. 1983. Mitochondrial DNA differentiation during the speciation process in *Peromyscus*. Molec. Biol. Evol. 1:38–56.

Avise, J. C., M. H. Smith, R. K. Selander, T. E. Lawlor, and P. R. Ramsey. 1974. Biochemical polymorphism and systematics in the genus *Peromyscus*. V. Insular and mainland species of the subgenus *Haplomylomys*. Syst. Zool. 23:226–238.

Bermingham, E., and J. C. Avise. 1986. Molecular zoogeography of freshwater fishes in the southeastern United States. Genetics 113:939–965.

Bermingham, E., T. Lamb, and J. C. Avise. 1986. Size polymorphism and heteroplasmy in the mitochondrial DNA of lower vertebrates. J. Hered. 77:249–252.

Bour, R., and A. Dubois. 1984. *Xerobates* Agassiz, 1857, synonyme plus ancien de *Scaptochelys* Bramble, 1982. (Reptilia, Chelonii, Testudinidae). Bull. Soc. Linnéenne Lyon 53:30–32.

Bramble, D. M. 1971. Functional morphology, evolution and paleoecology of gopher tortoises. Ph.D. Diss. Univ. California, Berkeley.

———. 1982. *Scaptochelys*: Generic revision and evolution of gopher tortoises. Copeia 1982:852–867.

BROWN, W. M. 1985. The mitochondrial genome of animals, pp. 95–130. *In* R. MacIntyre (ed.), Molecular Evolutionary Genetics. Plenum, N.Y.

BROWN, W. M., M. GEORGE, JR., AND A. C. WILSON. 1979. Rapid evolution of animal mitochondrial DNA. Proc. Nat. Acad. Sci. USA 76:1967–1971.

DENSMORE, L. D., J. W. WRIGHT, AND W. M. BROWN. 1985. Length variation and heteroplasmy are frequent in mitochondrial DNA from parthenogenetic and bisexual lizards (genus *Cnemidophorus*). Genetics 110:689–707.

DIXON, W. J. 1981. Statistical Software. Univ. California Press, Berkeley.

FELSENSTEIN, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783–791.

FERRIS, S. D., R. D. SAGE, E. M. PRAGER, U. RITTE, AND A. C. WILSON. 1983. Mitochondrial DNA evolution in mice. Genetics 105:681–721.

GENTRY, H. S. 1982. Sinaloan deciduous forest. Desert Plants 4:73–77.

JENNINGS, R. D. 1985. Biochemical variation of the desert tortoise, *Gopherus agassizi*. M.S. Thesis. Univ. New Mexico, Albuquerque.

LANSMAN, R. A., R. O. SHADE, J. F. SHAPIRA, AND J. C. AVISE. 1981. The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. III. Techniques and potential applications. J. Molec. Evol. 17:214–226.

LUCCHITTA, I. 1972. Early history of the Colorado River in the Basin and Range Province. Geol. Soc. Amer. Bull. 83:1933–1948.

———. 1979. Late Cenozoic uplift of the southwestern Colorado Plateau and adjacent lower Colorado River region. Tectonophysics 61:63–95.

METZGER, D. G. 1968. The Bouse Formation (Pliocene) of the Parker-Blythe-Cibola area, Arizona and California. U.S. Geological Survey Professional Paper, 600-D. Geol. Surv. Res. 1968:D126–D136.

MOODIE, K. B., AND T. R. VAN DEVENDER. 1979. Extinction and extirpation in the herpetofauna of the southern high plains with emphasis on *Geochelone wilsoni* (Testudinidae). Herpetologica 35:198–206.

MOOSER, O. 1972. A new species of Pleistocene fossil tortoise, genus *Gopherus,* from Aguascalientes, Aguascalientes, Mexico. Southwest. Natur. 17:61–65.

NEI, M. 1987. Molecular Evolutionary Genetics. Columbia Univ. Press, N.Y.

NEI, M., AND W.-H. LI. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Nat. Acad. Sci. USA 76:5269–5273.

NEIGEL, J. E., AND J. C. AVISE. 1986. Phylogenetic relationships of mitochondrial DNA under various demographic models of speciation, pp. 515–534. *In* E. Nevo and S. Karlin (eds.), Evolutionary Processes and Theory. Academic Press, N.Y.

PATTON, J. L., AND S. Y. YANG. 1977. Genetic variation in *Thomomys bottae* pocket gophers: Macrogeographic variation. Evolution 31:697–720.

SAUNDERS, N. C., L. G. KESSLER, AND J. C. AVISE. 1986. Genetic variation and geographic differentiation in mitochondrial DNA of the horseshoe crab, *Limulus polyphemus*. Genetics 112:613–627.

SHAFIQULLAH, M., P. E. DAMON, D. J. LYNCH, S. J. REYNOLDS, W. A. REHRIG, AND R. H. RAYMOND. 1980. K-Ar geochronology and geologic history of southwestern Arizona and adjacent areas. Arizona Geol. Soc. Dig. 12:201–260.

SHIELDS, G. F., AND A. C. WILSON. 1987. Calibration of mitochondrial DNA evolution in geese. J. Molec. Evol. 24:212–217.

SMITH, P. B. 1970. New marine evidence for a Pliocene marine embayment along the lower Colorado River area, California and Arizona. Geol. Soc. Amer. Bull. 81:1411–1420.

SNEATH, P. H. A., AND R. R. SOKAL. 1973. Numerical Taxonomy. Freeman, San Francisco, CA.

TAJIMA, F. 1983. Evolutionary relationship of DNA sequences in finite populations. Genetics 105:437–460.

VAN DEVENDER, T. R., K. B. MOODIE, AND A. H. HARRIS. 1976. The desert tortoise (*Gopherus agassizi*) in the Pleistocene of the northern Chihuahuan Desert. Herpetologica 32:288–304.

WEINSTEIN, M. N., AND K. H. BERRY. 1987. Morphometric Analysis of Desert Tortoise Populations. Report and contribution no. CA950-CT7-003. U.S. Department of the Interior, Bureau of Land Management, Riverside, CA.

WILSON, A. C., R. L. CANN, S. M. CARR, M. GEORGE, JR., U. B. GYLLENSTEN, K. M. HELM-BYCHOWSKI, R. G. HIGUCHI, S. R. PALUMBI, E. M. PRAGER, R. D. SAGE, AND M. STONEKING. 1985. Mitochondrial DNA and two perspectives on evolutionary genetics. Biol. J. Linn. Soc. 26:375–400.

YOUNG, R. A., AND W. J. BRENNAN. 1974. Peach Springs tuff: Its bearing on structural evolution of the Colorado Plateau and development of Cenozoic drainage in Mohave County, Arizona. Geol. Soc. Amer. Bull. 85:83–90.

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