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Phylogenetic Relationships among the Salamanders of the *Bolitoglossa macrinii* Species Group (Amphibia: Plethodontidae), with Descriptions of Two New Species from Oaxaca (México)

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ABSTRACT.—The *Bolitoglossa macrinii* species group (restricted geographically to southern Oaxaca and southwestern Guerrero, México) is a monophyletic assemblage of five species, *B. macrinii*, *Bolitoglossa riletti*, *Bolitoglossa hermosa*, *Bolitoglossa oaxacensis* sp. nov., and *Bolitoglossa zapoteca* sp. nov. DNA sequences totaling 1164 base pairs for the mitochondrial genes 16S ribosomal RNA and cytochrome *b* were analyzed to generate a phylogenetic hypothesis for the relationships within the group. Our hypothesis is in agreement with previous morphological and allozyme analyses. Divergence within the group is great (to 18.6% for cytochrome *b*, to 7.0% for 16S rRNA) and two clades are well supported: one including *B. riletti*, *B. hermosa*, and *B. zapoteca* and the other including *B. macrinii* and *B. oaxacensis*. These clades likely diverged beginning in mid-Miocene times. We describe two new species, *B. oaxacensis*, for inland Oaxacan populations previously assigned to *B. macrinii*, and *B. zapoteca* for the easternmost populations of the clade. The new species are diagnosed by less interdigital webbing, distinctive coloration (each with subdued or no white spotting but differing from each other in pattern and hue) and by extensive differentiation in allozymes (*B. oaxacensis*) and mitochondrial DNA sequences (both species). The new taxa are the third and fourth species of *Bolitoglossa* endemic to the State of Oaxaca.

RESUMEN.—El grupo de *Bolitoglossa macrinii* (limitado geográficamente al sur de Oaxaca y el suroeste de Guerrero, México) es un conjunto monofilético constituido por cinco especies, *B. macrinii*, *Bolitoglossa riletti*, *Bolitoglossa hermosa*, *Bolitoglossa oaxacensis* sp. nov., and *Bolitoglossa zapoteca* sp. nov. Se analizaron secuencias de ADN con un total de 1164 pares de bases de los genes mitocondriales 16S ARN ribosomal y citocromo *b* con objeto de generar una hipótesis filogenética sobre las relaciones de los miembros del grupo. Nuestra hipótesis concuerda con análisis previos de morfológicos y aloenzimas. La divergencia dentro del grupo es alta (hasta el 18.6% en el citocromo *b*, y hasta el 7.0% en el 16S rRNA). En nuestros análisis existe apoyo para dos clados: uno que incluye a *B. riletti*, *B. hermosa*, y *B. zapoteca*, y otro que incluye a *B. macrinii* y *B. oaxacensis*. Estos dos clados probablemente comenzaron su divergencia a mediados del Mioceno. Se describen dos especies nuevas, *B. oaxacensis*, que incluye las poblaciones del interior de Oaxaca previamente asignadas a *B. macrinii*, y *B. zapoteca* que integra las poblaciones más orientales del clado. Las especies nuevas se diferencian por su membrana interdigital reducida, su coloración distintiva (ambas sin moteado blanco, o con éste casi borrado, aunque difieren entre sí en el tono y el patrón general de coloración), y por su clara diferenciación aloenzimática (*B. oaxacensis*) y de ADN mitocondrial (ambas especies). Los nuevos taxa son la tercera y cuarta especie de *Bolitoglossa* endémicas del Estado de Oaxaca.

The *Bolitoglossa macrinii* group is one of the most readily diagnosed and distinctive clades within the large genus *Bolitoglossa* (Wake and Lynch, 1976; Papenfuss et al., 1983). All members of this clade have unusual osteological characters: weak premaxillary bones, often with incomplete frontal processes, and small premaxillary teeth, even in adult males. Mental glands are absent in males (Papenfuss et al., 1983). Based on the presence of a complicated tail base morphology, the group was included in the ''beta'' section of *Bolitoglossa* (Wake and Lynch, 1976). Papenfuss et al. (1983) noted, however, that the beta tail structure (Wake and Dresner, 1967) of these salamanders is not that typical of other beta *Bolitoglossa* but involves large individual variation and intraindividual asymmetry, thus calling into question the value of the tail structure character for diagnosing a beta section. The large *Bolitoglossa morio* clade that occurs on the opposite (southeast) side of the Isthmus of Tehuantepec may be the closest relative of the *Bolitoglossa macrinii* clade, but rela-

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tionships within *Bolitoglossa* remain poorly understood. Members of the two clades share similarities in skull structure (Wake and Brame, 1969; Papenfuss et al., 1983), and a similar generalized external morphology, with hand and foot webbing varying from moderate to extensive.

The *B. macrinii* group at present includes three described species, all restricted to the Sierra Madre del Sur in southern México, northwest of the Isthmus of Tehuantepec. *Bolitoglossa macrinii* (Lafrentz 1930) occurs along the Pacific slopes of the Sierra Madre del Sur in Oaxaca, in the vicinity of San Miguel Suchixtepec and San Gabriel Mixtepec. *Bolitoglossa riletti* Holman 1964 also occurs along the Pacific slopes of the Sierra Madre del Sur in Oaxaca but further to the west, near Putla. *Bolitoglossa hermosa* Papenfuss, Wake, and Adler 1983 may be restricted to low elevations along the drainage of the Río Atoyac in Guerrero, although there is an unconfirmed record of its occurrence at high elevation (Adler, 1996). Despite the low frequency of capture of specimens, and the narrow geographic ranges of the species, the group is well studied with respect to morphology and allozymes (Papenfuss et al., 1983).

Specimens examined from south of Sola de Vega, in an inner valley of the Sierra Madre del Sur of Oaxaca, differ from typical *B. macrinii* in coloration, interdigital webbing, and allozymes, suggesting that they might represent a different species (Papenfuss et al., 1983). These authors chose not to describe a new species because of the possible existence of geographically intermediate populations that might establish genetic continuity with typical *B. macrinii* across the mountains to the south. Recent fieldwork along the Pacific slopes of the Sierra Madre del Sur uncovered a population that is closer geographically to typical *B. macrinii* than to the Sola de Vega populations, permitting a test of the previous hypothesis.

Another population from the vicinity of Quiegolani, the easternmost locality for the group, was assigned tentatively to *B. macrinii* by Papenfuss et al. (1983), although freshly collected specimens were not available. Recently, we have obtained specimens representing these eastern populations and have incorporated these specimens in our new analysis.

In this paper, we use partial sequences of the mitochondrial genes 16S rRNA (henceforth 16S) and cytochrome *b* (henceforth cyt *b*) to generate phylogenetic hypotheses and to evaluate previous hypotheses of geographic structure and taxonomy within the *B. macrinii* group.

Materials and Methods

Isolation, Amplification, and Sequencing of DNA.—We examined mitochondrial DNA (mtDNA) derived from 15 specimens, including samples from throughout the geographic range of the *B. macrinii* group. These represent five ingroup taxa from 10 localities (for detailed map see fig. 1 in Papenfuss et al., 1983) and three outgroups. Many of these samples were used in the allozymic studies of Papenfuss et al. (1983). Localities of origin, museum collection numbers and GenBank accession numbers are given in Table 1. Genomic mtDNA was extracted from small amounts of frozen tissue, fresh tail tips, or protein extracts using NaCl following a protocol modified from Miller et al. (1988). Fragments of 647 base pairs, corresponding to codons 7 (part)-223 (part) of the *Xenopus* cyt *b* gene (Roe et al., 1985), and of approximately 517 bp of the 16S gene, corresponding to positions 2510–3059 in the human mitochondrial genome (Anderson et al., 1981), were amplified via the polymerase chain reaction (Saiki et al., 1988), using the primers MVZ 15 and MVZ 18 (Moritz et al., 1992) for cyt *b*, and 16Sar and 16Sbr (Palumbi et al., 1991) for 16S. PCR reactions consisted of 38 cycles with a denaturing temperature of 92°C (1 min), annealing at 48–50°C (1 min) and extension at 72°C (1 min) in a Techne PHC-1 thermocycler. PCR reactions were run in a total volume of 25 μl, using 0.6 units of Taq polymerase (Cetus) in tubes containing 0.5 pmol of each primer, 0.75 mM dNTPs, and 1.5 mM $MgCl_2$ in a pH 8.4 buffer with 50 mM KCL and 10 mM Tris HCl (final concentrations). Both heavy- and light-strand primers were used for PCR amplifications and sequencing.

Double-stranded templates were cleaned using MicroSpin S-300 HR columns (Pharmacia Biotech). Four μl of double-strand product were used as the template for cycle sequencing reactions in 10 μl total volume with the Perkin-Elmer Ready Reaction Kit™ to incorporate dye-labeled dideoxy terminators. Thermal cycling was performed using standard conditions. Cycle sequencing products were purified using ethanol precipitation and separated by electrophoresis on a 6% polyacrylamide gel using an ABI 377 DNA sequencer (Applied Biosystems).

Partial sequences of cyt *b* were read from both strands and aligned to each other by eye in the program Sequence Navigator™ version 1.0.1 (Applied Biosystems). The resulting partial 16S sequences were checked and aligned using CLUSTAL in the program Sequence Navigator™ version 1.0.1 (Applied Biosystems). Computer-generated alignments were refined by eye and by comparing them to published secondary structure models for 16S (Ortí and Meyer, 1997). Sequence divergences were estimated using the Kimura two-parameter (K2p) distance (Kimura, 1980; determined using PAUP* 4.0b5, D. Swof-

TABLE 1. Samples used in this study, locality, voucher specimen number, GenBank numbers, and sequences obtained.

Sample no.	Species	Locality	Museum no.	Cyt *b*	16S
1	*B. hermosa*	México: Guerrero: 4.5 km NE Río Santiago	MVZ 143804	—	AF416685
2	*B. hermosa*	México: Guerrero: 11.3 mi NE Atoyac	MVZ 163690	AF416678	AF416686
3	*B. macrinii*	México: Oaxaca: San Gabriel Mixtepec	MVZ 158523	—	AF416687
4	*B. macrinii*	México: Oaxaca: San Gabriel Mixtepec	MVZ 158524	—	AF416688
5	*B. macrinii*	México: Oaxaca: San Gabriel Mixtepec	13800	AF416680	AF416689
6	*B. macrinii*	México: Oaxaca: 40 km N Pochutla	MVZ 158515	AF416679	—
7	*B. oaxacensis*	México: Oaxaca: 40 km N San Gabriel Mixtepec	IBH 13374	AF416681	AF416690
8	*B. riletti*	México: Oaxaca: 19.5 km NE Putla	MVZ 146774	—	AF416691
9	*B. riletti*	México: Oaxaca: 19.5 km NE Putla	MVZ 146775	—	AF416692
10	*B. riletti*	México: Oaxaca: 19.5 km NE Putla	MVZ 146777	—	AF416693
11	*B. riletti*	México: Oaxaca: 6.2 km NE Putla	MVZ 146767	—	AF416694
12	*B. riletti*	México: Oaxaca: 6.1 km S Putla	MVZ 146778	—	AF416695
13	*B. riletti*	México: Oaxaca: 20.9 km NE Putla	MVZ 194328	AF416682	AF416696
14	*B. yucatana*	México: Quintana Roo	MVZ 197507	AF212980	AF218485
15	*B. morio*	Guatemala: San Marcos	MVZ 143677	AF212986	AF218495
16	*B. subpalmata*	Costa Rica: Puntarenas: Monteverde	MVZ 229172	AF212094	AF416697
17	*B. zapoteca*	México: Oaxaca: Santa María Ecatepec	IBH 13375	AF416683	AF416698
18	*B. zapoteca*	México: Oaxaca: Santa María Ecatepec	IBH 13376	AF416684	AF416699

TABLE 2. Corrected sequence divergence (Kimura two-parameter distance) for cyt *b* (above diagonal) and 16S (below diagonal). Specimen numbers correspond to Table 1.

		1	2	3	4	5	6	7	8	9	10	11
1	*B. hermosa* (1, 2)	***	—	0.1831	0.1697	0.1859	0.0803	—	0.2578	0.2307	0.2378	0.1518
2	*B. macrinii* (3)	0.0627	***	—	—	—	—	—	—	—	—	—
3	*B. macrinii* (4)(6)	0.0570	0.0022	***	0.0318	0.0601	0.1667	—	0.2191	0.2161	0.2090	0.1429
4	*B. macrinii* (5)	0.0591	0.0044	0.0019	***	0.0620	0.1539	—	0.2107	0.2209	0.2050	0.1409
5	*B. oaxacensis* (7)	0.0615	0.0239	0.0217	0.0238	***	0.1798	—	0.1987	0.2263	0.1973	0.1387
6	*B. riletti* (8, 10, 11, 12, 13)	0.0339	0.0678	0.0614	0.0614	0.0551	***	—	0.2427	0.2211	0.2400	0.1441
7	*B. riletti* (9)	0.0359	0.0702	0.0636	0.0636	0.0573	0.0019	***	—	—	—	—
8	*B. yucatana* (14)	0.1086	0.1109	0.1027	0.1003	0.0937	0.1017	0.1040	***	0.2484	0.2394	0.2232
9	*B. morio* (15)	0.0837	0.0831	0.0775	0.0798	0.0644	0.0906	0.0929	0.0685	***	0.2456	0.2245
10	*B. subpalmata* (16)	0.1077	0.0986	0.0943	0.0944	0.0878	0.0944	0.0967	0.0925	0.1064	***	0.2382
11	*B. zapoteca* (17, 18)	0.0482	0.0582	0.0508	0.0529	0.0489	0.0567	0.0589	0.0905	0.0750	0.0987	***

ford, Smithsonian Institution) to correct for multiple hits.

Phylogenetic Analyses.—Phylogenetic inference was based on maximum parsimony analyses for independent and combined datasets (MP; PAUP*, vers. 4, D. L. Swofford, Sinauer Assoc., Sunderland, MA, 2000). MP phylogenies were estimated using the exhaustive algorithm. Each base was treated as an unordered character with four alternative states. Gaps in the 16S data were treated as missing. MP analyses were conducted without the steepest descent option and with accelerated character transformation optimization (ACCTRAN), tree bisection-reconnection branch swapping (TBR), save minimal trees (MULPARS), and zero-length branches collapsed to yield polytomies. We used nonparametric bootstrapping (1000 pseudoreplicates, 50% majority rule, heuristic search) and decay indices to assess the stability of internal branches in cladograms (Felsenstein, 1985; Felsenstein and Kishino, 1993). Transversion (tv) to transition (ti) weights of 4:1 and 10:1 were used for the analysis to increase resolution, especially at the base of the tree (Moritz et al., 1992). Trees were rooted by outgroup comparisons with sequences of three distantly related species of *Bolitoglossa*. We included two representatives of the beta assemblage (Wake and Lynch, 1976), *Bolitoglossa morio*, from the *B. morio* group, a possible closest relative of the *B. macrinii* group, and *Bolitoglossa yucatana*, representing the *Bolitoglossa platydactyla-mexicana* clade (García-París et al., 2000a). A representative of *Bolitoglossa* alpha (García-París et al., 2000b), *B. subpalmata*, was used as the most basal outgroup to root the trees.

Maximum Likelihood Analysis (ML: Felsenstein, 1981) was used with the heuristic algorithm. The tree found during the MP searches was used as the starting tree for ML. Based on empirical frequencies and five rate categories, the probabilities of the possible nucleotide transformations, the proportions of invariable sites, and the gamma ''shape'' parameter of the gamma distribution of rate heterogeneity across nucleotide positions (Yang, 1996) were fixed to the empirical values calculated from the starting tree in a search for a better ML tree (i.e., a tree with a higher log-likelihood value), under the general time-reversible model of nucleotide substitution (Yang, 1994; Gu et al., 1995; Swofford et al., 1996).

Morphological Descriptions.—External morphology was studied for all representatives of the *B. macrinii* group, including all features studied earlier by Papenfuss et al. (1983): coloration, 12 external measurements (snout–vent length, tail length, snout to gular fold length, head width, axilla-groin length, forelimb length, hind-limb length, shoulder width, foot width, head depth, interorbital width, and internarial width) and tooth counts, determined using standard methodologies.

Results

We obtained partial sequences of 16S (517 bp) for 17 samples, and cyt *b* (647 bp) for 10 samples. The combined dataset consisted of 1164 bp for 9 taxa. The ratio of transitions to transversions (ti:tv) for all pairwise comparisons ranged from 1.5 to 9.5. For the 16S dataset the alignment of the ingroup required the introduction of 1 to 2 gaps per sequence. Insertion/deletion (indel) events affected a maximum of four postions. Indels were 1 bp in length.

Corrected sequence divergence (K2p; Kimura, 1980) among taxa for cyt *b* and 16S fragments (Table 2) ranged from 6.0 to 18.6% (cyt *b*) and between 2.2 and 7.0% (16S) among species of the ingroup. Sequence divergence among species of the *B. macrinii* group and the outgroups was very high, 19.7–25.8% (cyt *b*) and 6.4–11.1% (16S). Substantial divergence was found also between the coastal (*B. macrinii*) and the more interior samples (henceforth, interior) from southwestern Oaxaca (6.0–6.2% for cyt *b* and 2.2–2.4% for 16S), and between *B. macrinii* and the easternmost population (henceforth, eastern) from near Quiegolani (14.1–14.3% for cyt *b* and 5.1–5.8% for 16S).

Maximum Parsimony Analysis of the combined datasets produced a single most parsimonious tree (Fig. 1; L = 591 steps; 205 characters were parsimony informative; CI = 0.704; RI = 0.526). Monophyly of the *B. macrinii* group was well supported (bootstrap values, bs, 96%, decay index 8), and there are two subclades. The first of these included the westernmost (*B. hermosa* and *B. riletti*) and easternmost (henceforth, eastern) populations from the *B. macrinii* group (bs 90%, decay 7), with the first two being sister taxa (bs 100%, decay 23). The second subclade included the two samples of *B. macrinii* and the interior population (bs 97%, decay 8).

All differential weighting schemes resulted in one most parsimonious tree that varied in length: L = 974 (for ti:tv downweighted 1:4) and L = 1736 (1:10). The only topological effect of weighting transversions four to 10 times over transitions is that *B. morio* and *B. yucatana* were not sister taxa, but the topology of the ingroup is identical to the equally weighted analysis.

In the ML analysis, we obtained a tree with −Ln = 4095.68 (not shown). The ingroup topology was identical to all parsimony analysis, but the relationships between the outgroup taxa (*B. morio, B. yucatana*) differed. Both MP and ML analyses of independent datasets always produced the same topology for the ingroup.

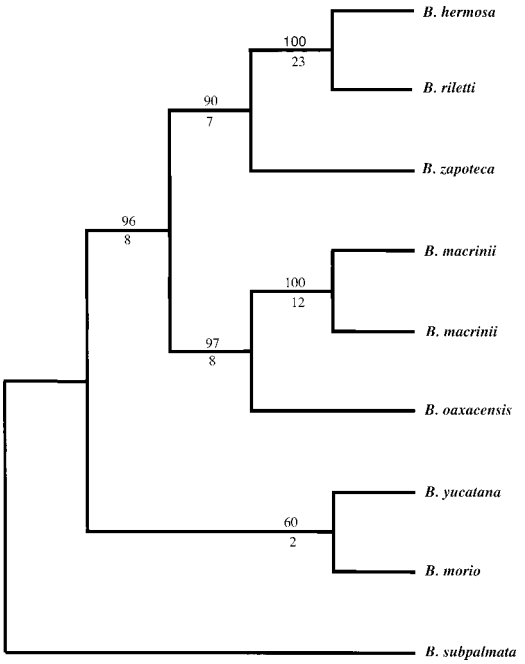

FIG. 1. Most parsimonious tree obtained for the equally weighted combined cyt *b* and 16S dataset (L = 591 steps; CI = 0.704; RI = 0.526). Bootstrap values in excess of 50% are shown above branches. Decay values are shown below.

We compared a topology in which the eastern population was in the *B. riletti-B. hermosa* subgroup (as in Fig. 1) to two alternative arrangements: (1) eastern population basal to the ingroup, and (2) eastern population a member of the *B. macrinii*—interior population subgroup. A Wilcoxson signed-ranks test (Templeton, 1983) indicated that, despite the strong bootstrap values support for the preferred tree (Fig. 1), these trees did not differ significantly ($N = 24$, $P = 0.1025$; $N = 57$ $P = 0.2332$, respectively).

DISCUSSION

Our phylogenetic analyses support the monophyly and genetic distinctiveness of the *B. macrinii* group, as previously argued by Papenfuss et al. (1983) but also including our new samples. These authors suggested that the close relatives of the group were representatives of the beta section of *Bolitoglossa* (Wake and Lynch, 1976). We used a representative of the ''alpha'' section of *Bolitoglossa* to root the trees and two representatives of other clades of the beta section to evaluate the monophyly of the *B. macrinii* group. Divergence of the group as a whole from all comparative taxa is great, and support for *B. morio*, or for a clade formed by *B. yucatana* plus *B. morio*, as a sister taxon of the *B. macrinii* group, is weak. The limited representation of *Bolitoglossa* included in this paper does not permit further discussion of relationships within this complicated lineage (a comprehensive study is in progress, unpubl.).

Phylogenetic relationships among species of the *B. macrinii* group are fully resolved in our analyses (Fig. 1). The two primary lineages within the clade are represented by a subclade that includes samples of *B. hermosa* from Guerrero, *B. riletti* from southwestern Oaxaca, and the eastern population from near Quiegolani, Oaxaca (however, this last population is not so securely associated with this subclade as the bootstrap values and decay index suggest, see results). The second subclade includes the central samples from southern Oaxaca, corresponding to coastal *B. macrinii* and the population of uncertain status from further inland. Sequence divergence between the two subclades is large (K2p for cyt *b* is 13.9–18.6%, for 16S is 3.4–7.0%), in concordance with the large genetic distances recorded ($D_{Nei} = 0.71–1.11$) in an electrophoretic study of 19 allozyme loci (Papenfuss et al., 1983). The lowest divergence between taxa (including those described later in this paper) for cyt *b* is 6.0%, with a maximal level of 18.6%, and for 16S the comparable values are 2.2% and 7.0%, showing that these taxa are all well diverged and probably relatively old. Within the second subclade maximal divergence between taxa for cyt *b* is 6.2% and for 16S is 2.4%. The eastern population is the most divergent, within the *B. macrinii* group, of all populations sampled (as great as 15.1% for cyt *b* and 5.9% for 16S).

We are unable to explain why the easternmost and westernmost populations consistently cluster together. Although the phylogeny (Fig. 1) is fully resolved, Wilcoxon Signed-rank tests are unable to reject the hypothesis that the eastern population is sister to a clade including all of the remaining members of the *B. macrinii* group, or even a member of the second subclade. If it were not for the eastern population, we would postulate that the formation of the basin of the Río Verde, a major river system in western Oaxaca, might have been a vicariant event separating these two lineages. This deep basin, which cuts through the Sierra Madre del Sur, is more than 1000 m in depth, and runs perpendicular to the coastal line from the Pacific Ocean to the vicinity of Asunción Nochixtlán. However, it is difficult to explain the position of the eastern population using this scenario, unless that population is a remnant of an early widespread form that was separated from its western relatives at the same time as the members of the other subclade were isolated from them.

We tentatively date the divergence of the lineages to mid-Miocene times, using the calibra-

tion of Tan and Wake (1995) for cyt *b* (0.8 % divergence per million years), and time estimates derived from allozyme divergence (Thorpe, 1982). A subsequent split within the western lineage, which separated current *B. riletti* and *B. hermosa*, also is old, as inferred from the large sequence divergence (8.0% cyt *b*) and genetic distance (D_{Nei} = 0.36). These two species are currently separated by a large geographic distance, with no evident major geographic features intervening.

There were five geographic samples assigned to *B. macrinii* by Papenfuss et al. (1983). One set, located around San Miguel Suchixtepec, includes the type locality of the species: ''Cerro Espino, 1000 m hoch, subtropischer Laubwald am Südhang der Sierra Madre del Sur, bei Concordia, Staat Oaxaca, Mexiko'' (Lafrentz, 1930). A second population occurs nearer to the Pacific Coast, in the Pluma Hidalgo region. A third group of populations is located in the vicinity of San Gabriel Mixtepec, about 65 km west of San Miguel Suchixtepec. Individuals from this locality are similar to the specimens from the region of the type locality, both in morphology and molecular characteristics. A fourth group, which corresponds to one new species, is located in the mountains south of Sola de Vega, a northern interior location, separated from San Gabriel Mixtepec by about 50 km (this is the northernmost locality mapped as *B. macrinii* by Papenfuss et al., 1983; Fig. 1). The fifth populational unit, the easternmost of the species, is located both well interior from the coast and to the east, around Quiegolani, approximately 50 km northeast of the type locality; it, too, is described as a new species.

Papenfuss et al. (1983) found *B. macrinii* to be deeply differentiated at the population level with respect to allozymes (D_{Nei} ranging from 0.09 to 0.8), and these authors suggested that more than a single species was present. In particular, the population from near Sola de Vega stood out from the rest (D_{Nei} ranges between 0.54 and 0.80 to other populations assigned to *B. macrinii*). However, the first and third populations also differed greatly (D_{Nei} 0.24; Papenfuss et al., 1983), although D_{Nei} of either to the second group is only 0.09. Differences also were found among the populations in external morphology.

We recently found an individual assignable on morphological grounds to the Sola de Vega unit from near the village of Puerto Portillo, Oaxaca. This specimen was located south of the Atoyac River on the northern side of the ridge that separates the valley of San Gabriel Mixtepec and that of the Río Atoyac. The Puerto Portillo population is located about 40 km north of the San Gabriel Mixtepec population of *B. macrinii*, but despite their geographic proximity, the cyt *b* sequences of the two differ by 6.2% of their base pairs (K2p). For a comparison, divergence between two populations of *B. macrinii* from San Gabriel Mixtepec (3-5) and San Miguel Suchixtepec (6), which are separated by about 65 km, is only around 3.2%. The split of *B. macrinii* from the interior populations is old, suggesting that the latter should be placed in a different taxon. Mitochondrial DNA genetic divergence between these two taxa is large (6.0–6.2% for cyt *b* and 2.2–2.4% for 16S), on the order of levels of divergence generally found among related species of Bolitoglossini (García-París and Wake, 2000; García-París et al., 2000a). On the basis of differences in external morphology, mainly extent of interdigital webbing and coloration, and on divergence in both mtDNA and allozymes, we conclude that specimens from near Sola de Vega and Puerto Portillo merit recognition as a new species. The inclusion of both the Sola de Vega and Puerto Portillo populations in a single species is based on their concordant morphological traits, because we have allozyme data only for Sola de Vega and mtDNA data only for Puerto Portillo (we have been unsuccessful in obtaining new material in the Sola de Vega region, Parra-Olea et al., 1999). The two localities are separated by the deep Río Atoyac basin; it is possible that they represent two independent evolutionary units, but available evidence suggests conspecificity.

The easternmost population was sampled only recently following several unsuccessful attempts. Because our sample is small (three old specimens plus two recently collected individuals), morphological analysis is largely precluded. However, there are apparent coloration differences from all other populations of the *macrinii* group. Because this population is the most differentiated in its mitochondrial DNA of any of the populations sampled within the entire *B. macrinii* group, we believe that it merits recognition as a separate species.

Description of Two New Species of the *Bolitoglossa macrinii* Group from Oaxaca, México

Bolitoglossa oaxacensis sp. nov.

Atoyac Salamander—Salamandra del Atoyac
Figures 2–3

Holotype.—MVZ 158533, an adult female collected 9.2 km south of Sola de Vega, Oaxaca, México, approximatrly 1800 m elevation, 16°28′23″N, 96°59′50″W, on October 16, 1981, by S. K. Sessions, D. Darda, J. F. Lynch, and D. B. Wake.

Paratypes.—IBH 13374, an adult male from near Puerto Portillo, 40 km (by road) north San Gabriel Mixtepec, Oaxaca, México, at ap-

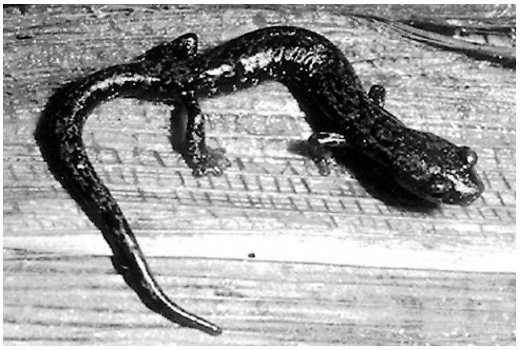

FIG. 2. An adult male of *Bolitoglossa oaxacensis* from near Puerto Portillo, Oaxaca, México (IBH 13374, paratype).

proximately 1920 m elevation, 16°14′13″N, 97°08′88″W, 11-VIII-1999, M. García-París, G. Parra-Olea, and T. J. Papenfuss leg.; UTA CV 5321-22, UTA CV A3657, one adult female and two adult males from 8.5 km southwest Sola de Vega, Oaxaca, México; BYU 42337, an adult female ''65 miles south of Oaxaca City'' (105 km), Oaxaca, México, V. Tipton leg.

Referred Specimens.—Five specimens from Santa Rosa near Lachao, Juquila Distr., Oaxaca, collected by T. MacDougall, October, 1969 (UCM 52386); April 4, 1971 (UCM 52563), and August, 1972 (UCM 52594–96).

Diagnosis.—A member of the *B. macrinii* species group based on osteological and molecular characters, distinguished from most other members of the group by having less interdigital webbing, distinctive coloration consisting of a uniformly dark brown general background, and differences in mitochondrial DNA sequences (cytochrome *b*, 16S rRNA) and protein allelomorphs.

Description.—*Bolitoglossa oaxacensis* is a medium-sized, slender salamander (snout-vent length, SVL 44.3–49.3, $\bar{x}$ = 46.1, in 3 males; 50.8 and 55.8 in two females) with a relatively flat, narrow head (head width/SVL = 0.14–0.16), that is well demarcated from the body. The eyes are of moderate size and only slightly protuberant. Nostrils are very small, and nasolabial protuberances relatively inconspicuous but most evident in males. Mental glands of males are absent. Grooving patterns of the head and neck region are typical of the genus. Vomerine teeth are in patched rows and are relatively numerous (26–38, x=32 in five individuals). Small maxillary teeth are present in series of moderate length (47–52, $\bar{x}$ = 49 N = 5). Premaxillary teeth are present in equal numbers in males and females, with no sexual dimorphism in size (4–9, $\bar{x}$ = 6.8 N = 5). Limbs are long, and when adpressed nearly overlap in males but somewhat less in females (separated by one-half costal interspace in the three males examined, and one to one and a half in the two females). Webbing of the hands and feet is moderate for the genus but slight for the *B. macrinii* group, and it includes little more than the first full phalanx in the four longest digits (Fig. 3). The four longest digits of the foot are not very disproportionate in length. Subdigital pads are large and well developed. Fingers in order of decreasing length: 1-2-4-3, toes: 1-5-4-2-3.

Measurements of the Holotype (in Millimeters).—Head width 8.0; snout to gular fold (head length) 12.5; head depth at posterior angle of jaw 4.0; eyelid length 3.0; eyelid width 1.7; anterior rim of orbit to snout 3.2; horizontal orbital diameter 2.5; interorbital distance 4.0; snout to forelimb 14.9; distance separating external nares 2.5; nostril diameter 0.3; snout projection be-

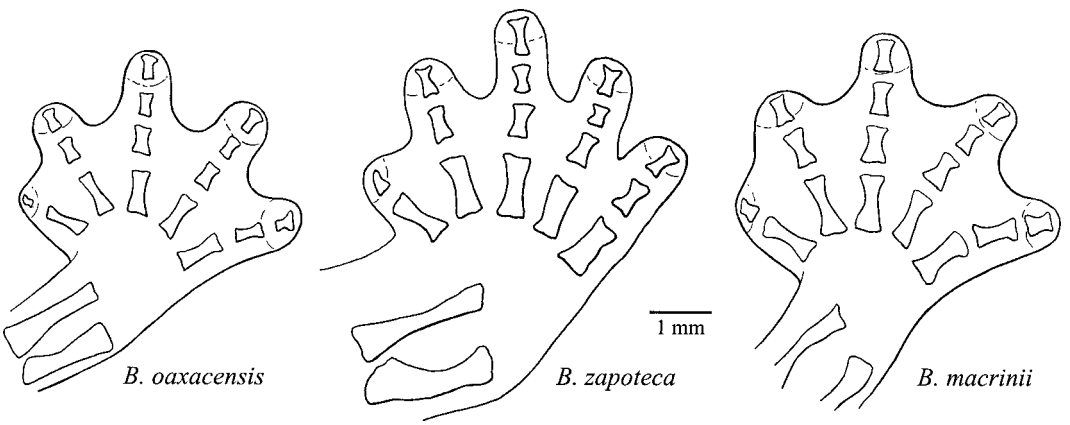

FIG. 3. Right foot of (A) *Bolitoglossa oaxacensis* (IBH 13374), (B) *Bolitoglossa macrinii* (GP 384), and (C) *Bolitoglossa zapoteca* (IBH 13375). Drawn from radiographs. Outlines of bony elements are shown. Also indicated are the limits of the well-formed subdigital pads.

yond mandible 1.2; snout to posterior angle of vent (SL) 50.8; snout to anterior angle of vent 45.3; axilla to groin 26.7; tail length 44.0; tail width at base 3.1; tail depth at base 3.1; forelimb length (to tip of longest digit) 11.1; hind limb length 11.5; width of right hand 4.0; width of right foot 6.0.

Tooth Counts.—Premaxillary teeth 4, maxillary teeth 50, vomerine teeth 33.

Coloration.—The holotype, preserved in alcohol, is dark reddish brown throughout, without markings, darker on tail and body, lighter ventrally, especially in the gular region. Coloration in life was recorded as ''very dark with little pattern'' (DBW field color notes). The specimen from Puerto Portillo (IBH 13374; Fig. 2) has a dark brown to blackish brown ground color, a little lighter ventrally, with a dorsolateral series of reddish-gold markings, sometimes fused to form an obscure, poorly defined band (so infiltrated with dark pigment that hard to perceive without submersion in alcohol) that extends from the posterior margin of the eye to the base of the tail. The band is most evident as a pair of dorsolateral stripes that are most prominent from the base of skull over shoulder. Each stripe is broken more posteriorly, and emarginate, almost scalloped. Scattered dark red-gold marks are present along the dorsal surface of the head, trunk, and tail, denser in the scapular region. A few small light yellow-golden spots are present on the flanks, most concentrated along the line separating ventral and lateral coloration. The venter is dark gray-brown with a few relatively large, dull whitish to golden marks scattered throughout the ventral region but more concentrated on the gular region and absent on the tail venter. There is no light coloration in the limb insertions, only dark blackish brown ground color. Hands and feet dark brown but ventral surfaces paler. Iris dark reddish brown.

Behavior.—The holotype was found under superficially crusted dirt in a relatively wet roadside bank. The specimen from Puerto Portillo was found under bark in a short, upright, wet, decaying stump.

Habitat.—The Sola de Vega specimens were found in road banks near dense humid oak forest with scattered pines. The Puerto Portillo individual was found in the remnants of a recently heavily logged pine forest. The populations exist at relatively high elevation (1800–1920 m), untypical for other members of the *B. macrinii* group.

Range.—The species is known from the Sierra Madre del Sur of Oaxaca, specifically from the mountains south of Sola de Vega, to immediately south of the Atoyac River Basin, in the vicinity of Puerto Portillo, Oaxaca, México. We have assigned poorly preserved specimens (UCM) from the vicinity of Santa Rosa, about 20 km north of San Gabriel Mixtepec, to *B. oaxacensis*, and it is possible that the new species may occur in sympatry with *B. macrinii* somewhere in this region. The BYU specimen has an inexact locality, but we estimate that the specimen was collected approximately 10 km south of Sola de Vega, which places it very near the type locality.

FIG. 4. An adult male of *Bolitoglossa zapoteca* from near Quiegolani, Oaxaca, México (IBH 13375, holotype).

Conservation Status.—*Bolitoglossa oaxacensis* is an uncommon salamander that is rarely encountered. The original habitat of the species, mesic dense oak and pine forest, is still present in the mountains in the Sola de Vega region (Parra-Olea et al., 1999), but extensive logging is affecting a large portion of potential habitat in the mountains south to the Río Atoyac Basin. The mesic forests in this area are now almost gone, but other species of the *B. macrinii* group persist in areas modified for nonintensive agriculture (small banana and coffee plantations).

Etymology—The species name is derived from ''Oaxaca,'' the name of the Mexican state to which it is restricted.

Bolitoglossa zapoteca sp. nov.

Zapotec Salamander—Salamandra Zapoteca
Figures 3–4

Holotype.—IBH 13375, an adult male collected 1 km east Santa María Ecatepec, Oaxaca, México, 1875 m elevation, 16°17.23′N, 95°53.30′W, on 19 September 2000, by G. Parra-Olea, M. García-París, and J. Hanken.

Paratypes.—IBH 13376, same data as holotype. AMNH 51182–84, Quiegolani, Oaxaca, Mexico, T. C. MacDougall leg., 1944.

Diagnosis.—A member of the *B. macrinii* spe-

cies group based on osteological and molecular characters, distinguished from other members of the group by its dark black coloration with small, scattered white spots, and differences in mitochondrial DNA sequences. The new species differs from *B. macrinii* and *B. hermosa* in lacking large whitish dorsal spots and blotches, and from *B. riletti* and *B. oaxacensis* in being black rather than brownish. It has more digital webbing than *B. oaxacensis*, but less than is typical of *B. riletti*.

Description.—*Bolitoglossa zapoteca* is a relatively large species (the holotype is 60.3 mm SVL; Taylor, 1949, reported a male from Quiegolani that is 69 mm SVL and a female that is 73 mm SVL) with a large, relatively flat head of moderate width (head width/SVL = 0.16 in male holotype; 0.20 in subadult female paratype), modestly demarcated from the body. The eyes are relatively small and only slightly protuberant, not extending laterally beyond jaws. Nostrils are very small. Nasolabial protuberances are little developed in the male holotype; the upper lip is moderately swollen and extended anteriorly. There is no mental gland in either the holotype or the largest known male (Taylor, 1949). Grooving patterns of the head and neck are typical of members of the genus. Vomerine teeth are in patched rows, and are relatively numerous (49 in holotype, 22 in paratype). Small maxillary teeth are few in number (41 in holotype, 5 in paratype) and in a short row that ends at middle of the eye. Premaxillary teeth are numerous and small (10 in holotype, 7 in paratype). Limbs are of moderate length, with limb interval of one and a half. Digits are moderately webbed, including all but 2 phalanges of the third toe and 1.5 or more phalanx of toes 2, 4, and 5; about 2 phalanges of the longest finger free of webbing, with more than 1 phalanx for the two next longest digits. Digits bear well-developed terminal pads. Fingers and toes not very disproportionate in length. Fingers in order of decreasing length: 1-2-4-3, toes: 1-5-4-2-3.

Measurements of the Holotype (in Millimeters).—Head width 9.5; snout to gular fold (head length) 13.9; head depth at posterior angle of jaw 4.7; eyelid length 4.0; eyelid width 2.7; anterior rim of orbit to snout 3.4; horizontal orbital diameter 3.0; interorbital distance 4.8; minimum distance separating base of eyelids 3.7; snout to forelimb 17.5; distance separating external nares 3.7; nostril diameter 0.3; snout projection beyond mandible 1.6; snout to posterior angle of vent (SVL) 60.3; snout to anterior angle of vent 55.3; axilla to groin 33.3; width of body across shoulders 7.9; tail length 39.9 (approximately 10 mm were removed before measurement); tail width at base 4.9; tail depth at base 4.6; forelimb length (to tip of longest digit) 14.2; high limb length 15.3; width of right hand 5.1; width of right foot 6.4.

Tooth Counts.—Premaxillary teeth 10, maxillary teeth 41, vomerine teeth 49.

Coloration (in Alcohol).—The two freshly collected specimens were similar in coloration, being dominantly dark glossy black dorsally, with somewhat lighter hands and feet. The venter, while lighter, is a dark gray black with the gular region being less dark that the venter of the trunk. No ventral or lateral white speckling; the ground color is generally immaculate with the exception of a few small whitish spots on the dorsal and lateral surfaces. These spots are diffuse and imprecisely bordered, forming whitish flecks or spotlike marks. The largest of these is on the base of the tail of the holotype and is about 1.2 mm in diameter. The holotype is marked with one small, obscure spot at the rear of each temple, two small spots over the shoulders, 14–15 widely scattered inconspicuous spots on the dorsal and dorsolateral surfaces of the trunk, 4–5 on the base of the tail, and 7–8 tiny flecks scattered on the dorsal and dorsolateral surfaces of the tail. There is one roughly circular amalgamation of diffuse spots 2.3 mm in diameter near the base of the right hind limb. The subadult paratype resembles the adult in being glossy dark black. As in the holotype the head lacks spotting. The whitish spots are few, inconspicuous and widely scattered. There is one small spot between the shoulders, five small spots on the trunk (the largest 1 mm in diameter), and 8–10 small spots on the base of the tail and proximal part of the tail. The eyes of both specimens are dark. Ventral surfaces are unmarked and are a lighter color than the rest of the animal but are dark gray-black.

Behavior.—The newly collected specimens (holotype and one paratype) were found in cracks in the drying soil of a roadside bank. The soil was relatively moist under the surface crust. The specimens were essentially immobile upon discovery.

Habitat.—The heavily disturbed environment above and to the east of Quiegiolani originally was a humid pine-oak forest. Although the forest is largely removed, some trees persist. Specimens were found in road banks near dense humid oak forest with scattered pines. Like *B. oaxacensis*, this species occurs at higher elevation than typical for other members of the *B. macrinii* group.

Range.—The species is known only from a small area in the eastern portion of the Sierra Madre del Sur of Oaxaca, specifically the mountains east of Quiegolani.

Conservation Status.—*Bolitoglossa zapoteca* is rare, and only five specimens have reached collections. Its small known range is in an area that

has been heavily logged and greatly disturbed by other human activities, and yet it persists. Prospects for its long-term survival are not good.

Etymology.—The species is named for the indigenous peoples who live in the mountains of southern Oaxaca.

Comments.—Taylor (1949) recorded information for this species but made the assumption that the specimens he studied were typical of *B. macrinii*, at that time known only from the types. His description combines features of *B. macrinii* and *B. zapoteca*. Arden H. Brame (letter to DBW, 8 October 1965) examined the specimens from Quiegolani and first suggested that they represented a new species, based on the slight webbing of the hands and feet as compared with most *B. macrinii*. Brame kindly shared data he took from the AMNH paratypes from Quiegolani. A large (72.9 SL) female (AMNH 51824) had 51 maxillary and 42 vomerine teeth, whereas a smaller one (53.3 SL, AMNH 51822) had 30 maxillary and 35 vomerine teeth. A male (67.9 SL) had 29 maxillary teeth and 30 vomerine teeth, and Brame noted that the male had female-size and shape teeth.

General Comparisons and Zoogeography.—*Bolitoglossa oaxacensis* and *B. zapoteca* differ from other species of the *B. macrinii* group by their reduced interdigital webbing, which is even less than in *B. macrinii*. Webbing is rather variable in *B. macrinii* (compare Fig. 4 with fig. 2 of Papenfuss et al., 1983) but is rarely as restricted as is typical for either of the new species. The color of *B. oaxacensis* differs from that of *B. hermosa, B. macrinii*, and *B. zapoteca* by its more subdued and nearly patternless nature. The other species are usually black with large cream-white, somewhat metallic, dorsal marks (Papenfuss et al., 1983), whereas *B. oaxacensis* is brownish with obscure dorsal patterning. *Bolitoglossa zapoteca* has small whitish spots, but it is black rather than brown. Although both *B. oaxacensis* and *B. riletti* are basically brown in coloration, *B. oaxacensis* is darker and has less evident light markings.

The two new species resemble other members of the *B. macrinii* group in having weakly developed premaxillary bones, and the adult male holotype of *B. zapoteca* lacks enlarged premaxillary teeth (in fact, it has a high number of small teeth on the bone) and has no evident mental gland. The frontal processes of the premaxillary (visible in a radiograph) of the holotype are weakly developed but complete, whereas those of the smaller paratype are discontinuous on one side. On both of the new species the transverse processes of the first caudal vertebrae are swept anterolaterally and expanded near their tips but not bifurcated in the pattern more typical of what Lynch and Wake (1976) called *Bolitoglossa* beta.

The *B. macrinii* group is geographically isolated from all other species groups in the genus. *Bolitoglossa hermosa* is the only species of the genus known from Guerrero (Adler, 1996), and the other four species of the group are endemic to Oaxaca. As a group, they represent the northern- and western-most populations known for *Bolitoglossa* along the Pacific coast of Mexico. The Isthmus of Tehuantepec appears to be a significant barrier for these generally upland salamanders. Both the *B. mexicana* and the *B. rufescens* groups of *Bolitoglossa* occur on both sides of the Isthmus, but both are primarily lowland in distribution. The *B. macrinii* group is unique in being endemic to Mexico north and west of the Isthmus. Accumulating molecular evidence (García-París et al., 2000a; unpubl. results) suggests that the *B. macrinii* group is separated from other clades within *Bolitoglossa* by long branches, and these data, combined with the osteological data, suggest to us that this clade might be one of the most basal clades in the genus. The genus appears to have originated in Nuclear Central America (Wake and Lynch, 1976), and this clade might have separated from its relatives early in the history of the present-day genus and it has undergone a small but significant radiation in the Sierra Madre del Sur of Oaxaca and uplands of adjacent Guerrero over what we think was a long span of time.

The State of Oaxaca has the most diverse salamander fauna in México. Casas-Andreu et al. (1996) report 22 endemic species, and we know of a number of additional species that are awaiting description. Furthermore, several of the existing species (e.g., *Thorius minutissimus*) are in need of revision, so the fauna is likely to grow considerably in the near future.

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