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Phylogeographic Structure and Color Pattern Variation among Populations of *Plethodon albagula* on the Edwards Plateau of Central Texas

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Texas populations of slimy salamanders are isolated from other members of the *Plethodon glutinosus* complex and are currently placed in the species *P. albagula* with populations from the Ozark Plateau and Ouachita Mountains of Missouri, Oklahoma, and Arkansas. We sequenced a portion of the mitochondrial protein-coding gene ND4 for 52 *P. albagula* from 25 localities in Texas and one locality in Missouri. Bayesian MCMC analysis identified five parapatric lineages of *Plethodon* in Texas. Several morphologically distinctive populations are phylogenetically embedded within the Texas radiation. Additional studies of the interactions among the lineages identified in this study will be necessary to resolve their taxonomic status.

THE *Plethodon glutinosus* complex is broadly distributed throughout much of eastern North America. This complex was once treated as a single species, but studies by Highton (1984) and Highton et al. (1989) showed that the complex was composed of numerous genetically (and sometimes morphologically) distinct, mostly parapatric species. Although the specific criteria used by these authors to delimit species has been criticized (e.g., Frost and Hillis, 1990; Tilley et al., 1990; Petranka, 1998), the evidence for multiple species in the complex is clear.

Despite the general consensus of many distinct species of slimy salamanders, the taxonomic status of the western populations of the complex (especially those isolated on the Edwards Plateau of central Texas) has been repeatedly questioned (Grobman, 1944; Highton, 1962; Highton et al., 1989; Frost and Hillis, 1990; Reddell, 2001; Taylor and Phillips, 2003). Grobman (1944) described a white-chinned morph of slimy salamander from the southeastern Balcones Escarpment of the Edwards Plateau as a subspecies (*P. glutinosus albagula*). Highton et al. (1989) elevated *P. albagula* to species status based on a unique combination of allozymes and suggested that the species was distributed in two broad, geographically separated areas: the Edwards Plateau of central Texas and the Ozark Plateau and Ouachita Mountains of Missouri, Arkansas, and northeastern Oklahoma, where it contacts other members of the *P. glutinosus* complex. A few scattered records for this species also have been recorded from intervening areas of eastern Texas, but the existence of populations in these areas needs to be confirmed (Anthony, 2005). Highton et al. (1989) did not consider the morphological character used by Grobman (1944) to be diagnostic for the species. Nonetheless, Highton et al. (1989) did note some genetic differentiation between their samples of light-chinned and dark-chinned *P. albagula* and, in an addendum, noted a high genetic distance between the samples of these two morphs (once an error had been corrected in the data matrix). Furthermore, Carr (1996) conducted multivariate morphological analyses and showed that *P. albagula* from Oklahoma did not cluster with *P. albagula* from Texas.

In central Texas, populations of *P. albagula* (*sensu* Highton et al., 1989) are distributed along the Balcones Escarpment of the Edwards Plateau, with a northern limit of Coryell and Bell counties and a western limit of eastern Edwards County (Fig. 1). Our collections extend the known range of *P. albagula* one county to the west and one county to the north of the range reported by Dixon (2000). Records reported by Dixon (2000) from Mason and San Saba counties are based on sight records by cavers and have not been confirmed with specimens (Fig. 1); these records are outside of the known or expected range of the species, and they are probably not based on observations of *P. albagula*. Within the central Texas distribution, however, there is a geographic break between Bell County and southern Williamson County (Fig. 1). The populations in Coryell and Bell counties (all located on the

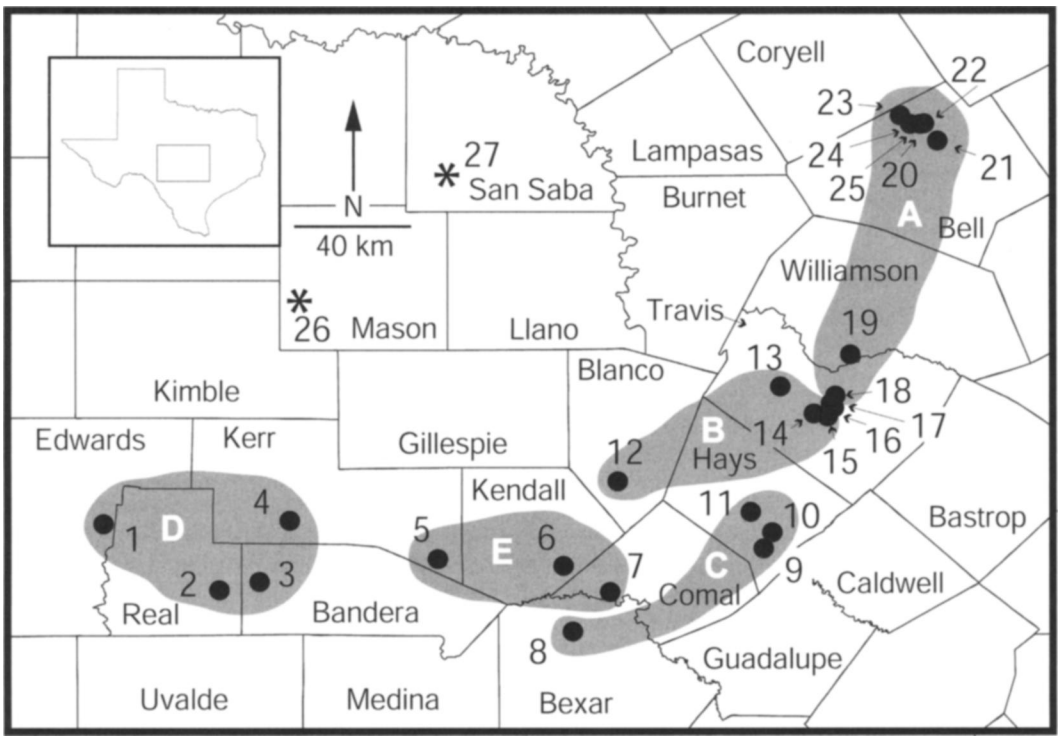

Fig. 1. Map showing collecting localities in Texas. Inset map shows location in central Texas. Shaded areas with white letters show clades as labeled in Figure 3. Numbers 1–25 refer to collecting localities of specimens used in this study: 1, FM 335, 20.6 km S of intersection of FM 335 and State Hwy 41; 2, FM 337, outside Blue Oak Ranch; 3, Can Creek Cave #2; 4, 10 km S of Hunt, on S side of Hwy 39, 15 m up a draw; 5, Cherry Creek Ranch, entrance to Antonio's Cave; 6, Grand Column Cave; 7, Washington Cave; 8, Low Priority Cave; 9, Wonder World Cave; 10, Rattlesnake Cave; 11, Fern Bank Spring; 12, T-Cave; 13, Lake Austin, Apache Shores; 14, S. Shore Colorado River at Red Bud Isles; 15, Brackenridge Field Lab; 16, 2609 Westover, Austin; 17, Reed Park, Tarryhollow Drive and Pecos Street, Austin; 18, 3-Holer Cave, Spicewood Springs Road at Mesa Drive; 19, Chaos Cave; 20, Treasure Cave; 21, Tweedledum Cave; 22, Lunch Counter Cave; 23, Violet Cave; 24, Buchanan Cave; 25, Bear Spring. Numbers 26 and 27 show the location of two questionable sight records that form the basis of county records reported by Dixon (2000; see text): 26, Glory Cave; 27, Chicken Cave.

Fort Hood Military Base) are found in an isolated karst region (Reddell, 2001; Taylor and Phillips, 2003). Individuals from the isolated Fort Hood populations have fewer white spots, and most specimens are almost completely black (Fig. 2). Given the morphological differences and geographic separation from other Texas populations, these populations have been hypothesized to represent a distinct species (Reddell, 2001; Taylor and Phillips, 2003).

Many additional color pattern differences are also evident among Texas populations of *P. albagula*, with considerable variation in the extent of white lateral and dorsal spotting and white pigmentation on the venter (Fig. 2). Grobman (1944) described differences between the dark-chinned morph (which he called *P. g. glutinosus*) and the light-chinned morph (which he named *P. g. albagula*) by noting the difference in the degree and distribution of black pigment in the midline area of the throat. In addition, Grobman (1944) noted that specimens with the light throat also had white pigment " . . . concentrated along the sides to form a broad lateral band." This pattern is seen in the animal shown in Figure 2B. He noted that this pattern differed from topotypic *P. glutinosus*, but that this pattern was found in other parts of the range of what was then considered to be *P. glutinosus*.

Highton (1962) noted that the two color morphs described by Grobman (light-chinned and dark-chinned) were both found on the Edwards Plateau of Texas, and the two morphs were essentially parapatric in this region. He suggested that the two morphs might represent two independent invasions of the Edwards

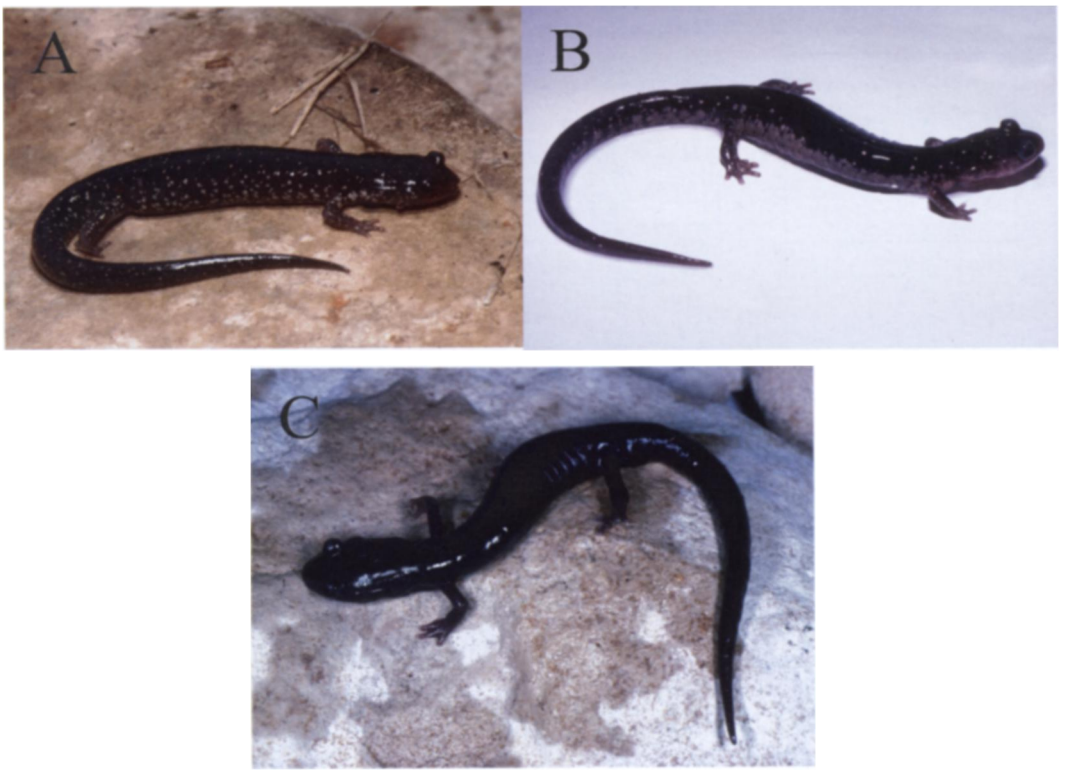

Fig. 2. Photos comparing the range of morphological variation in Texas populations of the *Plethodon glutinosus* complex. (A) Spotted morph from Real County, representative of Group D (most Group E populations also have a similar pattern); (B) morph with white lateral stripe from Hays County, representative of Group C (most Group B populations also have a similar pattern; this is the phenotype that Grobman [1944] described as representative of *P. g. albagula*); (C) unspotted morph from Fort Hood, representative of Group A.

Plateau by *Plethodon* from the Interior Highlands (Ozarks and Ouachitas) of Missouri, Arkansas, and Oklahoma.

The primary goal of this study was to examine the phylogeographic relationships of *P. albagula* in Texas based on mitochondrial DNA (mtDNA) variation. We examined the relationships of the various morphologically differentiated and geographically isolated populations of *P. albagula* in Texas, with particular emphasis on the morphologically distinct Fort Hood populations, and the light- versus dark-chinned morphs. Additionally, if the color pattern differences diagnose distinct phylogenetic groups (based on independent data from mitochondrial DNA), previous hypotheses of systematic diversity in Texas will be supported.

Materials and Methods

Sampling.—We sampled *Plethodon albagula* from each county in Texas where there is a credible record of their occurrence. A total of 50 individuals representing 25 unique localities in Texas and two individuals from a single locality in Missouri were sampled (Table 1). Voucher specimens were deposited in the Texas Natural History Collection at the University of Texas at Austin. *Plethodon glutinosus* from Sevier County, Tennessee (Mahoney, 2001) was included in our analyses for comparison to other members of the *P. glutinosus* complex, based on relationships reported by Highton et al. (1989). We used other species in the *P. glutinosus* species group (but outside of the *P. glutinosus* complex) as outgroups to root our tree. These included *P. jordani, P. fourchensis*, and *P. ouachitae* (Highton, 1995; Mahoney, 2001).

DNA extraction and sequencing.—DNA was isolated from frozen liver tissue using either a Qiagen DNeasy kit or Viogene DNA extraction kit. Two primers, ND4 and LEU (Arevalo et al., 1994) were used to amplify and sequence an approxi-

TABLE 1. LOCALITIES OF TAXA SAMPLED IN THIS STUDY. Numbers in parentheses next to locality name correspond to population numbers listed in Figure 1. TNHC = Texas Natural History Collection (University of Texas) catalog number; DMH = David M. Hillis field number; DCC = David C. Cannatella field number.

County	Locality	Number of individuals	Voucher number	GenBank accn. number
Bandera	Can Creek cave #2 (3)	1	TNHC 61453	DQ284539
Bell	Treasure Cave (20)	2	TNHC 62450, TNHC 61364	DQ284546, DQ284574
	Lunch Counter Cave (22)	1	TNHC 62449	DQ284541
	Bear Spring (25)	1	TNHC 64163	DQ284554
	Buchanan Cave (24)	1	TNHC 62451	DQ284552
	Violet Cave (23)	1	TNHC 62453	DQ284551
	Tweedledum Cave (21)	1	TNHC 63848	DQ284581
Bexar	Low Priority Cave (8)	2*	TNHC 62458, TNHC 61452	DQ284561, DQ284556
Blanco	T-cave (12)	2	TNHC 64210–64211	DQ284577, DQ284572
Comal	Washington Cave (7)	2	TNHC 62459–62460	DQ284547, DQ284549
Edwards	FM 335, 20.6 km S of intersection of FM 335 and State Hwy 41 (1)	2	TNHC 63679–63680	DQ284570, DQ284571
Hays	Fern Bank Spring (11)	1	TNHC 64202	DQ284567
	Wonder World Cave (9)	2	TNHC 64196–64197	DQ284566, DQ284568
	Rattlesnake Cave (10)	2	TNHC 61377, TNHC 61382	DQ284573, DQ284575
Kendall	Grand Column Cave (6)	2	TNHC 62454–62455	DQ284560, DQ284562
Kerr	Hunt, 10 km SW on S side of TX Hwy 39, 15 m up a draw (4)	1	TNHC 63854	DQ284583
	Cherry Creek Ranch (5)	2	TNHC 64178–64179	DQ284569, DQ284590
Real	FM 337, outside Blue oak ranch (2)	1	TNHC 63849	DQ284582
Travis	3-holer cave (18)	4	DMH 92-56–DMH 92-58, TNHC 64213	DQ284576, DQ284584, DQ284585, DQ284586
	Westover road (16)	2	TNHC 64148, TNHC 64149	DQ284542, DQ284544
	Brackenridge field lab (15)	4	TNHC 64151–64154	DQ284557, DQ284555, DQ284539, DQ284548
	Red Bud Isles (14)	4	TNHC 64155–64157, TNHC 64191	DQ284550, DQ284545, DQ284564, DQ284543
	Reed Park (17)	3	TNHC 64026–64028	DQ284587–DQ284589
	Apache Shores (13)	4	TNHC 64180–64181, TNHC 64183, TNHC 64185	DQ284553, DQ284559, DQ284565, DQ284558
Williamson	Chaos Cave (19)	2	TNHC 63855–63856	DQ284579, DQ284580
Ozark (MO)	Bear Cave	2	DCC 3587, DCC 3588	DQ284540, DQ284563
Sevier (TN)–*P. glutinosus*		1		AF329332
Polk (AR)–*P. fourchensis*		1		AF329331
Macon (NC)–*P. jordani*		1		AF370017
LeFlore (OK)–*P. ouachitae*		1		AF329335

* Locality questionable for one individual. This specimen was brought to the authors labeled as having been collected in Bandera County at Can Creek Cave, along with another individual for that Bandera County location and a specimen from Low Priority Cave in Bexar County. We believe one of the two specimens labeled as collected in Bandera County actually was collected in Bexar County, as it shares the same haplotype with the individual labeled from Bexar County and is highly divergent from the Bandera County specimen. All specimens were collected on the same trip and given to the authors at the same time, so a labeling error is likely.

mately 900-bp region of the mitochondrial genome. The amplified region consists of part of the ND4 gene, as well as three tRNA coding regions (histidine, serine, and leucine). This region has previously been shown to be variable on inter- and intraspecific levels in *Plethodon* (Mahoney, 2001, 2004). PCR products were cleaned using a Viogene gel extraction kit and subsequently used in standard sequencing reactions using Big Dye version 3.0 (Applied Biosystems). Sequencing reactions were cleaned using Sephadex spin columns and products were sequenced on an ABI 3100 automated sequencer (Applied Biosystems). All samples were sequenced and read in both primer directions. Sequences were analyzed using DNAstar software version 2 and aligned by eye using MacClade. The partial ND4 region of 664 bp in length, of which a contiguous stretch of 532 bp contained no missing data across all samples, was used in phylogenetic analyses. Individuals with shared haplotypes were represented in the phylogenetic analyses by a single sequence.

Morphological examination.—After preservation in formalin and subsequent transfer to 70% ethanol, all specimens were examined under a dissecting microscope to determine the throat pigmentation patterns (as differentiated by Grobman, 1944). Data were also recorded on the extent and location of white spotting.

Phylogenetic analysis.—The best-fitting model of evolution was assessed for our sequence data using Modeltest version 3.06 under the Akaike Information Criterion (Posada and Crandall, 1998). We used this model (GTR + I + Γ) in a Bayesian phylogenetic analysis conducted with MrBayes version 3.0b4 (Ronquist and Huelsenbeck, 2003). We analyzed 3×10^6 generations (sufficient for convergence), using one cold and three heated Markov chains, and sampled every 100 generations after a burnin of 50,000 generations (determined by evaluating the variance in likelihood scores). Likelihood scores were stable after the burnin period.

RESULTS

Our results are consistent with the monophyly of *P. albagula* (as recognized by Highton et al., 1989; Bayesian posterior probability 0.99), although more samples outside of Texas are required for a more rigorous test of species monophyly. The Texas populations of *P. albagula* are also supported as monophyletic (posterior probability 0.94; Fig. 3). The Texas populations are genetically diverse and the mtDNA divergence among some of the Texas populations of *P. albagula* is greater than the differences between Ozarkian populations of *P. albagula* and our sample of *P. glutinosus* (Fig. 3, Table 2). After the burnin period of 50,000 generations, the average log likelihood score for the Bayesian analysis was −2631.6689 with a variance of 10.267.

The Texas samples grouped into five major parapatric groups (groups A–E, Figs. 1 and 3). The monophyly of three of these groups (A, B, and D) is strongly supported (with posterior probabilities of 1.0). Group C consists of genetically and morphologically very similar populations from the vicinity of the type locality of *P. albagula* in Bexar County, but this group has no significant support for monophyly (Fig. 3). Group E consists of two strongly supported subgroups (posterior probability of monophyly for each subgroup = 1.0), but the two subgroups are united with less support (PP = 0.76).

Groups B and C consist of populations of the light-chinned morph, as described by Grobman (1944), although (as noted by Grobman) occasional individuals from these populations have somewhat darker throat coloration. Most individuals in Groups B and C also have the extensive white lateral coloration that Grobman (1944) considered typical of *P. albagula* (see Fig. 2B). However, we found little support for monophyly of Groups B and C from the mitochondrial DNA analysis; these two groups are in a polytomy with Group A in our majority-rule consensus tree of the Bayesian samples (Fig. 3). The light-chinned coloration, and the extensive white lateral pigmentation, could be considered synapomorphies of Groups B and C; this is not in conflict with the mitochondrial analysis. However, our analysis does not support the monophyly of the various dark-chinned groups (Groups A, D, and E) in Texas. Rather, the light-chinned groups (B and C) from the southeastern Balcones Escarpment appear to be most closely related to the dark-chinned Group A (from the Jollyville Plateau and Fort Hood region along the northeastern portion of the Balcones Escarpment). In addition to the dark chins, some populations in Group A (in particular, some of the northernmost populations at Fort Hood) exhibit the darkest overall coloration seen in Texas *P. albagula*, and most individuals appear to be solid black (Fig. 2C).

Groups D and E are morphologically similar, but genetically are the most divergent Texas populations (Table 2). Individuals from these regions are typically dark-chinned (although the throat coloration in most juvenile specimens is lighter). Group E is composed of samples from

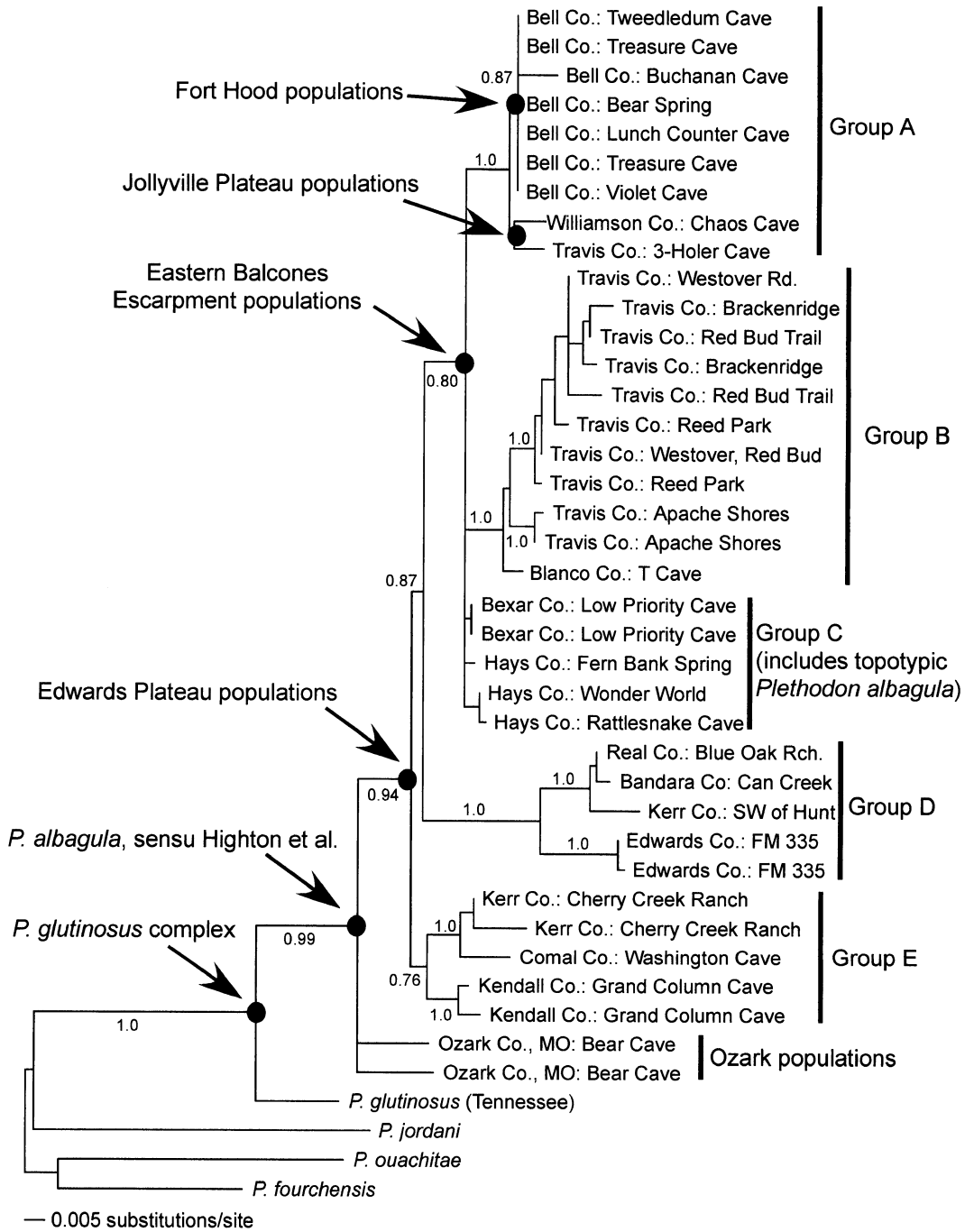

Fig. 3. Bayesian phylogenetic analysis of populations of *P. albagula* and related species, based on an analysis of ND4 sequences. Support values at nodes are Bayesian posterior probabilities.

Comal, Kendall, and eastern Kerr counties, immediately to the south and west of the distribution of Group B. The westernmost group (D) consists of individuals from Edwards, Real, Bandera, and western Kerr counties.

Of the 14 Texas populations containing multiple specimens, only six of these show genetic variation among representatives. The uncorrected percent divergence among members of the same population in all cases is below

TABLE 2. UNCORRECTED PERCENT DIVERGENCE (*p* DISTANCES) FOR MEMBERS OF EACH PHYLOGENETIC GROUP. Outgroups are also shown for comparison. Numbers shown are the range of distances for all individuals in each clade.

	Group A	Group B	Group C	Group D	Group E	Ozark pop.	*P. glutinosus*	*P. jordani*	*P. fourchensis*	*P. ouachitae*
Group A	–									
Group B	0.023–0.041	–								
Group C	0.012–0.027	0.013–0.030	–							
Group D	0.048–0.060	0.047–0.071	0.038–0.056	–						
Group E	0.022–0.045	0.024–0.047	0.018–0.035	0.050–0.064	–					
Ozark pop.	0.034–0.045	0.038–0.051	0.032–0.040	0.052–0.066	0.032–0.041	–				
P. glutinosus	0.052–0.055	0.059–0.069	0.052–0.054	0.063–0.077	0.047–0.054	0.047–0.050	–			
P. jordani	0.113–0.117	0.116–0.126	0.110–0.112	0.125–0.135	0.109–0.120	0.111	0.107	–		
P. fourchensis	0.092–0.096	0.094–0.102	0.090–0.094	0.098–0.112	0.090–0.093	0.091–0.093	0.084	0.097	–	
P. ouachitae	0.107–0.117	0.110–0.125	0.102–0.109	0.129–0.132	0.112–0.118	0.108–0.111	0.100	0.096	0.083	–

1.2%. This is far below the range of divergence between individuals of different phylogenetic groups (Table 2).

DISCUSSION

Morphological variation is often used as the sole criterion for describing novel species in a wide variety of taxa. Within amphibians, analysis of genetic data has shown that some species described on the basis of color pattern differences are merely color morphs within reproductively connected species (e.g., Feder et al., 1978; Titus et al., 1989). In our analysis of *P. albagula*, the color pattern morphs are geographically and somewhat phylogenetically cohesive (Fig. 3). However, the solid-black morph consists of a genetically nearly uniform subset from within Group A, and populations of the light-chinned morph are phylogenetically embedded within three groups of dark-chinned populations (Groups A, D, and E). The monophyly of the Texas populations (Groups A–E) does not support the hypothesis (Highton, 1962) of two separate invasions of *Plethodon* onto the Edwards Plateau from central highlands.

Highton et al. (1989) noted that the dark- and light-chinned individuals appeared to be genetically distinguishable based on allozymes (although they cautioned that this needed further study because of low sample size). Based on geographic proximity, one of their samples of the dark-chinned populations would fall in our Group E and one would fall in Group B. The light-chinned sample from the Highton et al. (1989) study would fall in our Group C. Thus, their comparison of light- and dark-chinned populations involved phylogenetically divergent groups (as based on the mitochondrial DNA analyses). In addition, the Group B sample of Highton et al. (1989) was collected a very short distance away from several of our specimens (population 17, Fig. 1); however, all of our Group B samples have light chins. This apparent incongruence may reflect intra-population variation, or it may indicate there are more complicated interactions among haplotype groups than can be revealed by mtDNA alone. A previous study on *Plethodon* species found that mtDNA was able to reveal more about recent evolutionary history and species barriers than allozymes could (Weisrock et al., 2005). The Weisrock et al. (2005) study found evidence for hybridization between *P. shermani* and members of the *P. glutinosus* complex with mtDNA, where allozyme data showed no support for hybridization or introgression. Our data show a similar pattern of finer-scale resolution with mtDNA than allozyme data.

Our analysis indicates a phylogenetic break between populations in northern and southern Travis County, represented by individuals in groups A and B. This break is closely consistent with the deep phylogenetic split among species of *Eurycea* north and south of the Colorado River (Chippindale et al., 2000). However, in the case of *Plethodon*, populations of Group B (which is mostly found south of the Colorado River) are also found within a few kilometers north of the Colorado River (in isolated woodland habitat within the city of Austin). These populations of Group B from north of the Colorado River occur south of the Asylum Terrace of the Colorado River; this terrace was deposited by the Colorado River during the Pleistocene, prior to down-cutting by the river to its present location (Quinn, 1957; Urbanec, 1963; Weber, 1968). Therefore, although these populations are currently north of the Colorado River, the areas in which they occur were south of the river until the Pleistocene. We have attempted to locate a contact zone between populations representing Groups A and B, but the closest populations we have found are separated by a few kilometers within developed areas of the city of Austin. In addition to habitat loss from development, the gravels and clay of the Asylum Terrace do not provide much appropriate habitat for these salamanders.

In contrast to the genetic break over a very short geographical distance between Groups A and B, the geographic break in the distribution between Bell and Williamson counties is not reflected in patterns of genetic diversity. Samples from either side of this geographic gap are only about 1% divergent in the sequences we examined, and all these samples are part of a well-supported monophyletic Group A.

Group E is found in the central portion of the range of *P. albagula* in Texas, in parts of Kendall, Comal, and Kerr counties (Figs. 1 and 3). Although this group occupies a central distribution, it appears to be phylogenetically the most divergent of the Texas *P. albagula* clades. Populations of Group E may come into contact with populations of Group B to the north, Group C to the east and south, and Group D to the west. However, we have not yet found populations from any of these groups in geographic contact. Populations in Group D are genetically highly divergent and form a very strongly supported and well differentiated mitochondrial lineage (Figs. 1 and 3).

We do not believe that the color pattern variation and mtDNA data presented here are sufficient for a definitive determination of the species boundaries in the Texas populations of the *P. glutinosus* complex at this time. Our analyses do, however, suggest several hypotheses that can be tested with additional data. First, Groups A through E may each represent distinct species, with little or no reproductive contact. Second, Groups B and C (the light-chinned populations) may represent a reproductively cohesive species, distinct from Group A to the north and Groups D and E to the west. Third, all of the populations on the Edwards Plateau of Texas may represent a genetically and morphologically diverse group of reproductively connected populations that are best treated as a single, highly polymorphic species. Fourth, Highton et al.'s (1989) concept of *P. albagula* also appears to apply to a monophyletic group of populations in the *P. glutinosus* complex including individuals from Texas and individuals from the Ozark Plateau and Ouachita Mountains of Missouri, Arkansas, and Oklahoma, although additional genetic sampling outside of Texas is needed to test this hypothesis fully.

Given the high genetic (this paper) and morphological (Carr, 1996) divergence between the Texas and Ozark/Ouachita populations, in addition to the considerable geographic separation of these two areas, it does not seem likely that Highton et al.'s (1989) concept of *P. albagula* (hypothesis 4 above) delimits a single cohesive evolutionary species. Nonetheless, we conclude that additional studies are needed to determine which of the remaining hypotheses is best supported. These new studies might include genetic analyses (using nuclear genes) at the contact zones among Groups A–E, behavioral assays of reproductive interactions among individuals from different groups, or detailed morphological assessments of the various populations. Until such studies have been conducted, we believe that it is premature to make taxonomic revisions to the populations currently assigned to *P. albagula*. However, biologists who study this group should be aware of the high degree of divergence (both molecular and morphological) among the populations currently placed in the species *P. albagula*.

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