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GENETIC DIFFERENTIATION AMONG PLETHODONTID SALAMANDERS (GENUS *BOLITOGLOSSA*) IN CENTRAL AND SOUTH AMERICA: IMPLICATIONS FOR THE SOUTH AMERICAN INVASION

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ABSTRACT: An electrophoretic survey of proteins from 14 populations representing eight species and two species groups of Central and South American *Bolitoglossa* examines patterns of intraspecific and interspecific genetic differentiation and the possible implications of these patterns for the question of the time of entry of plethodontid salamanders into South America. These species are very old, as evidenced by the great genetic differentiation among species (including presumed close cladistic relatives), the high degree of genetic subdivision within species, and the very high levels of genetic variation within individual populations. The frequency of polymorphic loci and mean heterozygosity recorded for some populations, particularly the Costa Rican species *B. subpalmata*, may be the highest levels yet recorded for vertebrates. Cluster analysis based on degree of genetic relatedness reveals only a weak distinction between South American and Central American species; furthermore, many component lineages may predate the establishment of the continuous, permanent Pliocene land connection between Panamá and Colombia. Thus, the recent South American fauna likely comprises descendants of several lineages that independently entered South America. Lastly, a specimen of *B. pandi* collected recently in Colombia indicates that this species, which previously was known from a single specimen collected near the turn of this century and feared extinct, still survives.

Key words: Amphibia; Caudata; *Bolitoglossa*; Allozymes; Biogeography; Electrophoresis; Panamanian Portal

SALAMANDERS present a classic example of a Holarctic distribution; eight of the nine extant families are found almost exclusively in the temperate regions of Europe, Asia, and North America. Only the supergenus *Bolitoglossa*, which comprises seven genera of the highly derived family Plethodontidae, has penetrated tropical latitudes to any significant degree, and only in the New World (Wake and Lynch, 1976). *Bolitoglossa* and *Oedipina*, two genera distributed mainly in Central America, have representatives in South America, and the range of *Bolitoglossa* extends as far as 17°S (Brame and Wake, 1963; Wake and Brame, 1966). However, while the South American salamander fauna is considerably richer than is generally acknowledged and occupies an enormous geographical area

(including much of the Amazon basin), fewer than 30 species are found in the entire continent, a number apparently no greater than that occurring in the small neighboring country of Panamá alone.

It is generally accepted that salamanders entered South America by dispersal from southern Central America, but the number of separate invasions of South America and their timing are debated. There are no fossils from tropical areas, so it has been necessary to infer details of the South American ingression indirectly from two sources: (1) the relative degree of morphological differentiation of related groups living on either side of the former gap between what is now lower Central America and northern South America, and (2) geologic evidence. Dunn (1926), in the first comprehensive discussion of this problem, suggested that the trans-isthmian migration of plethodontid salamanders occurred from the late Miocene to the Pliocene, or as much as 12–15 million years ago. Ideas of fixed continents prevailed in Dunn's time, and his suggestion reflects then-accepted dates of the establishment of a land connetion between the two American continents. Darlington (1957) proposed that salamanders entered South America following a mid to late Pliocene land connection. Recent studies which incorporate views of continental drift estimate a permanent land connection between Panamá and South America beginning 2 to 5 (Woodring, 1966), 5 to 7 (Holden and Dietz, 1972), or approximately 3 (Keigwin, 1978; Marshall et al., 1979) million years ago, thus supporting the notion of a later, Pliocene crossing.

Brame and Wake (1963) proposed multiple invasions of South America by salamanders of several different lineages, beginning in the late Pliocene and extending into the Pleistocene. Their suggestion was based on the differing degrees of morphological similarity between many South American taxa and their presumed Central American relatives. Brame and Wake argued that most of the recent *Bolitoglossa* fauna is derived from several lineages which separately invaded South America relatively early and in advance of a much more recent entry by *Oedipina* and the *phalarosoma* group of *Bolitoglossa*.

Wake and Lynch (1976) subsequently modified this hypothesis, suggesting that as few as two large-scale movements are sufficient to explain the present distribution of salamanders in South America. The initial invasion from southern Central America, quickly following the closure of the Panamanian (Bolívar) Portal in the mid to late Pliocene, was made by a morphologically generalized *Bolitoglossa* possibly resembling *B. savagei* of montane northern Colombia; an adaptive radiation by descendants of this single stem lineage, producing the relatively great morphological and ecological diversity that is displayed by the recent South American fauna, was then evolved in situ. The second, and much more recent, movement across the isthmian land connection by *Oedipina*, moving south, and members of the *B. phalarosoma* and *B. sima* species-groups, moving north as far as western Panamá, would account for the present distribution of the remaining South American salamander element in the isthmian region. A third possibility—and, to our knowledge, one not yet formally proposed—is that one lineage of *Bolitoglossa* and one of *Oedipina* entered South America soon following closure of the Panamanian Portal. The former underwent considerable speciation and morphological diversification, the latter did not.

Biochemical techniques have been used to analyze the effects of the establishment of the isthmian land connection on the distribution and diversification of marine groups, including fishes (Gorman and Kim, 1977; Gorman et al., 1976; Vawter et al., 1980) and sea urchins (Lessios, 1979, 1981). These procedures are particularly valuable in providing an independent assessment of alternate hypotheses of phylogenetic relationship that are de-

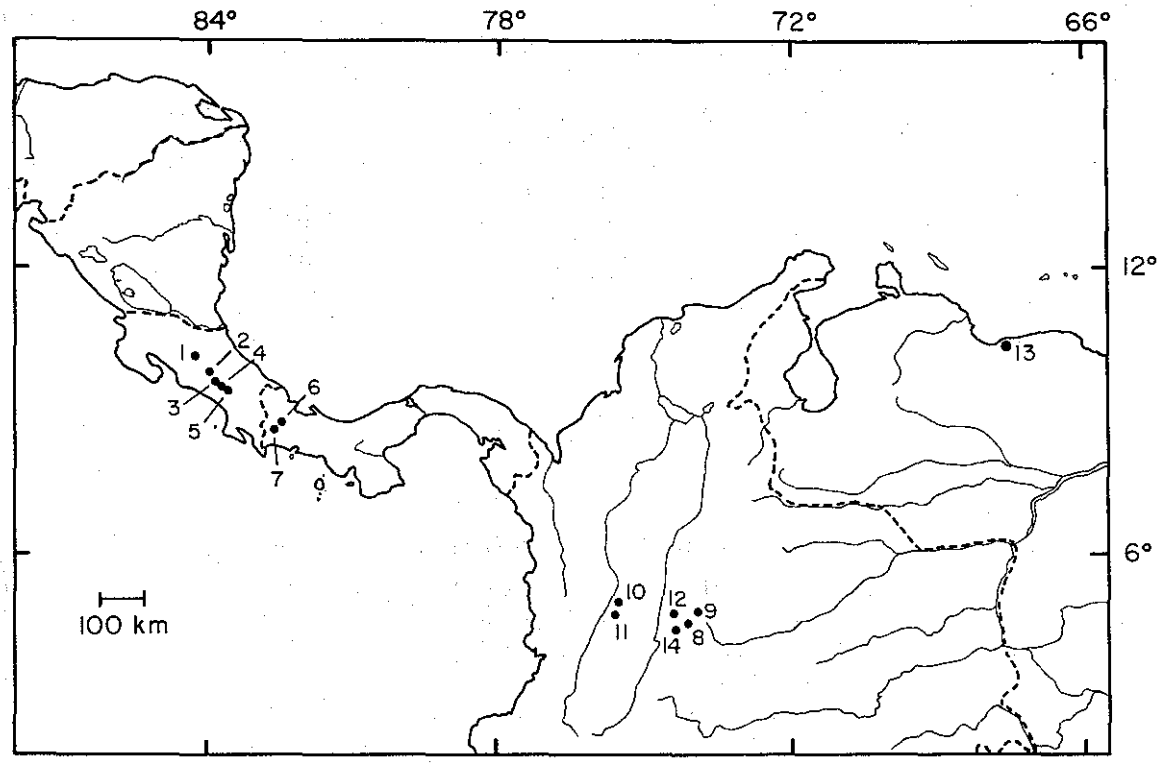

FIG. 1.—Map showing collection localities of *Bolitoglossa* in southern Central America and northwestern South America. 1–5, *B. subpalmata*; 6, *B. marmorea*; 7, *B. nigrescens*; 8–9, *B. adspersa*; 10–11, *B. vallecula*; 12, *B. pandi*; 13, *B. borburata*; 14, "Soacha."

rived from other sources, such as morphology. Thus, we took advantage of the recent collection of representatives of several species of *Bolitoglossa* from northern South America—where most salamander species generally are rare or otherwise difficult to obtain—to make electrophoretic comparisons with congeners from Panamá and Costa Rica. Our South American sample includes representatives of the largest and most diverse of the five species-groups of *Bolitoglossa* found on the continent; our Central American sample includes members of one of the five closely related species-groups of *Bolitoglossa* occuring from western Panamá to México. We hoped to identify implications of the biochemical data for the biogeography of salamanders in the isthmian region, but because we lack samples of other species groups of *Bolitoglossa*, and *Oedipina*, we cannot fully resolve the biogeographic questions. Nevertheless, we can examine the genetic relationships among a number of South and Central American species as a first step in identifying the origins of the entire South American salamander fauna. Specifically, our goals are: (1) to determine the patterns of intraspecific and interspecific differentiation in South American and in Talamancan Central America, (2) to examine the possibility that the South American species show a uniform degree of genetic differentiation relative to those in Central America, as would be expected if all South American *Bolitoglossa* are derived from a single lineage with respect to Central American species, and (3) to estimate divergence times between taxa in order to date the entry of salamanders into South America.

TABLE 1.—Populations of *Bolitoglossa* included in electrophoretic analysis.

Population	Species	Locality	Sample size
1	*B. subpalmata*	Volcán Poás, Costa Rica	7
2	*B. subpalmata*	El Empalme, Costa Rica	11
3	*B. subpalmata*	22 km S of El Empalme, Costa Rica	16
4	*B. subpalmata*	6 km W of Villa Mills, Costa Rica	4
5	*B. subpalmata*	Villa Mills, Costa Rica	19
6	*B. marmorea*	Volcán Chiriquí, Panamá	16
7	*B. nigrescens*	Volcán Chiriquí, Panamá	3
8	*B. adspersa*	Cruz Verde, Colombia	20
9	*B. adspersa*	Guasca, Colombia	20
10	*B. vallecula*	San Felix, Colombia	20
11	*B. vallecula*	Santa Rosa, Colombia	1
12	*B. pandi*	Albán, Colombia	1
13	*B. borburata*	Rancho Grande, Venezuela	1
14	"Soacha"	Soacha, Colombia	6

MATERIALS AND METHODS

Three Central American and five South American species of *Bolitoglossa* were represented in the analysis (Fig. 1; Table 1). The Central American sample represented five populations of *B. subpalmata* from Costa Rica (one from Volcán Poás [the type locality] and four from the Cordillera de Talamanca [these populations variously have been assigned to *B. pesrubra* and *B. torresi*, but are assigned to *B. subpalmata* by Vial, 1968]), one population of *B. marmorea* and one population of *B. nigrescens*. All three Central American species belong to the *subpalmata* species-group. The South American sample represented two populations of *B. adspersa* from the Cordillera Oriental of Colombia, two populations of *B. vallecula* from the Cordillera Central of Colombia, one population of *B. pandi* from the Cordillera Oriental of Colombia, one population of *B. borburata* from near Rancho Grande from the Cordillera de la Costa, Venezuela, and one population of an undescribed species from the Cordillera Oriental of Colombia. The latter species will be referred to by its locality (i.e., "Soacha"). All five South American species are members of the *adspersa* group (Wake and Lynch, 1976). Sample sizes varied from 20 in several populations to only single specimens for others (Table 1). Our single specimen of *B. pandi* is only the second specimen of this rare species ever collected (see below). The sample of *B. nigrescens* extends the known distribution of this species, which previously was known from a single specimen from the Cordillera Central of Costa Rica (Taylor, 1949).

The electrophoretic data comprise a survey of 18 presumptive loci which were assayed using standard horizontal starch gel electrophoresis and staining procedures (Ayala et al., 1972; Harris and Hopkinson, 1976; Selander et al., 1971; Table 2). Salamanders were collected in the field and transported living to the laboratory. Animals were sacrificed and whole tissue samples were stored at −76 C until used. Tissue samples consisted of aqueous extracts of viscera and of the musculature of the ventral body wall. Alleles are designated by letters, with *a* being the fastest migrant. Polymorphism estimates were based on all observed variants, and heterozygosities were recorded from direct counts. Carcasses were retained as voucher specimens and have been deposited in the Museum of Vertebrate Zoology.

Two standard estimates of genetic differentiation between populations, Nei's D (Nei, 1972, 1978) and Rogers' S (Rogers, 1972), were computed from observed

TABLE 2.—Electrophoretic methods.

Locus	Buffer	No. of electromorphs
Aconitase (*Acon*)	TC 7.0	3
Adenosine deaminase (*Ada*)	TM	7
Glucosephosphate isomerase (*Gpi*)	GPI Phosphate	6
Glutamate oxaloacetate transaminase (*Got*)	TC 7.0	7
Glyceraldehyde phosphate dehydrogenase (*Gapdh*)	TC 8.0	5
Glycerol-3-phosphate dehydrogenase (*α-Gpd*)	TC 8.0	4
Isocitrate dehydrogenase-1 (*Idh*-1)	TC 8.0	8
Isocitrate dehydrogenase-2 (*Idh*-2)	TC 8.0	5
Lactate dehydrogenase-1 (*Ldh*-1)	Poulik	8
Lactate dehydrogenase-2 (*Ldh*-2)	Poulik	3
Leucine aminopeptidase (*Lap*)	TC 7.0	3
Malate dehydrogenase-1 (*Mdh*-1)	TC 7.0	6
Malate dehydrogenase-2 (*Mdh*-2)	TC 7.0	5
Mannose phosphate isomerase (*Mpi*)	TC 8.0	5
Phosphoglucomutase (*Pgm*)	TC 7.0	7
Peptidase-1 (*Pep*-1)	GPI Phosphate	7
Peptidase-2 (*Pep*-2)	GPI Phosphate	6
Superoxide dismutase (*Sod*)	Poulik	6

electromorph frequencies. Dendograms were derived from matrices of D using UPGMA (Sneath and Sokal, 1973) and from matrices of $1 - S$ ($= D_R$) using the methods of Fitch and Margoliash (1967) and of Wagner (Farris, 1967, 1970; Kluge and Farris, 1969).

RESULTS

Electrophoretic Variation

Electromorph frequencies at 18 loci were surveyed (Table 3). No locus is fixed for the same electromorph in all populations, nor does any single variant of a given locus even predominate in all populations. The frequency of polymorphic loci per population ranges from 11.1 to a very high 77.8 percent. In two populations the number of electromorphs per locus ranges as high as 5 (*Pep*-1, population 2; *Pep*-2, population 10), while the number of electromorphs per locus averaged across all populations, including those with sample sizes of one, varies from 1.1 (*Gapdh*) to 2.3 (*Pep*-2). Mean heterozygosity per population ranges from 0.058 to 0.278. The above maximal values greatly exceed those previously recorded for natural populations of vertebrates (Nevo, 1978).

Genetic Differentiation

Estimates of genetic differentiation among populations are also great, whether considered within the entire sample, within geographic regions, or within single species (Table 4). Overall, interspecific measures of Nei's D range from 0.25 ("Soacha" to *B. adspersa* from Guasca) to nearly 2.30 (*B. pandi* to population 4 of *B. subpalmata*); comparisons among Central American or South American species commonly exceed D = 1.0.

Intraspecific comparisons within South American species (*B. adspersa, B. vallecula*) indicate only slight differentiation (maximum D = 0.069), but genetic distances among the five populations of *B. subpalmata* in Costa Rica are as great as D = 0.627. The pattern in *B. subpalmata* resembles an "isolation by distance" phenomenon—genetic distance generally increases with geographic distance between any two populations—although the geographic distance needed to achieve considerable genetic differentiation may be slight (e.g., D = 0.286 for populations 2 and 4, which are separated by only a few km). These high estimates of genetic differentiation among populations of *B. subpalmata* are associated

TABLE 3.—Electromorph frequencies.

Locus	Electromorph	Population 1	2	3	4	5	6	7	8	9	10	11	12	13	14	No. electromorphs per locus
Aconitase	a	0.07	1.0	0.94	1.0	0.87	1.0				0.85	1.0		1.0		1.4
	b							0.50	1.0	1.0	0.15				1.0	
	c	0.93		0.06		0.13		0.50					1.0			
Adenosine deaminase	a						0.94									1.5
	b										0.03					
	c			0.16	0.25	0.16	0.06	1.0	1.0	0.95			1.0		1.0	
	d													1.0		
	e									0.05	0.97	1.0				
	f	0.50	1.0	0.84	0.75	0.85										
	g	0.50														
Glucosephosphate isomerase	a		0.05	0.16		0.11			1.0	1.0					1.0	1.6
	b												1.0			
	c	1.0	0.95	0.81	0.87	0.89	0.94				0.97	1.0		0.50		
	d				0.13		0.06									
	e							0.83			0.03			0.50		
	f			0.03				0.17								
Glutamate oxaloacetate transaminase	a						0.94			0.25						1.9
	b		0.09			0.03										
	c									0.23	0.60	0.50				
	d	1.0	0.73	0.44		0.05	0.03	1.0					1.0	1.0	0.92	
	e					0.18			1.0	0.52	0.40	0.50				
	f		0.18	0.56	1.0	0.74										
	g						0.03								0.08	
Glycerol-3-phosphate dehydrogenase	a			0.06		0.13										1.4
	b	1.0	1.0	0.94	1.0	0.81	1.0			0.08						
	c					0.03										
	d					0.03		1.0	1.0	0.92	1.0	1.0	1.0	1.0	1.0	
Isocitrate dehydrogenase-1	a	0.71														1.7
	b			0.16	0.13											
	c														0.67	
	d						1.0	1.0								
	e	0.29	0.95	0.68	0.37	0.63			0.25					1.0		
	f									1.0	0.97	1.0				
	g		0.05	0.16	0.50	0.37			0.75				1.0		0.33	
	h										0.03					

TABLE 3.—Continued.

Locus	Electromorph	Population 1	2	3	4	5	6	7	8	9	10	11	12	13	14	No. electromorphs per locus
Isocitrate dehydrogenase-2	a												1.0			1.3
	b						0.59									
	c	1.0	1.0	1.0*	1.0	1.0	0.41		1.0	1.0	0.10			0.50	1.0	
	d										0.08					
	e							1.0			0.82	1.0		0.50		
Lactate dehydrogenase-1	a	0.79	0.59	0.31										1.0		1.8
	b					0.03										
	c										1.0	1.0				
	d					0.26			1.0	1.0			1.0		1.0	
	e		0.18	0.25	0.13											
	f			0.09			1.0									
	g	0.21	0.23	0.35	0.62	0.50		1.0								
	h				0.25	0.21										
Lactate dehydrogenase-2	a			0.38	0.13	0.05	0.47		1.0	1.0	0.05		1.0		1.0	1.4
	b	1.0	1.0	0.62	0.87	0.95	0.53	1.0			0.90	1.0		1.0		
	c										0.05					
Leucine aminopeptidase	a	1.0		0.66	0.62	0.26										1.4
	b		1.0	0.34	0.38	0.71	1.0	1.0	1.0	1.0	0.82	1.0	1.0	1.0	1.0	
	c					0.03					0.18					
Malate dehydrogenase-1	a					0.13	1.0									1.4
	b		0.25†	0.41	0.75	0.53										
	c								1.0	1.0	1.0	1.0	1.0		1.0	
	d	1.0	0.55†	0.59	0.25	0.34										
	e		0.20†													
	f							1.0						1.0		
Malate dehydrogenase-2	a							0.17	1.0	1.0	0.05					1.2
	b												1.0		1.0	
	c			0.91	1.0	1.0	1.0									
	d							0.83			0.95	1.0		1.0		
	e	1.0	1.0	0.09												
Mannose phosphate isomerase	a					0.08										1.5
	b	0.07	1.0	1.0	1.0	0.92	1.0			0.03						
	c										0.70	1.0				
	d	0.93						1.0	1.0	0.92	0.30		0.50	1.0	0.92	
	e									0.05			0.50		0.08	

TABLE 3.—Continued.

Locus	Electro-morph	Population 1	2	3	4	5	6	7	8	9	10	11	12	13	14	No. electro-morphs per locus
Peptidase-1	a		0.04													1.8
	b	0.36	0.41													
	c	0.64	0.18													
	d		0.23	0.19			0.78									
	e						0.06									
	f		0.14	0.72	1.0	1.0	0.03	1.0	0.05					1.0		
	g			0.09			0.13		0.95	1.0	1.0	1.0	1.0		1.0	
Peptidase-2	a		0.09				0.97									2.3
	b		0.50	0.09	0.25	0.08	0.03				0.27	0.50				
	c	0.93	0.18	0.09				1.0	0.77	0.85	0.28		1.0		1.0	
	d	0.07	0.23	0.66	0.75	0.84			0.23	0.15	0.27			1.0		
	e										0.08	0.50				
	f			0.16		0.08					0.10					
Phosphoglucomutase	a									0.20			0.50		0.33	1.9
	b	0.21	0.05	0.19	0.38	0.58			0.95	0.80						
	c							1.0								
	d	0.79	0.90	0.81	0.62	0.42			0.05		0.97	1.0	0.50	1.0	0.50	
	e										0.03					
	f		0.05				0.97								0.17	
	g						0.03									
Superoxide dismutase	a								1.0	0.65						1.1
	b												1.0		0.33	
	c							1.0							0.67	
	d									0.35	1.0	1.0		1.0		
	e	1.0	1.0	1.0	1.0	1.0										
	f						1.0									
Glyceraldehyde phosphate dehydrogenase	a								1.0	1.0	1.0	1.0	1.0		1.0	1.1
	b													1.0		
	c	1.0	1.0	1.0	1.0	1.0	1.0									
	d							0.83								
	e							0.17								
% loci polymorphic		44.4	44.4	77.8	50.0	72.2	44.4	22.2	27.8	27.8	66.7	11.1	11.1	11.1	27.8	
$\bar{H}$		0.151	0.183	0.267	0.278	0.225	0.094	0.222	0.058	0.092	0.161	0.111	0.111	0.111	0.111	

* $n = 13$.
† $n = 10$.

TABLE 4.—Coefficients of Nei's Distance (above the diagonal) and Rogers' Similarity (below the diagonal) between populations.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	—	0.333	0.435	0.627	0.622	1.366	1.267	1.702	1.694	1.515	1.739	1.476	1.008	1.354
2	0.662	—	0.173	0.286	0.248	0.762	1.561	1.800	1.861	1.145	1.130	1.879	0.742	1.518
3	0.579	0.753	—	0.058	0.069	0.701	1.662	1.626	1.720	1.325	1.390	1.932	0.826	1.536
4	0.508	0.676	0.843	—	0.032	0.751	1.600	1.706	1.899	1.359	1.396	2.297	0.969	1.920
5	0.499	0.696	0.831	0.876	—	0.709	1.463	1.396	1.555	1.287	1.329	2.038	0.852	1.699
6	0.267	0.458	0.483	0.463	0.466	—	1.824	2.086	1.900	1.563	1.524	2.284	1.626	1.967
7	0.293	0.237	0.215	0.226	0.261	0.183	—	1.122	1.125	1.146	1.248	1.031	0.654	0.852
8	0.206	0.199	0.220	0.202	0.254	0.136	0.320	—	0.069	0.956	1.131	0.533	1.429	0.254
9	0.223	0.206	0.220	0.185	0.242	0.162	0.323	0.882	—	0.740	0.897	0.575	1.460	0.245
10	0.249	0.353	0.314	0.293	0.318	0.237	0.321	0.371	0.454	—	0.023	1.014	0.688	0.927
11	0.203	0.348	0.273	0.262	0.283	0.230	0.299	0.325	0.410	0.912	—	1.128	0.754	1.105
12	0.245	0.185	0.175	0.133	0.163	0.122	0.353	0.564	0.547	0.364	0.328	—	1.447	0.284
13	0.344	0.449	0.395	0.358	0.399	0.207	0.505	0.234	0.235	0.474	0.459	0.237	—	1.232
14	0.286	0.255	0.245	0.189	0.233	0.156	0.417	0.746	0.753	0.388	0.341	0.722	0.293	—

with exceptionally high levels of genetic variability overall. Specifically, 15 of the 18 loci examined are polymorphic in at least one of the five populations of this species, and as many as seven electromorphic variants occur at a single locus (*Ldh*-1; mean for all loci = 3.3).

South American populations are not clearly differentiated as a group from those in Central America. Although the average pairwise genetic distance between South American species and those in Central America is greater than the average pairwise distance among the South American species themselves ($\bar{D}$ = 1.45 versus $\bar{D}$ = 0.90), certain individual species are most similar genetically to species on the opposite side of the isthmian link. Specifically, *B. nigrescens* from western Panamá showed a slightly lower average genetic distance to the South American than to the other Central American populations (D = 0.83–1.25, $\bar{x}$ = 1.03; versus D = 1.27–1.82, $\bar{x}$ = 1.3). This species and *B. borburata* from Venezuela are closer to each other than either is to any other species.

Because of the diverse nature of our sample and the unexpectedly great genetic differentiation observed among many populations, analysis of patterns of shared electromorphic variants (Wake et al., 1978) is appropriate for only a few comparisons. For example, the two populations of *B. adspersa* show a fixed variant difference at only one locus (*Idh*-1); these populations share the most common variants at all other loci, although a few unique variants occur at low frequency in each population. The Santa Rosa population of *B. vallecula*, although represented by only a single specimen, closely resembles the much larger conspecific sample from San Felix (D = 0.024); all of its electromorphs are represented in the larger sample.

The five populations of *B. subpalmata* present a complicated pattern of variant sharing because of their extreme genetic variation and relatively great genetic differentiation. They share only one unique fixed variant (*Sod*), but 13 additional electromorphs absent in other species are shared by at least two, and often all, of the five populations. Interestingly, these populations from Costa Rica share a number of electromorphs with the morphologically distinct *B. marmorea* from western Panamá (the minimal D to *B. marmorea* is only slightly greater than the maximal value among populations of *B. subpalmata*—0.70 versus 0.63). Population 1 from Volcán Poás does not differ from the four remaining conspecific populations in any fixed variant, but major frequency differences at several loci

(e.g., *Idh*-1, *Acon*-1, *Pep*-2) contribute to a relatively large average genetic distance to these populations (0.50). Population 2 is closer to population 1 than is any other sample of *B. subpalmata*, and it is geographically also closest genetically. Populations 2, 3, 4 and 5 are geographically close and, not surprisingly, share many variants. However, even such nearby populations as 4 and 5 show major differences in electromorph frequency at some loci (e.g., *Ldh*-1, *Gpd*, *Got*-1). This phenomenon is particularly striking in the case of populations 2 and 3 which are genetically well differentiated (D = 0.17), yet are separated by a map distance of less than 22 km. Given the small geographic distance involved and the existence of continuous suitable intervening habitat, the differentiation between these populations must be judged as great.

Discussion

North American plethodontid salamanders consistently display high levels of genetic differentiation among populations, both within and between species (e.g., Duncan and Highton, 1979; Feder et al., 1978; Highton and Larson, 1979; Larson, 1980; Larson and Highton, 1978; Larson et al., 1981; Tilley and Schwerdtferger, 1981; Tilley et al., 1978; Wake et al., 1978; Yanev, 1978). Frequently, comparisons of congeneric species yield values of genetic distance that exceed those seen between genera of other vertebrate classes. Based on these large levels of differentiation, and the presumed great amount of time required to attain them, many North American plethodontid taxa are judged to be of great antiquity.

Bolitoglossa of Central and South America demonstrate a similar pattern. For example, genetic differentiation within *B. subpalmata* is great (see above and Table 4). The maximal D among populations is 0.627, a value larger than that recorded for many sympatric species of the genus *Plethodon* (Highton and Larson, 1979). It might be argued that *B. subpalmata* is a superspecies (sensu Mayr, 1970) rather than an old, genetically well-differentiated species; indeed, the populations from the Talamancan region have been placed in separate species by some authors, based on slight differences in external coloration (*B. pesrubra* and *B. torresi*, Taylor, 1952). However, Vial (1968) concluded that all populations are conspecific because of the lack of morphological features distinguishing any population. The electrophoretic data support Vial's conclusions. Although genetic differentiation in many pairwise comparisons is great, the overall pattern of relationships is to be expected in a species with limited gene flow among populations. Specifically, the Volcán Poás population (1) shows the least genetic distance to the geographically closest population in the Talamancan range (El Empalme, 2), and there is a general correlation between genetic distance and map distance to the remaining populations. We consider *B. subpalmata* a single, yet highly subdivided, species that has experienced restricted gene flow among populations in the past.

Bolitoglossa adspersa and *B. vallecula*, the two South American species, are members of the same *adspersa* species-group. These species are considered to be close relatives based on great morphological similarity and geographical proximity (Brame and Wake, 1963, 1972; Wake et al., 1970). The two populations of each species demonstrate little intraspecific differentiation (D = 0.07 and 0.02, respectively). However, measures of genetic distance between populations of these two "sister species" range from D = 0.74 to 1.13. Thus, despite relative morphological similarity, these species are well-differentiated genetically, suggesting that they have had long independent histories.

We examined patterns of genic relationship among species by constructing dendrograms, based on genetic distance, using several different algorithms. The EVOLVE program written by Walter Fitch produced Fitch-Margoliash trees

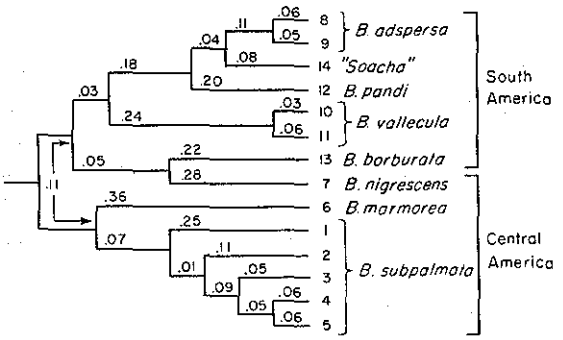

FIG. 2.—Dendrogram constructed from matrix of D_R by method of Fitch and Margoliash. Numbers refer to populations listed in Table 1. % SD = 8.53.

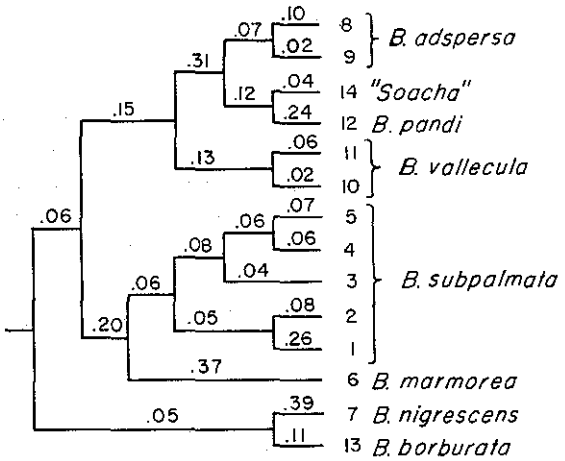

FIG. 3.—Dendrogram constructed by the distance Wagner method applied to the matrix of D_R.

from D_R; the tree with the lowest % SD (8.53) is illustrated in Fig. 2. In this tree, South American species cluster apart from Central American species, with the exception of *B. borburata* and *B. nigrescens* which first cluster together before joining the remaining South American species. Among the Colombian species, *B. adspersa*, *B. pandi*, and "Soacha" form a relatively tight cluster which is well separated from *B. vallecula*; in the Central American cluster, all five populations of *B. subpalmata* join together before this species joins *B. marmorea*. A second tree (not illustrated), with nearly as good a "fit" using the % SD criterion (Prager and Wilson, 1978), has the *B. borburata–B. nigrescens* cluster first joining *B. vallecula* (populations 10 and 11) rather than the entire South American cluster (% SD = 8.93). In constructing the Fitch-Margoliash trees we were unable to identify a clear outgroup and, therefore, were unable to designate a root. Accordingly, the EVOLVE program rooted the dendrogram near the center of each tree, assuming equal rates of increase of genetic distance along all branches. However, it is evident from the branch lengths in Fig. 2 that asymmetries are present. Thus, the particular root established in these trees may be only approximate.

A Wagner tree based on the matrix of D_R is illustrated in Fig. 3. The general similarity to the Fitch-Margoliash tree (Fig. 2) is striking, although the position of *B. borburata* and *B. nigrescens* has changed; these two species form a cluster that is an outgroup to all the remaining species. Marked asymmetries in branch lengths from common nodes are common here also.

A UPGMA tree based on the full matrix of D is illustrated in Fig. 4. This method assumes equal rates of molecular evolution along all branches, an assumption which, as we have already shown, is not valid for the *Bolitoglossa* data set. However, we present the UPGMA tree to facilitate comparison with many studies in the literature which have used the technique. There is close similarity to the other trees (Figs. 2 and 3), with the exception, once again, of the troublesome cluster of *B. borburata* and *B. nigrescens* which here is linked to *B. vallecula*, as in the second-best Fitch-Margoliash tree.

If the hypothesis that all South American species are derived from a single lineage is correct, and if that lineage also gave rise to the *B. subpalmata* group in Central America, we would expect all the South American species to be clearly separated as a group from the *B. subpalmata* group. This pattern is never seen, although it is approached in several trees. Specifically, three species, *B. adspersa*, *B. pandi* and "Soacha", cluster together consistently and appear to represent a

single lineage which has diversified in South America. However, because no consistent pattern of relationship emerges between the *B. nigrescens–B. borburata* cluster and the remaining South and Central American species, we cannot determine from these data the number of additional lineages that have separately entered South America. Sample sizes for *B. nigrescens* and *B. borburata* are small (three and one, respectively) and the inconsistent pattern of relationships of these species vis-à-vis the remaining species may be due partly to sampling error in genetic distance estimates (see discussion of this problem by Gorman and Renzi, 1979). Thus, identification of the true affinities of these species, which are necessary for a final test of the above hypothesis, must await the collection of additional, larger samples.

Observed amounts of genetic differentiation have been used to estimate divergence times between populations. These estimates are based on the correlation between D and the elapsed time since separation derived by various indirect methods, most frequently microcomplement fixation of albumin (see Sarich, 1977; Vawter et al., 1980). For salamanders, attempts to calibrate a molecular "clock" have yielded an estimate of 1 D = 14 million years of separation (Maxson and Maxson, 1979). At values of D = 1 or more, variance in time estimates is great; because of this, together with our demonstration (above) of variation in the rate of molecular evolution in different lineages of *Bolitoglossa*, we can at best estimate only the order of magnitude of times of separation rather than precise timing.

The smallest genetic distance between any pair of South and Central American species is D = 0.65 (*B. borburata* versus *B. nigrescens*, both represented by small samples), yet even this distance far exceeds those recorded for so-called geminate pairs of Atlantic and Pacific Ocean fishes by Vawter et al. (1980) and Gorman et al. (1976) (maximal D = 0.36 and 0.15,

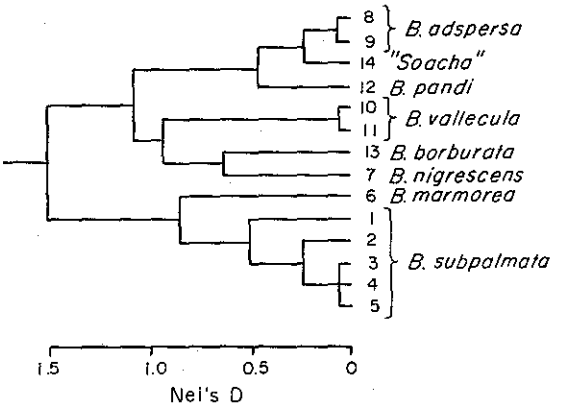

FIG. 4.—Dendrogram constructed by unweighted pair-group method (UPGMA) applied to the matrix of D.

respectively). In these studies, the initial divergence between species pairs was attributed to the closure of the Panamanian Portal. In fact, most pairwise comparisons between South and Central American species of *Bolitoglossa* yield much higher values. The mean genetic distance between trans-isthmian species (excluding that between *B. borburata* and *B. nigrescens*) is approximately D = 1.3; based on the calibration of genetic distance with time that is claimed for other salamander genera (Maxson and Maxson, 1979), this represents a divergence time between the faunas of South and Central America of over 18 million years. Even allowing for the increased variance at this large genetic distance, this suggests that divergence between South and Central American faunas likely preceded the closure of the portal. Further support is provided by the unexpectedly large estimated divergence times among South American species. For example, the genetic distance observed between populations of the morphologically similar species *B. adspersa* and *B. vallecula* (D = 0.74–1.13) corresponds to a divergence time on the order of 10–15 million years.

No recent author has estimated a time of origin of the permanent land connection between Central and South America

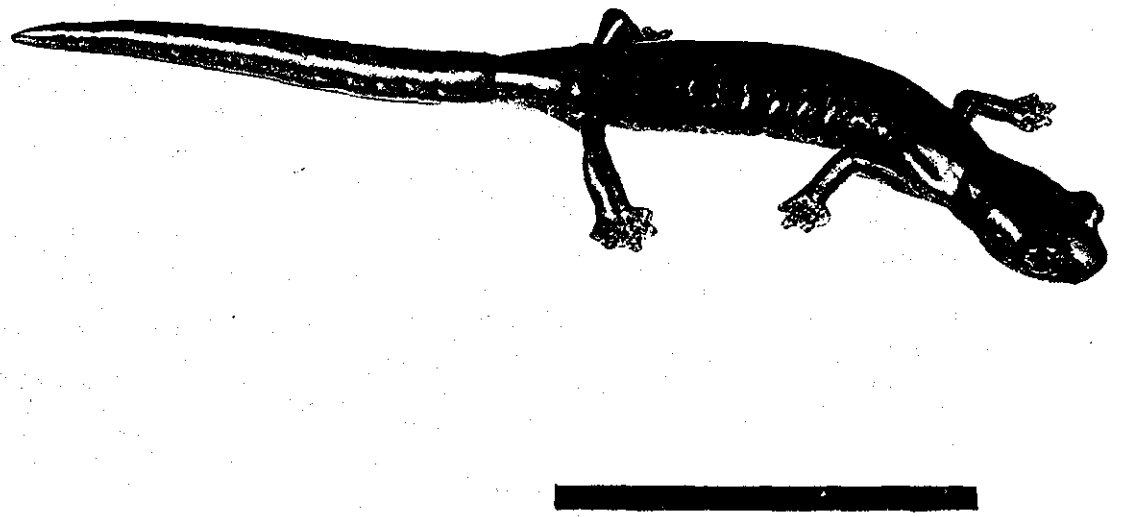

FIG. 5.—*Bolitoglossa pandi*, second specimen of this poorly known species, collected near Albán, Cundinamarca, Colombia. Scale is 25 mm.

greater than about 7 million years ago (see above). Thus, these divergence times (1) are discordant with an hypothesis that cladogenic events were correlated with the establishment of land continuity between the two American continents in the late Pliocene, and (2) imply that most lineages, and even some extant species, in our sample were in existence prior to the establishment of land continuity. Assuming that these divergence times are accurate, we suggest that several lineages (even within what we now call the *B. adspersa* species-group) existed in southern Central America by the early Pliocene; many of these lineages subsequently dispersed southward with the establishment of the land connection in the late Pliocene. We consider the alternate scenario, that diversification occurred in South America from an ancestral stock that somehow managed to bridge the water gap before the establishment of the continuous land connection, less likely in view of the intolerance of most plethodontid salamanders for marine environments, but it is not impossible. An "island hopping" mechanism has been invoked to explain the appearance of several North American mammals, reptiles and anurans in South America as early as the late Cretaceous (see review by Webb, 1978). Under this alternative scenario, salamanders would have been present and differentiated into separate lineages (even within the single *adspersa* species-group) prior to the establishment of a permanent land connection.

Our sample comprises only a few of the species of *Bolitoglossa* in southern Central and South America; 2 of the 10 described species-groups are represented. Furthermore, because of the observed high level of genetic variability, seen most clearly in *B. subpalmata*, and because our analysis was based on only 18

loci, the resulting distance measures should be viewed as only approximations. Gorman and Renzi (1979) have shown that when variation within populations is low, accurate information can be derived from very small population samples, even from a single specimen. With increased variation, either the sample size, the number of loci examined, or preferably both, should be increased to minimize measurement error. Nevertheless, we feel that our results justify the following general conclusions. First, South American species of *Bolitoglossa* are well differentiated genetically at levels comparable to those observed both among congeneric species in southern Central America and among species of other plethodontid genera in North America. Second, the magnitude of genetic distances observed between South American and Central American species pairs, and among South American species, suggests an early origin of the South American salamander fauna. Specifically, divergence times derived from the currently favored calibration of genetic distance with time imply that many South American lineages, and even recent species, existed prior to the establishment of the Panamanian isthmus in the Pliocene.

Status of Bolitoglossa pandi

We take this opportunity to note that *B. pandi* is not extinct as was suggested by Wake and Lynch (1976). This species is one of the most poorly known salamanders; even the description of the species was based on a single, partly dissected specimen collected near the turn of the century (Brame and Wake, 1963). The specimen used in our electrophoretic analysis was collected by Pere Alberch and Miguel Escallón from a bromeliad in a primary cloud forest 6 km N Albán, Cundinamarca, Colombia, elevation 2400 m, on 14 July 1977. It closely resembles the type specimen of *B. pandi* in all essential features (Fig. 5) and is, therefore, assigned to that species.

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Literature Cited

Ayala, F. J., J. R. Powell, M. L. Tracey, C. A. Mourão, and S. Pérez-Salas. 1972. Enzyme variation in the *Drosophila willistoni* group. IV. Genetic variation in natural populations of *Drosophila willistoni*. Genetics 70:113–139.

Brame, A. H., Jr., and D. B. Wake. 1963. The salamanders of South America. Nat. Hist. Mus. Los Angeles Cty. Contrib. Sci. 69.

———. 1972. New species of salamanders (genus *Bolitoglossa*) from Colombia, Ecuador, and Panama. Nat. Hist. Mus. Los Angeles Cty. Contrib. Sci. 219.

Darlington, P. J., Jr. 1957. Zoogeography: The Geographical Distribution of Animals. John Wiley, New York.

Duncan, R., and R. Highton. 1979. Genetic relationships of the eastern large *Plethodon* of the Ouachita Mountains. Copeia 1979:95–110.

Dunn, E. R. 1926. The Salamanders of the Family Plethodontidae. Smith College, Northampton, Massachusetts.

Farris, J. S. 1967. The meaning of relationship and taxonomic procedure. Syst. Zool. 16:44–51.

———. 1970. Methods for computing Wagner trees. Syst. Zool. 19:83–92.

Feder, J. H., G. Z. Wurst, and D. B. Wake. 1978. Genetic variation in western salamanders of the genus *Plethodon*, and the status of *P. gordoni*. Herpetologica 34:64–69.

Fitch, W. M., and E. Margoliash. 1967. Construction of phylogenetic trees. Science 155:279–284.

Gorman, G. C., and Y. J. Kim. 1977. Genotypic evolution in the face of phenotypic conversativeness: *Abudefduf* (Pomocentridae) from the Atlantic and Pacific sides of Panama. Copeia 1977:694–697.

Gorman, G. C., Y. J. Kim, and R. Rubinoff. 1976. Genetic relationships of three species of *Bathygobius* from the Atlantic and Pacific sides of Panama. Copeia 1976:361–364.

Gorman, G. C., and J. Renzi, Jr. 1979. Genetic distance and heterozygosity estimates in electrophoretic studies: effects of sample size. Copeia 1979:242–249.

Harris, H., and D. A. Hopkinson. 1976. Hand-

book of Enzyme Electrophoresis in Human Genetics. North-Holland, Amsterdam.

HIGHTON, R., AND A. LARSON. 1979. The genetic relationships of the salamanders of the genus *Plethodon*. Syst. Zool. 28:579–599.

HOLDEN, J. C., AND R. S. DIETZ. 1972. Galapagos core, NazCoPac triple junction and Carnegie/Cocos ridges. Nature London 235:266–269.

KEIGWIN, L. D., JR. 1978. Pliocene closing of the Isthmus of Panama, based on biostratigraphic evidence from nearby Pacific Ocean and Caribbean sea cores. Geology 6:630–634.

KLUGE, A. G., AND J. S. FARRIS. 1969. Quantitative phyletics and the evolution of anurans. Syst. Zool. 18:1–32.

LARSON, A. 1980. Paedomorphosis in relation to rates of morphological and molecular evolution in the salamander *Aneides flavipunctatus* (Amphibia: Plethodontidae). Evolution 34:1–17.

LARSON, A., AND R. HIGHTON. 1978. Geographic protein variation and divergence in the salamanders of the *Plethodon welleri* group (Amphibia, Plethodontidae). Syst. Zool. 27:431–448.

LARSON, A., D. B. WAKE, L. R. MAXSON, AND R. HIGHTON. 1981. A molecular phylogenetic perspective on the origins of morphological novelties in the salamanders of the tribe *Plethodontini* (Amphibia, Plethodontidae). Evolution 35:405–422.

LESSIOS, H. A. 1979. Use of Panamanian sea urchins to test the molecular clock. Nature London 280:599–601.

———. 1981. Divergence in allopatry: molecular and morphological differentation between sea urchins separated by the Isthmus of Panama. Evolution 35:618–634.

MARSHALL, L. G., R. F. BUTLER, R. E. DRAKE, G. H. CURTIS, AND R. H. TEDFORD. 1979. Calibration of the great American interchange. Science 204:272–279.

MAXSON, L. R., AND R. D. MAXSON. 1979. Comparative albumin and biochemical evolution in plethodontid salamanders. Evolution 33:1057–1062.

MAXSON, L. R., R. HIGHTON, AND D. B. WAKE. 1979. Albumin evolution and its phylogenetic implications in the plethodontid salamander genera *Plethodon* and *Ensatina*. Copeia 1979:502–508.

MAYR, E. 1970. Populations, Species, and Evolution. Harvard University Press, Cambridge, Massachusetts.

NEI, M. 1972. Genetic distance estimates between populations. Am. Nat. 106:2833–2892.

———. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89:583–590.

NEVO, E. 1978. Genetic variation in natural populations: patterns and theory. Theor. Popul. Biol. 13:121–177.

PRAGER, E. M., AND A. C. WILSON. 1978. Construction of phylogenetic trees for proteins and nucleic acids: empirical evaluation of alternative matrix methods. J. Mol. Evol. 11:129–142.

ROGERS, J. S. 1972. Measures of genetic similarity and genetic distance. Stud. Genet. 7, Univ. Texas Publ. 7213:145–153.

SARICH, V. M. 1977. Rates, sample sizes, and the neutrality hypothesis for electrophoresis in evolutionary studies. Nature London 265:24–28.

SELANDER, R. K., M. H. SMITH, S. Y. YANG, W. E. JOHNSON, AND J. B. GENTRY. 1971. Biochemical polymorphism and systematics in the genus *Peromyscus*. I. Variation in the old-field mouse (*Peromyscus polionotus*). Stud. Genet. 6, Univ. Texas Publ. 7103:49–90.

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

TAYLOR, E. H. 1949. New salamanders from Costa Rica. Univ. Kans. Sci. Bull. 33:279–288.

———. 1952. The salamanders and caecilians of Costa Rica. Univ. Kans. Sci. Bull. 34:695–791.

TILLEY, S. G., R. B. MERRITT, B. WU, AND R. HIGHTON. 1978. Genetic differentiation in salamanders of the *Desmognathus ochrophaeus* complex (Plethodontidae). Evolution 32:93–115.

TILLEY, S. G., AND P. M. SCHWERDTFERGER. 1981. Electrophoretic variation in Appalachian populations of the *Desmognathus fuscus* complex (Amphibia: Plethodontidae). Copeia 1981:109–119.

VAWTER, A. T., R. ROSENBLATT, AND G. C. GORMAN. 1980. Genetic divergence among fishes of the eastern Pacific and the Caribbean: support for the molecular clock. Evolution 34:705–711.

VIAL, J. L. 1968. The ecology of the tropical salamander, *Bolitoglossa subpalmata*, in Costa Rica. Rev. Biol. Trop. 15:13–115.

WAKE, D. B., AND A. H. BRAME, JR. 1966. Notes on South American salamanders of the genus *Bolitoglossa*. Copeia 1966:360–363.

WAKE, D. B., A. H. BRAME, JR., AND C. H. MYERS. 1970. *Bolitoglossa taylori*, a new salamander from cloud forest of the Serrania de Pirre, eastern Panama. Am. Mus. Novit. 2430.

WAKE, D. B., AND J. F. LYNCH. 1976. The distribution, ecology, and evolutionary history of plethodontid salamanders in tropical America. Nat. Hist. Mus. Los Angeles Cty. Sci. Bull. 25.

WAKE, D. B., L. R. MAXSON, AND G. Z. WURST. 1978. Genetic differentiation, albumin evolution, and their biogeographic implications in plethodontid salamanders of California and southern Europe. Evolution 32:529–539.

WEBB, S. D. 1978. A history of savanna vertebrates in the New World. Part II: South America and the great interchange. Ann. Rev. Ecol. Syst. 9:393–426.

WOODRING, W. P. 1966. The Panama land bridge as a sea barrier. Proc. Am. Philos. Soc. 110:425–433.

YANEV, K. P. 1978. Evolutionary studies of the

plethodontid salamander genus *Batrachoseps*. Ph.D. Dissertation, University of California, Berkeley (Diss. Abstr. No. 79-14821).

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