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## A PLASMA PROTEIN MARKER FOR POPULATION GENETIC STUDIES OF THE DESERT TORTOISE (*XEROBATES AGASSIZI*)

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ABSTRACT.—Fifty-seven individual plasma samples from desert tortoises (*Xerobates agassizi*) representing 10 separate populations were analyzed by polyacrylamide gel electrophoresis using alkaline buffers. An albumin-like protein was found to be polymorphic for two electromorphs in northern populations inhabiting the Mohave Desert Province, while Sonoran Desert populations to the south were monomorphic. The genetic divergence demonstrated in this survey is similar to earlier studies and provides evidence for the Colorado River as a potential barrier to gene flow among tortoise populations. These data suggest that tortoise plasma, examined by various electrophoretic methods, may provide a nondestructive means of determining the broad regional origin of desert tortoises.

Desert tortoises (*Xerobates agassizi*) presently inhabit two regions of southwestern Utah, separated east and west by the Beaver Dam Mountains. The population dynamics of the Beaver Dam Slope tortoises (west of the mountains) have been severely impacted by both human and animal activities during the past several decades. Despite both federal and state protective regulations, tortoise numbers in the slope region are probably at an all-time low (Mike Coffeen, UDWR, personal communication). Two stable populations near St. George, east of the Beaver Dam Mountains, face new human development projects that threaten the future of these previously isolated populations. The eastern populations are found in Paradise and City Creek canyons, and relocation of some tortoises from these populations is presently under consideration by the Utah Division of Wildlife Resources. However, several relocation issues remain unresolved, including methods of collection, conditioning, transport, sex ratios, and specimen numbers involved. One problem to be addressed is the question of genetic compatibility of Utah's tortoise populations, especially if any of the tortoises east of the Beaver Dam Mountains are translocated to the western slope.

Few data are available comparing the physiology or morphology of Utah's separate tortoise populations. Rainboth et al. (1989) electrophoretically analyzed whole blood homogenates of 146 desert tortoises from two separate localities in California for allozyme expression at 23 loci. The two populations were quite similar, as each locality contained unique elements only when allozyme combinations were used and there was a considerable degree of overlap in allozyme frequency. Lamb et al. (1989) included five tortoises from Paradise Canyon in an analysis of phylogeographic patterns in mitochondrial DNA (mtDNA) of the gopher tortoise complex. Their data indicated that these individuals fit within an eastern Mohave "clone" representing the northern Arizona and eastern Nevada region. Also, Jennings (1985) included five tortoises from the Beaver Dam Slope region

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TABLE 1. Genotypes and variability estimates for the polymorphic GP-1 locus resolved in 10 localities of *Xerobates agassizi*.

| Locality | N | Number of GP-1 genotypes | A* | H** |
|---|---|---|---|---|
| 1. Pima Co., AZ | 3 | 3AA | 1.0 | 0.0 |
| 2. Tucson, AZ | 6 | 6AA | 1.0 | 0.0 |
| 3. Pinal Co., AZ | 4 | 4AA | 1.0 | 0.0 |
| 4. Maricopa Co., AZ | 4 | 4AA | 1.0 | 0.0 |
| 5. Kingman, AZ | 3 | 2AA:1BB | 1.3 | 0.0 |
| 6. Beaver Dam Slope, AZ | 12 | 9AA:2AB:1BB | 1.5 | 0.083 |
| 7. Paradise Canyon, UT | 12 | 3AA:6AB:3BB | 1.5 | 0.208 |
| 8. Lincoln Co., NV | 2 | 1AA:1AB | 1.5 | 0.250 |
| 9. Riverside Co., CA | 4 | 1AA:3AB | 1.5 | 0.375 |
| 10. San Bernardino Co., CA | 7 | 3AA:4AB | 1.5 | 0.214 |

* = mean number of alleles/locus.
** = mean heterozygosity-direct count.

in biogeographic investigations comparing blood and tissue enzymes using horizontal starch gel electrophoresis. His study included no specimens from east of the Beaver Dam Mountains in Utah but showed that a north-to-south variation was evident and that the Arizona Beaver Dam Slope specimens fit within the northern (Mohavean) group.

Mitochondrial DNA and isozyme studies are costly and may involve traumatic (to the tortoise) and labor-intensive biopsies of internal tissue or the sacrificing of specimens for necropsy. The purpose of this study was to determine whether general proteins in tortoise plasma could be used to detect geographical differences in tortoise populations. Our primary interest was to compare plasma proteins among Utah's aforementioned tortoise populations using alkaline polyacrylamide gel electrophoresis. However, as electrophoretic profiles of plasma or serum from *Xerobates agassizi* have not been previously reported, we examined plasma profiles from tortoises from Arizona, California, and Nevada as well. This report follows the taxonomic grouping of gopher tortoises by Bramble (1982) and the nomenclature revision of Bour and Dubois (1984), applying *Xerobates* as the genus for the desert tortoise.

## MATERIALS AND METHODS

### Study Area and Sampling

A total of 68 desert tortoises were collected from 11 localities in Arizona (AZ), California (CA), Nevada (NV), and Utah (UT), representing most of the range of this species in the United States. Plasma samples were collected in heparinized tubes (3 ml) by venapuncture (jugular vein or antebrachial sinus) from 15 tortoises from the Beaver Dam Slope, AZ, and Paradise Canyon, UT. Additional plasma samples were donated by colleagues involved in desert tortoise projects in other parts of AZ, and in CA and NV. Localities and sample sizes for the populations sampled are listed in Table 1, and their geographic locations are plotted in Fig. 1.

### Electrophoresis

Plasma samples were lyophilized and stored at 4 C. Polyacrylamide gel electrophoresis (PAGE) was run in a Bio-Rad vertical-slab electrophoresis cell, Model 220. The gel was 1.5 mm thick. Sample wells 10 mm long were formed with Canalco stacking gel (2.5% acrylamide). The 7% acrylamide separating gel was 80 mm in length. Samples were electrophoresed toward the anode (+) at 20 mA constant current and were stopped 10 mm from the bottom of the gel. The electrophoresis buffer used was 0.025 M Tris/0.192 M glycine, pH 8.3; bromophenol blue was used as tracking dye. The gel was fixed in 10% acetic acid/40% isopropanol/50% water for 60 min, stained with coomassie blue (0.05%) in 10% acetic acid/10% isopropanol/80% water for 60 min, and destained in 10% acetic acid/10% isopropanol/80% water, using three or four changes of solution over a 24-hr period.

Only qualitative differences were examined in this study, and only the faster migrating proteins were used, since alkaline PAGE is not the preferred method for resolving differences in basic (globulin-like) proteins, due to their short migration distances. The

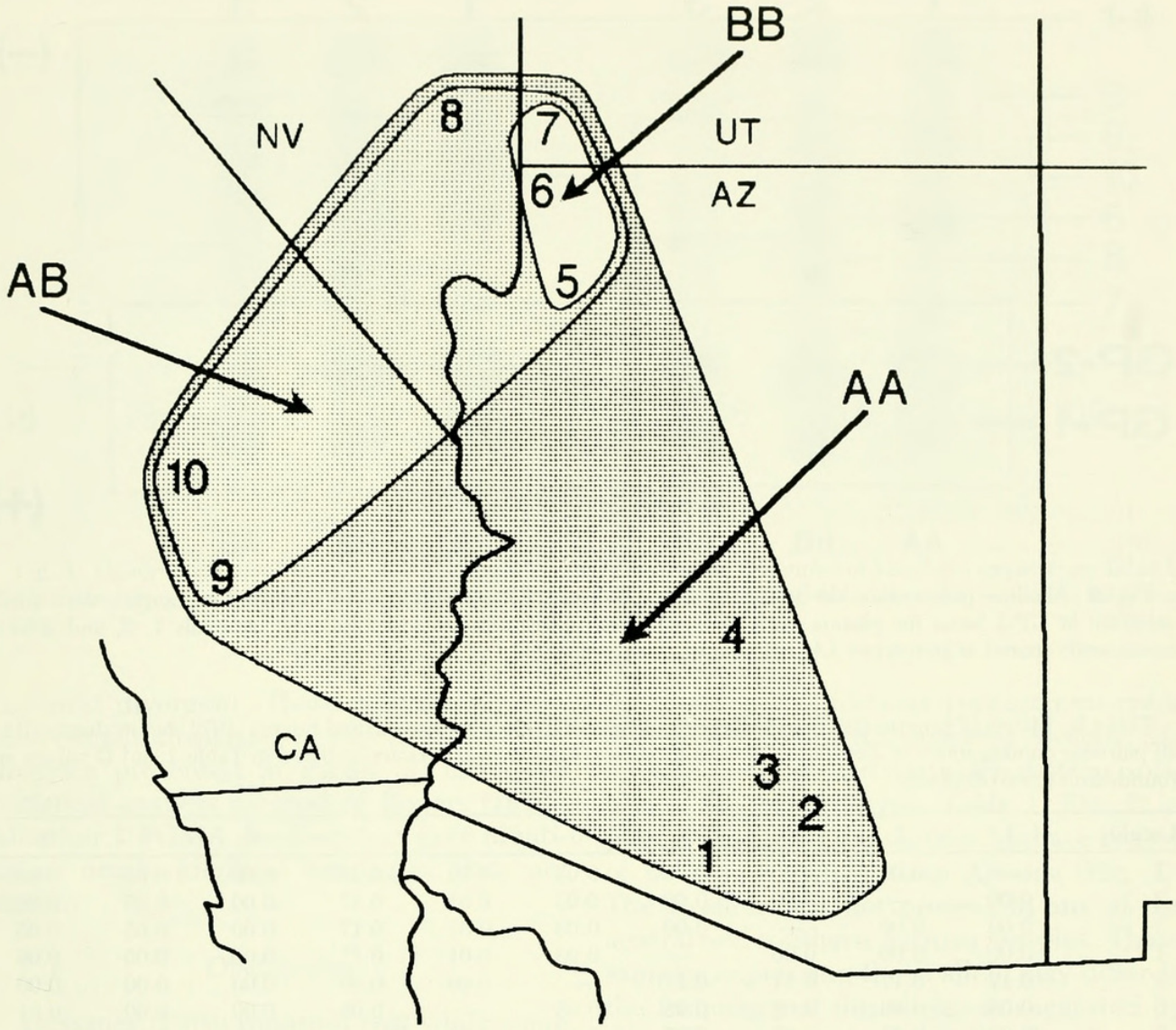


Fig. 1. Distribution of collection localities of tortoise genotypes AA, BB, and AB listed in Table 1. Numbers indicate localities.

electrophoretic mobilities of rattlesnake (*Crotalus atrox*) plasma albumin and bovine serum albumin were used as markers and compared with the albumin-like protein(s) of the desert tortoise samples. Both albumin markers migrated at faster rates than the tortoise albumin-like proteins analyzed in this study.

## Genetic Analysis

Two protein loci, identified in order of decreasing anodal mobility, were designated "general proteins" (GP) 1 and 2. Allelic data at both loci were recorded as individual genotypes for analysis with the BIOSYS-1 program of Swofford and Selander (1981). Measures of genetic variability computed for each population sample included average locus heterozygosity (H, direct count) and the mean number of alleles per locus (A). The genetic distance and similarity coefficients of Nei (1972, 1978) and Rogers (1972) were calculated for all pairwise combinations of samples (corrected for small samples sizes as described by Levene [1949]), and all such matrices were clustered by the UPGMA algorithm of Sneath and Sokal (1973). Genotype ratios from the largest samples (localities 6 and 7, n = 12 for both) were tested for conformance to Hardy-Weinberg proportions by the $X^2$ goodness-of-fit option of BIOSYS, again corrected for small sample sizes (Levene 1949).

## Results

Sixty-eight individual samples were analyzed from four states, including 11 localities

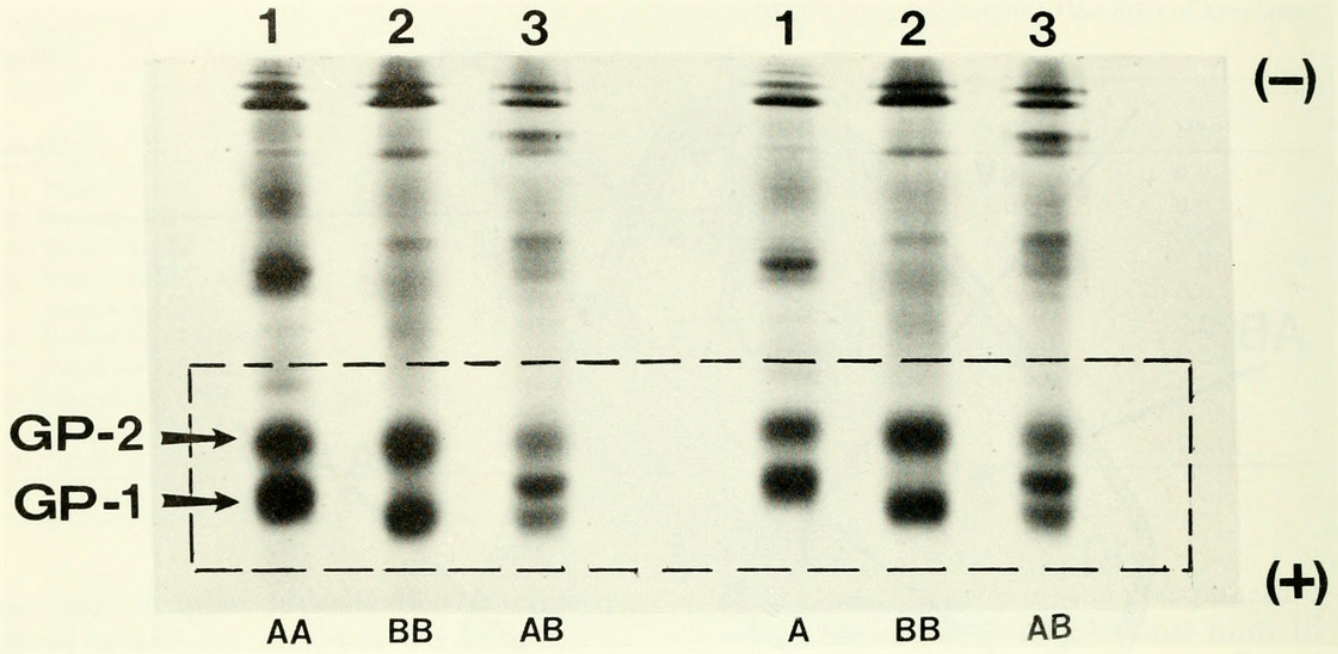


Fig. 2. Alkaline polyacrylamide gel illustrating GP-1 and GP-2 loci (enclosed in dotted rectangle), with allelic variation at GP-1 locus for plasma samples from three individuals (run in duplicate). Animals 1, 2, and 3 were consistently scored as genotypes AA, BB, and AB, respectively.

TABLE 2. Matrix of genetic distance coefficients of Nei (1978, above diagonal) and Rogers (1972, below diagonal) for all pairwise combinations of *Xerobates agassizi* localities; locality numbers are as listed in Table 1 and D values are rounded off to two decimals.

| Locality | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | — | 0.00 | 0.00 | 0.00 | 0.03 | 0.01 | 0.17 | 0.00 | 0.05 | 0.06 |
| 2 | 0.00 | — | 0.00 | 0.00 | 0.03 | 0.01 | 0.17 | 0.00 | 0.05 | 0.06 |
| 3 | 0.00 | 0.00 | — | 0.00 | 0.03 | 0.01 | 0.17 | 0.00 | 0.05 | 0.06 |
| 4 | 0.00 | 0.00 | 0.00 | — | 0.03 | 0.01 | 0.17 | 0.00 | 0.05 | 0.06 |
| 5 | 0.17 | 0.17 | 0.17 | 0.17 | — | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 6 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | — | 0.08 | 0.00 | 0.00 | 0.01 |
| 7 | 0.27 | 0.27 | 0.27 | 0.27 | 0.10 | 0.19 | — | 0.01 | 0.00 | 0.00 |
| 8 | 0.13 | 0.13 | 0.13 | 0.13 | 0.04 | 0.04 | 0.15 | — | 0.00 | 0.00 |
| 9 | 0.19 | 0.19 | 0.19 | 0.19 | 0.02 | 0.10 | 0.08 | 0.06 | — | 0.00 |
| 10 | 0.18 | 0.18 | 0.18 | 0.18 | 0.01 | 0.10 | 0.09 | 0.05 | 0.01 | — |

(considered as separate populations). The protein profiles present in 7 of 9 plasma samples from the Desert Tortoise Natural Area (CA) and 2 samples from Utah were unique but were very likely artifacts due to their badly hemolyzed condition. These 11 samples were excluded from the data analysis. This reduced the number of samples to 57 and the number of localities to 10 (Table 1). General protein-1 was polymorphic for two electromorphs (designated A and B) in several samples (Fig. 2). Table 1 summarizes the ratios of GP-1 genotypes across these 10 localities and the estimates of variability across both loci. Genotype ratios at localities 6 and 7 conformed to Hardy-Weinberg expectations ($X^2 = 2.789$, $P = .095$; $X^2 = .503$, $P = .478$, respectively, $df = 1$ in both cases), suggesting that this is a simple Mendelian co-dominant system with two alleles segregating in some populations. Table 2 summarizes pairwise comparisons of two genetic distance coefficients (Rogers 1972, Nei 1978) and shows that between-sample divergence was minimal. Nei's D values, for example, range from 0.00 to 0.17. Four of the Arizona samples (Maricopa Co., Pinal Co., Pima Co., and Tucson) are identical (Nei's D = 0.00). Five localities having the B allele at GP-1 formed a distinct separate cluster, albeit the total degree of divergence from the monomorphic populations was slight (D = 0.05). Within this group, the Arizona and California populations were nearly identical, while the Paradise Canyon (UT) samples were

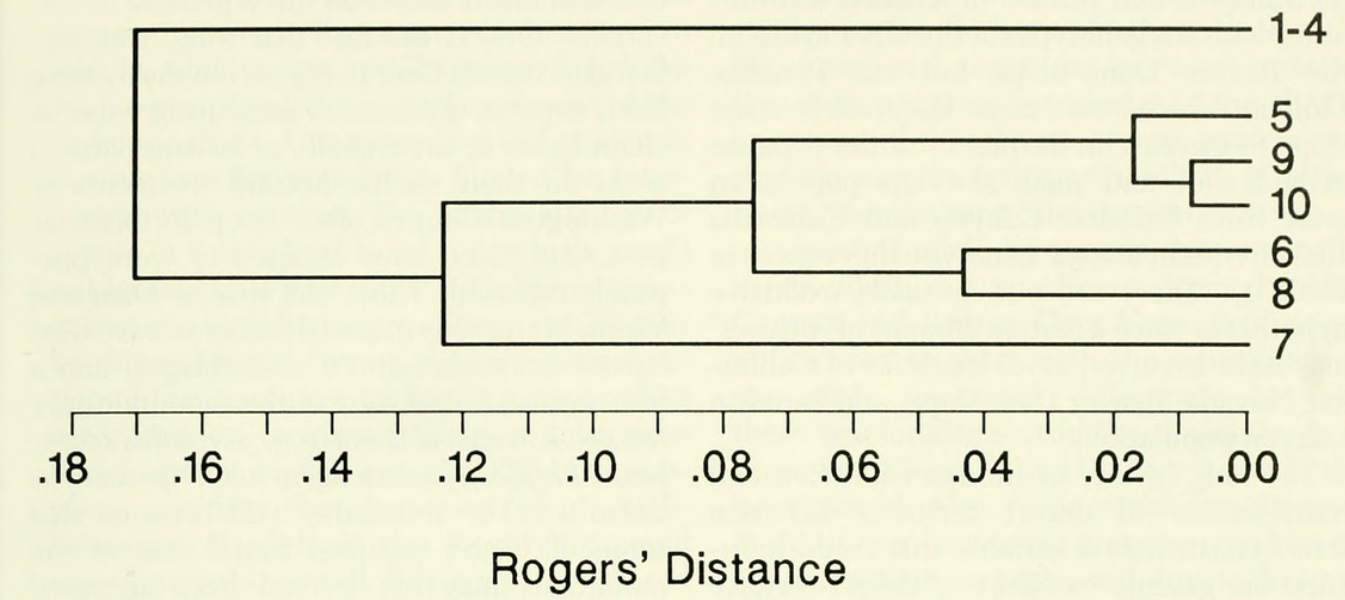


Fig. 3. Dendrogram based on Rogers' (1972) genetic distance values for 10 samples of *Xerobates agassizi* (see Table 1, Fig. 1). Clustering was by the UPGMA algorithm of Sneath and Sokal (1973), and the cophenetic correlation value was 0.826.

the most divergent. These relationships are also visually displayed in the UPGMA dendrogram presented in Figure 3, using the statistical analysis method of Rogers (1972). All other UPGMA dendrograms gave identical or nearly identical topologies (data not shown).

## DISCUSSION

Dessauer (1970) reported that while some blood proteins in reptiles are relatively conservative, others are quite polymorphic. Many species can be readily distinguished by the electrophoretic mobilities of blood proteins, and certain subspecies and populations can also be distinguished by differences in plasma albumin-like proteins (Dessauer and Fox 1958, Masat and Dessauer 1968). Masat and Dessauer (1968) also found that the albumin-like proteins of the Testudines have slower migration rates in alkaline buffers than do these same proteins in most other reptiles (and mammals). We also found that under alkaline conditions the desert tortoise albumin-like proteins migrate at slower rates than rattlesnake plasma albumin and bovine serum albumin.

The results of this investigation suggest geographical differences in genetic variability of the albumin-like protein (GP-1) of desert tortoises. The northern (Mohavean) populations were polymorphic, whereas the southern (Sonoran) populations were monomorphic at the GP-1 locus. An east-west Mohave difference was observed due to the eastern isolation of the BB genotype (Table 1, Fig. 2) in populations from the eastern Mohave region of Utah and northwestern Arizona (Fig. 1). The B allele was not present in any of the central and southern Arizona samples. Those samples expressing the B allele may differ in the frequency of this allele, as suggested by the differentiation between Paradise Canyon and the Beaver Dam Slope, but the present sample sizes are too small for accurate determination. Despite the small sample sizes, the heterozygosity estimates are more similar for the Paradise Canyon population and the three Mohavean populations (8, 9, and 10) than for the Paradise Canyon and Beaver Dam Slope populations (Table 1). If the Paradise Canyon tortoises differ from the Beaver Dam Slope population(s) in frequency of the B allele and are in fact more similar to California tortoises, this could reflect: (a) a divergence of allele frequencies between the slope and Paradise populations in allopatry, with allele frequencies at Paradise simply drifting to values similar to California populations (similarity by convergence); (b) transport of tortoises from California or Nevada to St. George and "dumping" into Paradise Canyon, but not at the slope (i.e., human-induced gene flow between California, Nevada, and Paradise); or

(c) transport and release of Arizona tortoises (most with AA genotypes at the GP-1 locus) on the Beaver Dam Slope but not Paradise Canyon, which would cause the A allele at the slope to increase in frequency at the expense of the B allele and "push" the slope population away from Paradise Canyon and California allele frequencies (see genotype differences in Table 1). These are not mutually exclusive hypotheses since a certain amount of "mixing" may have occurred at all localities of California, Nevada, Beaver Dam Slope, and Paradise Canyon populations.

The long history of human collection and translocation of desert tortoises between states constitutes a variable that could influence the genetic structure of desert tortoise populations, especially when comparing allele frequencies between populations well known as captive release sites. Hundreds, perhaps thousands, of tortoises have been picked up along roadways and released in different regions over the past several decades (Mike Coffeen and Eric Coombs, UDWR, personal communication). This logistical displacement of tortoises continues at present. Certain localities have been popular release sites, e.g., regions of southern California (Desert Tortoise Natural Area), Arizona (McDowell Mountain region near Phoenix), Nevada (near Las Vegas and recreation areas), and Utah (St. George). The most common avenues of translocation by motorists crossing the Mohave Desert are east to west and vice versa. Favorable habitats for tortoises exist from Washington County in southwestern Utah to southern California—a region heavily traveled over a major interstate highway for decades. In addition to the release of tortoises in this region by motorists and local citizenry, Utah's Beaver Dam Slope population(s) have been the site of approximately 200 captive release tortoises, regulated by the Utah Division of Wildlife Resources since 1970 (Mike Coffeen, UDWR, personal communication).

Although Utah's tortoise populations located east of the Beaver Dam Mountains are often regarded as "captive escapees" in a nonindigenous setting, there are no scientific data confirming this view. The hypothesis seems to have originated from several sources, e.g., magazine and newspaper articles, and the opinions of a few naturalists and herpetologists. Support for the introduced status is based on three general observations. One is the fact that some tortoises found in the St. George region obviously have been captive specimens, exhibiting rope or chain holes in their shells or having painted areas on their shells. Second, residents of Washington County often keep tortoises as pets, and some have escaped or were purposely released. Third, the Beaver Dam and Virgin Mountains presently form an east-west barrier between natural assemblages, and a few reptiles found west of the mountains are not present east of them (e.g., *Crotalus scutulatus*, *Phyllorhynchus decurtus*, *Dipsosaurus dorsalis*). The possibility still remains that some of Utah's tortoises found east of the mountains may be derived from ancestral stock of naturally occurring tortoise populations that have since mixed with captive released specimens. Support for the natural population relies on the fact that the Mohave Desert Province extends into this region, and many other Sonoran life-zone animals found on the western slope are also found east of the mountains and are natural assemblages. Like the desert tortoise, some of the reptiles are life-zone specific and occur on both sides, for example, the banded gecko (*Coleonyx variegatus*), Gila monster (*Heloderma suspectum*), and sidewinder (*Crotalus cerastes*).

The geographical differences observed in this investigation are similar to those found in the allozyme survey by Jennings (1985), the mtDNA survey by Lamb et al. (1989), and the morphometric analysis of tortoise remains by Weinstein and Berry (1987). Specifically, the present study supports the earlier molecular investigations of Lamb et al. (1989), which showed divergence between tortoise populations north and west of the Colorado River and those to the south and east. Their report provided good evidence that tortoise populations now isolated on opposite sides of the Colorado River have likely been separated from each other for several million years. The mtDNA lineages from central and southern Arizona formed a single haplotype that differed from the northern haplotypes in CA, NV, UT, and extreme northwestern AZ by a minimum of 17 restriction site changes (see Fig. 2 in Lamb et al. [1989]). This is one of the highest levels of intraspecific genetic divergence reported for any animal species and exceeds that reported for many interspecific comparisons.

The exception in our study was the small sample from near Kingman, AZ (locality 5 in Fig. 1), which genotypically grouped with the Mohavean populations north and west of the Colorado River. Consequently, the genotypic composition for population 5 must be interpreted with caution. The single BB homozygote in a sample of three individuals would not be expected unless the B allele was segregating at a high frequency. These results may be due to several factors, e.g., the translocation of this specimen by humans, a sampling error for a low-frequency allele, or a degradational artifact in this sample. If future sampling verifies the presence of a high-frequency B allele at this locality, it could represent an ancestral polymorphism shared with Mohavean populations north of the Colorado River. This anomalous result underscores the need for statistically adequate sample sizes in all future genetic studies of the desert tortoise. Therefore, identifying the specific origin of any individual tortoise on the basis of nuclear gene markers may be difficult. Weinstein and Berry (1987) suggest using a combination of physiological and morphometric screening methods to designate regional types. They analyzed shell morphology of adult (> 180 mm) desert tortoises of both sexes by using morphometric data gathered by the Bureau of Land Management at 31 different localities. These measurements were collected from tortoise remains by several persons over a 48-year period. These authors noted that live tortoises were not used in their analysis and that shell morphology of tortoise remains does incur some shrinkage over time following death. They recommend further studies comparing live tortoises. None of these authors suggested that the differences observed in their investigations justify subspecific designation for any of the regional populations, and thus *Xerobates agassizi* remains a monotypic species.

Alkaline PAGE was useful in examining the albumin-like proteins in tortoise plasma but does not resolve slight differences in the electrophoretic mobility among the majority of the plasma proteins. However, this method did detect the polymorphic nature of the albumin-like (GP-1) protein, and this protein may be one "marker" that could be used for designation of broad regional types. Since the results of this investigation are, with the possible exception of locality 5 noted above, very similar to the findings of others in that the broad regional genotypes in desert tortoises are approximately concordant, the PAGE screening of the plasma protein marker may provide one inexpensive method of objectively determining the regional origin of tortoises. If allele frequency data are to be used, additional specimens from Paradise Canyon and Beaver Dam Slope populations are needed to determine the significance of the allele frequency differences between these two localities. Additional samples from throughout Nevada and California would also be required. Also, some variations observed in the slower-migrating proteins could be examined with more high-resolution techniques (e.g., isoelectric focusing, two-dimensional electrophoresis) combined with bio-image analytical instrumentation that can quickly and accurately scan and record qualitative differences in electrophoretic profiles. Morphometric data from live tortoises should be collected to compare Paradise Canyon and western slope populations in conjunction with future molecular analyses. In fact, external morphology (such as shell shape) could be a more functional criterion for the survivability of Paradise Canyon tortoises on the Beaver Dam Slope (see Weinstein and Berry [1987]), since genetic differences between these populations appear to be slight.

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