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PHYLOGENY OF SOUTH AMERICAN TRIAD CORAL SNAKES (ELAPIDAE: *MICRURUS*) BASED ON MOLECULAR CHARACTERS

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ABSTRACT: We present new molecular data (mtDNA sequences and allozymes) for several species of South American "triad" coral snakes. We present a phylogenetic hypothesis for the group based on analysis of molecular characters using both maximum parsimony (MP) and maximum likelihood (ML) optimality criteria. Results from both methods are generally congruent and suggest a basal position for the morphologically and ecologically unique *Micrurus surinamensis*. The *M. frontalis* complex is shown to be paraphyletic with respect to several other species in the parsimony and likelihood trees, but a topology constraining this group to monophyly cannot be statistically rejected compared to the best MP or ML trees. All analyses provide strong support for polyphyly of *M. "lemniscatus"*, and in this case, the alternative monophyly topology is strongly rejected under both optimality criteria. These results underscore the need for a more detailed analysis of populations currently assigned to *M. lemniscatus*.

Key words: *Micrurus*; Coral snakes; Phylogeny; Mitochondrial DNA; Allozymes; South America

CORAL snakes (Serpentes, Elapidae) constitute a taxonomic group of more than 120 named entities (species and subspecies), which to date have been divided into three genera: *Leptomicrurus* Schmidt, 1937; *Micruroides* Schmidt, 1928; and *Micrurus* Wagler, 1824 (Roze, 1967, 1970, 1983, 1987, 1989, 1994, 1996; Roze and Bernal-Carlo, 1987; Scrocchi, 1990). They occur in a variety of habitats, ranging from deserts to cloud forests (Campbell and Lamar, 1989; Roze, 1996). The smallest genera include *Leptomicrurus* (three species), restricted to the northwestern and western Amazon basin, and the monotypic genus *Micruroides* from the southwestern United States and northwestern Mexico. *Micrurus* is the largest genus and contains 57 species distributed from the southern United States through Mexico and Central America to Argentina, and it attains its greatest species diversity in tropical latitudes (Campbell and Lamar, 1989; Roze, 1996).

Most species of *Micrurus* possess a color pattern of some combination of red (or orange), white (or yellow), and black body rings. The most common color pattern in South America is represented by body rings in a red-black-white-black-white-black-red (triad pattern) sequence (Campbell and Lamar, 1989; Roze, 1996; Savage and Slowinski, 1992; Slowinski, 1995). The range of the South American triad coral snakes extends from Panama to southern Argentina and currently is thought to include the following recognized species: *M. ancoralis, M. altirostris, M. baliocoryphus, M. bocourti, M. brasiliensis, M. decoratus, M. diana, M. dissoleucus, M. filiformis, M.*

frontalis, M. frontifasciatus, M. hemprichii, M. ibiboboca, M. isozonus, M. lemniscatus, M. meridensis, M. pyrrhocryptus, M. sangilensis, M. spixii, M. surinamensis, M. tricolor, and *M. tschudii* (Campbell and Lamar, 1989; Jorge da Silva, 1993, 1995; Jorge da Silva and Sites, 1999; Roze, 1994, 1996; Roze and Jorge da Silva, 1990; Scrocchi, 1990; Slowinski, 1995). Two species of triads are non-South American, including *M. elegans* (southeastern Mexico and Guatemala) and *M. laticollaris* (southwestern Mexico; Campbell and Lamar, 1989; Roze, 1996).

In North and Central America, coral snakes occur alone or in sympatry with only one congener. However, in South America, especially in the Amazon basin, up to nine species may be sympatric in some places (Dixon and Soini, 1986). Because they possess highly neurotoxic venoms, aposematic coloration, and serve as models for a number of colubrid snake Batesian (or Müllerian) mimics (Brodie, 1993; Greene and McDiarmid, 1981; Hinman et al., 1997; Mertens, 1956, 1957; Pough, 1988; Savage and Slowinski, 1992; Smith, 1975, 1977), coral snakes are interesting from many ecological and evolutionary perspectives.

The first study to present an explicit hypothesis of relationships among coral snakes was that of Roze and Bernal-Carlo (1987); they developed a phylogeny based on 27 characters (morphological, immunological, and paleontological), but the data were not reported in their paper, nor were the sample sizes, total number of species examined, or tree reconstruction methods described. Nevertheless, Roze and Bernal-Carlo (1987) recognized *Leptomicrurus* as a separate genus, and distinct *mipartitus* and South American triad groups within *Micrurus.*

The most recent phylogenetic (= cladistic, sensu Hennig, 1966) study of coral snakes is that of Slowinski (1995) based on biochemical and morphological data. Slowinski's analysis recovered the South American triad coral snakes as an incompletely resolved but unambiguously monophyletic group, consisting of the species *M. dissoleucus, M. frontalis, M. ibiboboca, M. lemniscatus, M. spixii,* and *M. surinamensis,* which he retained under the name *Micrurus* along with several other monophyletic groups. Slowinski's (1995) study initially included a total of 18 ingroup species examined for both allozyme and morphological characters, but the taxonomic distribution of the parsimony informative morphological characters lead him to hypothesize that the monophyletic South American triad group would also include *M. ancoralis, M. hemprichii, M. isozonus,* and *M. tschudii*; all of these species possess the slightly bilobed, short, stout hemipenis which was one of the synapomorphies that defined the group in the phylogenetic analysis. Slowinski further hypothesized that, on the basis of their similar coloration, relatively short tails, and distributions, the following species would also likely be members of this clade: *M. decoratus, M. filiformis, M. frontifasciatus, M. pyrrhocryptus, M. rondonianus,* and *M. sangilensis.* With few exceptions, most meristic and cranial osteological features are conserved within the entire genus (Jorge da Silva and Sites, 1999; Roze, 1983; Savitzky, 1978; Slowinski, 1995), and therefore they provide relatively few characters useful for phylogeny reconstruction. Slowinski (1995) retained the previously recognized genus *Micruroides* and synonymized *Leptomicrurus* with *Micrurus.*

The major distinction between these alternatives is the arrangement of the major groups relative to each other; both recognize the genus *Micruroides* as monotypic and basal to all other New World coral snakes, but this is the only shared feature between the two topologies. The Roze and Bernal-Carlo (1987; their Fig. 12) topology is ((*Micruroides*) (*Leptomicrurus*) ((*mipartitus* group) (((simple band group—*M. nigrocinctus*) (Mexico/Central American triads)) (South American triads)))), while the Slowinski (1995; his Fig. 6) topology is ((*Micruroides*) (((South American triads) ("*Leptomicrurus*")) ((*mipartitus* group) (monad + bicolor group)))). Roze (1996) recently resurrected the genus *Leptomicrurus* from *Micrurus,* but without refuting the phylogenetic analysis of Slowinski (1995).

The present study is part of an ongoing morphological and molecular investigation of the South American triad coral snakes, and it extends some of the findings included in a recent monographic review of the *M. frontalis* complex (Jorge da Silva and Sites, 1999). In that study, all available type specimens were redescribed and extensive accounts were given for each of the seven species recognized. These accounts provided complete synonomies, descriptions of variation in color patterns, meristic and morphometric variables, cranial and hemipenial morphology, and geographic distributions. Jorge da Silva and Sites (1999) did not, however, attempt any phylogenetic analysis in that review. Here we use two molecular data sets, protein allozyme loci and mtDNA sequences, to reconstruct a more detailed phylogenetic hypothesis for the South American triad group of *Micrurus*. This study includes some of the species recognized by Jorge da Silva and Sites (1999), and also some that were unavailable to Slowinski (1995). We recognize the limitations of the present work due to incomplete sampling (Wheeler, 1992), but several species of South American triads are difficult to obtain and were not available to us at the time this work was undertaken. Despite this limitation, the phylogenetic relationships presented in this paper provide important confirmation and extension of the studies of Slowinski (1995) and Jorge da Silva and Sites (1999), and they can be applied to tests of other comparative questions.

MATERIAL AND METHODS

Taxa Collected

We collected a total of 11 ingroup species, represented by 1–14 individuals per species, from 1988–1994; the South American monad species *M. corallinus* was used as an outgroup. The following acronyms denote collections where museum vouchers were deposited or institutions that donated specimens and/or tissue samples (acronyms follow Leviton et al., 1985). AMNH—American Museum of Natural History, New York, New York, U.S.A.; CEPB—Centro de Estudos e Pesquisas Biológicas—Universidade Católica de Goiás, Goiânia, Goiás, Brazil; FPZSP—Fundação Parque Zoológico de São Paulo, São Paulo, Brazil; HRL—Hospital Regional de Leticia, Colombia; IB—Instituto Butantan, São Paulo, São Paulo, Brazil; IM—Instituto Malbrán, Argentina; IVB—Instituto Vital Brazil, Niterói, Rio de Janeiro, Brazil; LSUMZ—Louisiana State University Museum of Zoology (tissue collection), Baton Rouge, Louisiana, U.S.A; MCP—Museu de Ciências e Tecnologia—Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil; MHNCI—Museu de História Natural Capão da Imbuia, Curitiba, Paraná, Brazil; MZUSP—Museu de Zoologia—Universidade de São Paulo, São Paulo, São Paulo, Brazil; and QCAZ—Museo de Zoología, Pontificia Universidad Católica del Ecuador, Quito, Ecuador. Table 1 summarizes the individuals used in this study, by species and geographic location.

Tissue Samples

Tissues were collected immediately after the administration of a lethal dose of B-Euthanasia (sodium pentobarbital). Blood was collected by sectioning the aortic arch and aspirating with microcapillary tubes (70 μl), followed by transfer to 2.0 ml cryogenic tube previously coated with EDTA-Na_2. Other tissues (heart, liver, stomach, small intestine, kidney, and skeletal muscle) also were extracted, transferred to individual cryogenic tubes, and all (including blood) immediately frozen in liquid nitrogen. These samples were transferred to individual tubes and preserved in 100% ethanol until use for mtDNA amplification and sequencing.

Protein Electrophoresis

We separated tissue samples into three classes for laboratory analysis: (1) skeletal muscle, (2) duodenum and stomach (flushed free of contents), and (3) liver and kidney, and stored these at −80 C until electrophoresis. Tissues were homogenized in equal volumes of grinding solution (0.01 M Tris), and subjected to horizontal electrophoresis in 12.5% (W/v)

TABLE 1.—South American coral snakes for which allozyme and mtDNA sequence data (*) were collected for this study; political units are: AR = Argentina, BR = Brazil, CO = Colombia, and EC = Ecuador, and within Brazil, the states are AL = Alagoas, AM = Amazonas, BA = Bahia, GO = Goias, MG = Mato Grosso, PR = Parana, RJ = Rio de Janeiro, RS = Rio Grande do Sul.

Taxa	Locality	Museum voucher
1. *Micrurus corallinus*-1*	Jurujuba Niterói, RJ, BR	CEPB 051
2. *Micrurus corallinus*-2	Itaipu, RJ, BR	IB 55599
3. *Micrurus frontalis*-1*	Guapo, GO, BR	CEPB 1587
4. *Micrurus frontalis*-2*	Senador Canedo, GO, BR	CEPB 2365
5. *Micrurus frontalis*-3	Ap. de Goiania, GO, BR	CEPB 604
6. *Micrurus frontalis*-4	Jatai, GO, BR	CEPB 336
7. *Micrurus frontalis*-5	Serranopolis, GO, BR	CEPB 1813
8. *Micrurus frontalis*-6	Jatai, GO, BR	CEPB 7404
9. *Micrurus frontalis*-7	Pontalina, GO, BR	CEPB 2285
10. *Micrurus frontalis*-8	Pontalina, GO, BR	CEPB 2363
11. *Micrurus frontalis*-9	Goiania, GO, BR	CEPB 2432
12. *Micrurus frontalis*-10	Goiania, GO, BR	CEPB 2433
13. *Micrurus altirostris*-1*	Campo Largo, PR, BR	MHNCL 4848
14. *Micrurus altirostris*-2*	Irati, PR, BR	MHNCL 4208
15. *Micrurus altirostris*-3*	Torres, RS, BR	MCP 5035
16. *Micrurus altirostris*-4*	Yaciretá, COR, AR	IB 55595
17. *Micrurus altirostris*-5	Cascavel, PR, BR	MHNCL 6255
18. *Micrurus altirostris*-6	Curitiba, PR, BR	MHNCL 6158
19. *Micrurus altirostris*-7	Guiba, RS, BR	MCP 3775
20. *Micrurus altirostris*-8	Porto Alegre, RS, BR	MCP 5259
21. *Micrurus altirostris*-9	Butia, RS, BR	MCP 4031
22. *Micrurus altirostris*-10	Yaciretá, AR	IB 55596
23. *Micrurus altirostris*-11	Yaciretá, AR	IB 55597
24. *Micrurus brasiliensis*-1*	Barreiras, BA, BR	CEPB 2301
25. *Micrurus brasiliensis*-2*	Desidério, BA, BR	IB 55385
25. *Micrurus baliocorryphus**	Santa Rosa, AR	MZUSP 10808
26. *Micrurus pyrrhocryptus*-1*	Santos Lugares, AR	MZUSP 10809
27. *Micrurus pyrrhocryptus*-2	Sauce Bajada, AR	CEPB 073
28. *Micrurus pyrrhocryptus*-3	Corrientes, AR	CEPB 074
29. *Micrurus pyrrhocryptus*-4	La Posta, AR	CEPB 1804
30. *Micrurus pyrrhocryptus*-5	Sgo. del Estero, AR	CEPB 2364
31. *M. l. lemniscatus**	Tabatinga, AM, BR	CEPB 2289
32. *M. l. carvalhoi*-1*	Vianópolis, GO, BR	CEPB 2687
33. *M. l. carvalhoi*-2*	Rio Preto, MG, BR	IB 55598
34. *M. l. carvalhoi*-3	Goiania, GO, BR	CEPB 3514
35. *M. l. carvalhoi*-4	Ouvidor, GO, BR	CEPB 2969
36. *M. lemniscatus*-1*	Quebrangulo, AL, BR	CEPB 2312
37. *M. lemniscatus*-2*	Arraial do Cabo, RJ, BR	IVB 1757
36. *Micrurus ibiboboca*-1*	Xingó, AL, BR	CEPB 024
37. *Micrurus ibiboboca*-2*	Xingó, AL, BR	CEPB 025
38. *Micrurus decoratus*-1*	Teresópolis, RJ, BR	IVB 2453
39. *Micrurus decoratus*-2	Teresópolis, RJ, BR	IVB 2450
40. *Micrurus h. hemprichii**	Ecuador, EC	QCAZ 2584
41. *Micrurus spixii obscurus*-1*	Tabatinga, AM, BR	CEPB 1807
42. *Micrurus spixii obscurus*-2	Leticia, CO	CEPB 2288
43. *Micrurus s. surinamensis*-1*	Leticia, CO	CEPB 1812
44. *Micrurus s. surinamensis*-2	Leticia, CO	CEPB 2290

starch gels (1:1 Sigma Starch—Electrostarch) at 5–7 C. Enzymes resolved and tissue-buffer combinations used for specific loci are given in Table 2. General histochemical staining protocols followed Murphy et al. (1996). We considered electromorphs of any given locus to be homologous if they had the same mobility, and designated those with differing mobilities as different electromorphs using al-

TABLE 2.—Enzymes and electrophoretic conditions used for allozyme studies of *Micrurus*. Names, locus acronyms, and enzyme commission (EC) follow the International Union of Biochemistry (1989). Tissue symbols: D = stomach and duodenum, L = liver and kidney, and M = skeletal muscle.

Enzyme	EC number	Locus	Tissue	Buffer
N-Acetyl-β-glucosaminidase	3.2.1.30	βGa	D, L	A, B
Aconitate hydratase	4.2.1.3	M-Acoh-A	L	B, C
		S-Acoh-A	L	B, C
Adenylate kinase	2.7.4.3	Ak-A	M	A, B, D
Aspartate aminotransferase	2.6.1.1	M-Aat-A	L	A, B, D
		S-Aat-A	L	A, B, D
Creatine kinase	2.7.3.2	Ck-A	M	A, B, D
Glucose-6-phosphate isomerase	5.3.1.9	Gpi-A	L	B, C, E
α-Glucosidase	3.2.1.20	αGlus	D	A, B
β-Glucuronidase	3.2.1.31	βGlur	D	A, B
Glutamate dehydrogenase	1.4.1.2	Gtdh	L	B, E
(s)-2-Hydroxy-acid oxidase	1.1.3.15	Haox	L	B, E
L-Iditol dehydrogenase	1.1.1.14	Iddh-A	L	B, C
Isocitrate dehydrogenase	1.1.1.42	M-Icdh-A	L	B, E
		S-Icdh-A	L	B, E
L-Lactate dehydrogenase	1.1.1.27	Ldh-A	L, M	C, D
		Ldh-B	L, M	C, D
Malate dehydrogenase	1.1.1.37	M-Mdh-A	L	B, E
		S-Mdh-A	L	B, E
Malate dehydrogenase (NADP*)	1.1.1.40	Mdhp	L	B, E
Mannose-6-phosphate isomerase	5.3.1.8	Mpi-A	L	B, C
	???	"Mpi-2"	L	B, C
Peptidases:	3.4.-.-			
L-leucylglycylglycine		Pep(Lgg)	M	B, C
L-leucyl-leucyl-leucine		Pep(Lll)	M	B, C
L-phenylalanyl-L-proline		Pep(Pap)	M	B, C
L-leucyl-L-alanine		Pep(La)	M	B, C
Phosphoglucomutase	5.4.2.2	Pgm-A	L	B, C
Phosphogluconate dehydrogenase	1.1.1.44	Pgdh-A	L	B, E
Phosphogluconate kinase	2.7.2.3	Pgk-A	M	A, B
Purine-nucleoside phosphorylase	2.4.2.1	Pnp-A	L	B, C
Superoxide dismutase	1.15.1.1	M-Sod-A	L	B, C
Triose-phosphate isomerase	5.3.1.1	Tpi-A	L	B, C
		"Tpi-2"	L	B, C

Buffers (from Appendix II of Murphy et al., 1996, pages 117–120):

A—Lithium borate/Tris citrate, pH 8.2–8.3.
B—Tris-citrate II, pH 8.0.
C—Tris-citrate/EDTA, pH 7.0.
D—Tris-citrate III, pH 7.0.
E—Tris-malate/EDTA, pH 7.4.

phabetic designations ("A"–"E") based on their order of appearance (the first electromorph resolved for any locus was designated "A", the second "B", and so forth). We compared and verified electromorph mobilities in separate side-by-side electrophoretic runs, and tested for hidden heterogeneity (cryptic enzyme variation; Coyne, 1982) by sequential electrophoresis. Thus, we varied either the starch-gel buffer system, or the electrode buffer, in order to resolve a greater portion of charge variation in the electromorphs (Highton and Hedges, 1995, for example). Appendix I gives all individual genotypes, by taxon, for all polymorphic loci.

Collection of Molecular Data

DNA was extracted by powdering approximately 100 mg of liver in a prechilled mortar and pestle under liquid nitrogen. The resulting powder (~100 mg) was

TABLE 3.—Primers used to amplify and sequence the *Micrurus* mitochondrial DNA region depicted in Fig. 1; ND4 and Leu-tRNA are from Arévalo et al. (1994).

Primer	Sequence (5′ to 3′)
ND4	CACCTATGACTACCAAAAGCTCATGTA-GAAGC
Leu-tRNA	CATTACTTTTACTTGGATTTGCACCA
Coral 1	CCCTGCCCAATCTAAAAAC
Coral 2	CTTCCTCCCATTTATTGTCC
Coral 3	CCTATTATCAAGGTCACAGC
Coral 4	TCTGGTTTTAGGGAGATTA
Coral 5	GGGAGGAATACATCAGTTTTTA
Coral 6	AAATCACTAATCGCATACTCTTCAAT
Coral 7	CTTTACAGGCGAACTCCTCATTGC
Coral 8	TGGTAAAGAGGGAGGTTGCAATGAGGAG
Coral 9	ATAGAAGAGTATGCAATTAGGGATTT

transferred to an Eppendorf tube and mixed with 500 µl of STE buffer (100 mM + 10 mM Tris + 1 mM EDTA, pH 7.5). This mixture was then lysed by adding 25 µl of a 10 mg/ml stock solution of proteinase K, and incubated for 2 h at 55 C. Lysis was followed by extraction in a phenol/chloroform/isoamyl alcohol solution (25:24:1), then ethanol precipitation, and final resuspension in TE buffer (10 mM Tris + 1 mM EDTA; Sambrook et al., 1989).

Approximately 0.1 ng of DNA was used to amplify a 812 base pair (bp) region of the mtDNA genome that included 672 bp of the 3′ end of the ND4 protein gene, and a 140 bp region that included the histidine, serine, and part of the leucine tRNA genes ($tRNA^{His}$, $tRNA^{Ser}$, and $tRNA^{Leu}$, respectively). Amplification of the segment was accomplished using the Polymerase Chain Reaction (PCR) technique (Erlich, 1989) and Sanger dideoxy sequencing (Sanger et al., 1977). Inital sequencing used the "ND4" and "Leu" primers described by Arévalo et al. (1994) followed by a combination of internal primers designed specifically for coral snakes (Table 3, Fig. 1). The 5′ ends of the ND4 and Leu primers anneal to positions 12900 and 13831, respectively, of the mitochondrial genome of *Xenopus laevis* (Roe et al., 1985).

The approach used was an initial amplification of double-stranded product with the ND4-Leu primer pair, or any combination of the additional internal primers (coral primers), followed by single-stranded amplifications of shorter segments using the internal coral snake primers (Table 3). A Perkin Elmer Cetus thermocycler was used with two protocols. Protocol A was used with ND4-Leu primers and consisted of denaturation at 94 C for 1 min, annealing at 35 C for 1 min, and extension at 72 C for 1 min in the first three cycles, followed by a change in the annealing temperature to 50 C for 1 min with 35 cycles. Relaxation of the annealing temperature was necessary due to mismatches of Leu primer to coral snake templates. Protocol B was used whenever double-stranded amplification was performed using any of the internal primers (94 C—1 min + 50 C—1 min + 72 C—1 min = 35 cycles) instead of ND4 and Leu.

PCR products were checked on a 1% agarose gel, purified by GeneClean® (Dynal, Olso, Norway), precipitated in 2.5 volumes of 95% ethanol and 0.1 volume of 3M Na Acetate, and resuspended into 14 µl of double-distilled water. These purified DNA samples were used for sequencing with a Sequenase version 2.0 Kit (USB, Cleveland, Ohio). Confirmation of base identity was made either by sequencing both strands of the same region, sequencing the same strand at least twice, and/or sequencing more than one conspecific individual from the same locality (*M. frontalis, M. ibiboboca*; Table 1). A total of 812 bases were confidently resolved in all taxa, and are available in GenBank (accessions: AF228424–AF228444).

Data Analyses

For phylogenetic analysis, each allozyme locus or nucleotide was treated as an independent character, and characters states were polarized by outgroup rooting (Nixon and Carpenter, 1993; Watrous and Wheeler, 1981), with *Micrurus corallinus* designated as the outgroup. PAUP* software (version 4.012; Swofford, 1999) was used to test for phylogenetic signal in both data sets and for most of the phylogenetic analyses. Phylogenetic signal was evaluated for each data set by calculating the skewness of the distribution of tree lengths (g1) for 10,000 randomly generated trees across

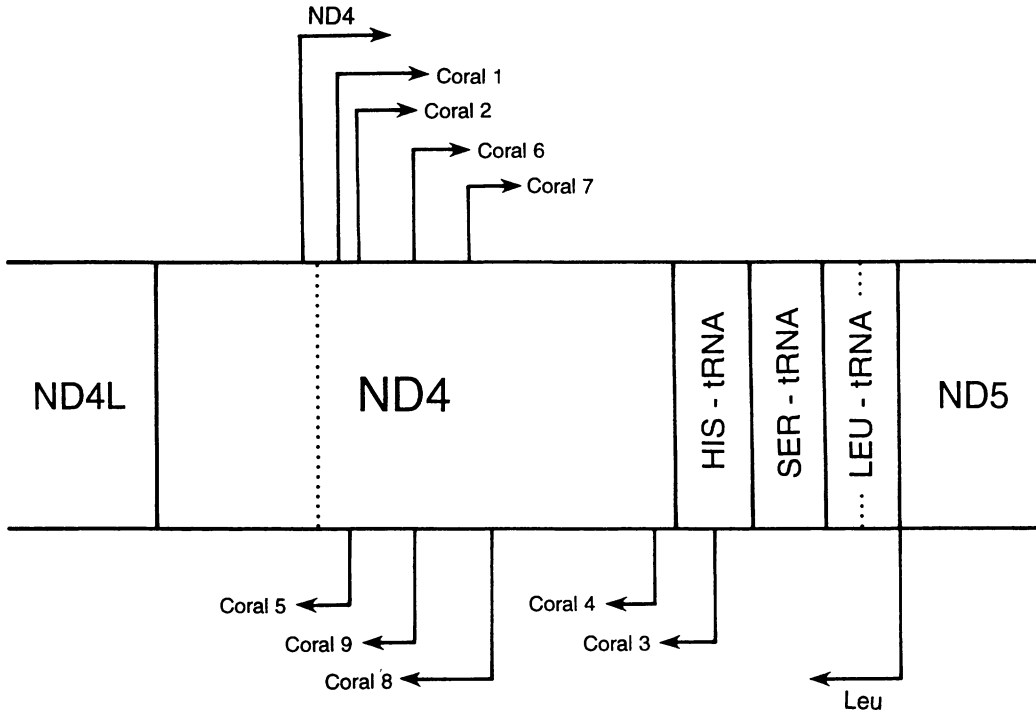

FIG. 1.—The mtDNA fragment of 812 bp sequenced for this study (between dotted lines), showing the approximate annealing positions of oligonucleotide primers given in Table 3.

the sequential deletion of taxa (Hillis, 1991), and using the probability values of Hillis and Huelsenbeck (1992; see Källersjo et al., 1992, for a dissenting opinion). Sequential deletion of taxa from the best supported clades provides an assessment of the distribution of phylogenetic signal in the shallow versus deep branches of the tree.

For all maximum parsimony (MP) analyses, the two data partitions (allozymes and mtDNA sequences) were first analyzed separately. The parsimony informative allozyme loci scored as "fixed" (i.e., no parsimony informative intraspecific polymorphism in our samples; $n = 14$ loci) were coded as individual characters with alleles as unordered character states. Four polymorphic loci (nos. 15–18 in Appendix I) were coded by transforming the electromorph frequencies of each locus into Manhattan distances which served as weighted values in a step matrix (Berlocher and Swofford, 1997; Wiens, 1995; step matrixes are given in Appendix II). This approach incorporates frequency information and permits the use of multistate characters, and it has been used in previous empirical studies (McGuire, 1996; Mendoza-Quijano et al., 1998). Wiens (2000) has shown, in a comparative study of phylogenetic methods for allozyme data, that frequency-based methods (whether parsimony, distance, or likelihood) give the most accurate estimates of phylogenies. Frequency parsimony did not perform as well as distance and likelihood methods in some cases, but it offers the advantages of permitting the inclusion of characters with missing data (not possible with current likelihood programs for frequency data; e.g., CONTML), combining allozyme data with other characters, and evaluating the contribution of individual characters to support of individual clades. Given these advantages and the overall acceptable performance of frequency methods under simulation and congruence studies (Wiens,

1998*a*, 2000; Wiens and Servedio, 1998), we adopted this method here.

Mitochondrial DNA sequences were aligned by eye against the *Sceloporus* sequences published by Arévalo et al. (1994) because the ND4 region contained no gaps. The tRNA sequences also contained no gaps and were aligned according to suggested vertebrate secondary structures described by Kumazawa and Nishida (1993).

Initial searches were based on the MP optimality criterion, with equal weighting of all characters (except for searches invoking the step matrix option). All MP searches were conducted with the following settings in place: accelerated character transformation (ACCTRAN), branch-and-bound (BANDB) searches with tree-bisection reconnection (TBR) branch swapping, save all minimum trees (MULPARS), random addition sequence, and zero-length branches collapsed to yield polytomies. Support for internal nodes was assessed by nonparametric bootstrapping [Felsenstein, 1985; 1000 pseudoreplicates for maximum parsimony, and 200 for maximum likelihood searches (see below)] as a conservative estimate of the stability of internal branches (Hillis and Bull, 1993).

We next implemented a maximum likelihood (ML) search for the mtDNA sequence data, by first testing for the best fit (i.e., most appropriate) model of molecular evolution, using the MODELTEST program (version 3.0; Posada and Crandall, 1998). The MODELTEST program requires input of a simple tree [we used the default suggestion of a neighbor-joining tree, which was based on distances estimated from a Jukes-Cantor (1969) substitution model], and uses this to test 56 alternative nested models of molecular evolution for optimum fit relative to the data matrix (see Huelseneck and Crandall, 1997; their Fig. 4). The program begins by testing the data for their fit to the simplest null model (equal base frequencies, equal rates of transitions and transversions, equal rates among sites; Jukes and Cantor, 1969) versus an alternative differing by only one of these parameters [unequal base frequencies, Felsenstein, 1981; this test has 3 degrees of freedom (df)], and it proceeds to the next level of model complexity after one of the original pair more "parameter simple" models has been rejected. Alternative models at the next level include another parameter (transition and transversion rates are equal, or not; 1 df), and successively more parameter-rich models (for example, mutation rates within transitions and within transversions are equal, or not) are sequentially tested until the best fit is found (see Fig. 1 of Posada and Crandall, 1998). The nested structure of the alternative models has the property, under a correct null hypothesis, of a likelihood ratio test (δ) which is asymptotically distributed as a χ^2 statistic with q degrees of freedom (where q is the difference in the number of free parameters between the null model and its alternative; see Posada and Crandall, 1998). To retain the nested structure of the models, likelihood scores are estimated from the same input tree, but tree topology appears to have little influence on the likelihood estimates for a given data set in these kinds of analyses (Yang et al., 1995), and for MODELTEST in particular (D. Posada, personal communication).

The likelihood ratio tests summarized in Table 4 show that the best-fit model for the *Micrurus* data is the modified HKY (see Hasegawa et al., 1985) + invariable sites (I) + rate heterogeneity (gamma, Γ; the HKY85 + I + Γ model of Gu et al., 1995), which was then used to estimate likelihood parameters for tree construction. The parameters input for the ML search (estimated from the default NJ tree) were as follows: Ti/Tv ratio = 3.7472 for the substitution model; base frequencies = 0.3215 (A); 0.2977 (C); 0.1266 (G); 0.2542 (T); proportion of invariable sites (I) = 0.5181; and the Gamma distribution shape parameter (Γ) = 0.8738. The ML analysis used a heuristic search strategy with TBR branch swapping, and random addition sequence for each of 100 replications; a molecular clock was not enforced. For both MP and ML trees, alternative topologies were tested for significance at the 95% level using the Templeton (1983) and Kishino-Hasegawa (1989)

TABLE 4.—MODELTEST analysis of 56 hierarchical substitution models for the *Micrurus* mtDNA data; -lnL scores were estimated under various models of evolution on a neighbor-joining tree, and compared for best fit to the sequences as described by Posada and Crandall (1998).

Null model (H_0)	H_0 vs. H_1	$-\ln L_0$	$-\ln L_1$	df	P
Equal base frequencies	JC69[a] vs. F81[b]	4328.90	4277.28	3	≪0.001
Equal ti/tv rates	F81[b] vs. HKY85[c]	4277.28	4081.66	1	≪0.001
Equal ti rates and equal tv rates	HKY85[c] vs. TrN[d]	4081.66	4081.34	1	0.4237
Unequal ti and tv rates	HKY85[c] vs. K3P[e]	4081.66	4080.09	1	0.0762
Equal rates among sites	HKY85[c] vs. HKY85 + Γ[f]	4081.66	3811.55	1	≪0.001
No invariable sites	HKY85[c] + Γ vs. HKY85 + Γ + I[g]	3811.55	3807.91	1	0.0070

[a] JC69, Jukes and Cantor (1969).
[b] F81, Felsenstein (1981).
[c] HKY85, Hasegawa et al. (1985).
[d] TrN, Tamura and Nei (1993).
[e] K3P, Kimura (1981).
[f] Γ, shape parameter of the gamma distribution.
[g] I, proportion of invariable sites.

tests, respectively, as implemented in PAUP*.

RESULTS

Patterns of Variation

Of the 32 allozyme loci resolved in this study, eight were monomorphic for the same electromorph across all ingroup and outgroup taxa (α-Glus, β-Glur, Haox, Gtdh, S-Icdh-A, Ldh-B, S-Mdh-A, and "Mpi-2"), and seven more were nearly so [single heterozygotes were resolved for Mdhp,and Pgm-A, and five loci displayed only "fixed" autapormophic states (Mpi-A, M-Aat-A, S-Aat-A, M-Icdh-A, Pgdh-A)]. The character matrix for all potentially parsimony informative loci, as well as the five with fixed autapomorphic electromorphs, is given in Appendix I.

The aligned 812 bp sequence of mtDNA for the 22 snakes sampled in this study included part of the ND4 gene (672 bp), in which a total of 233 positions (34.7%) varied in one or more of the ingroup taxa. Of the total variable positions, 150 (64,4%) corresponded to third, 21 (9.0%) to second, and 62 (26.6%) to first base positions. Of the variable second positions, eight (38.1%) were transitions and 13 (61.9%) were transversions; of the variable first positions, 45 (72.6%) were transitions and 17 (27.4%) were transversions. For the tRNA genes (140 bp), a total of 15 sites (10.7%) was variable, and of these, 11 (73.3%) were transitions and four (26.7%) were transversions.

Tests for Phylogenetic Signal

The allozyme data set was analyzed for phylogenetic signal by comparing the shortest trees recovered from a MP analysis against the lengths of 10,000 randomly distributed trees; this was done first for all taxa, and then iterated by sequential deletion of taxa from different hierarchical levels in consensus trees recovered in subsequent MP searches. The first search recovered nine equally parsimonious trees for 14 taxa (TL = 3752), which were significantly shorter than the distribution of random trees ($g1 = -1.0084$, $P < 0.01$, using the critical values for binary character data given by Hillis and Huelsen-

TABLE 5.—Input character matrix used for MP analysis of collapsed terminal units for the 14 "fixed" allozyme loci (nos. 1–14 in Appendix I), for all named entities of *Micrurus* used in this study. The numbers in parentheses identify the entities from Appendix I that were combined to create the terminals listed here; upper case letters refer to fixed or highest frequency electromorph for each of the genotypes given in Appendix I.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
M. corallinus (1+2)	C	D	A	A	C	C	A	B	B	E	A	A	A	A
M. frontalis (1–10)	A	A	A	A	A	A	A	A	A	A	A	A	A	B
M. altirostris (1–11)	A	A	A	A	A	A	A	A	A	A	A	A	C	B
M. brasiliensis (1+2)	A	A	A	C	A	A	A	A	A	A	A	A	A	A
M. baliocoryphus	A	A	A	A	B	A	A	A	A	A	A	A	A	A
M. pyrrhocryptus (1–5)	A	B	A	A	B	A	A	A	A	A	A	A	C	B
M. l. lemniscatus	A	-	B	C	B	A	B	-	B	C	B	A	A	B
M. l. carvalhoi (1–4)	A	-	B	C	B	A	B	A	B	C	B	A	A	B
M. lemniscatus (1+2)	A	A	A	C	A	A	A	-	A	C	A	B	A	B
M. ibiboboca (1–2)	A	A	B	C	A	A	A	A	A	A	A	A	A	A
M. decoratus (1+2)	A	B	A	B	B	-	A	A	C	D	A	A	B	B
M. hemprichii	-	A	A	-	B	B	A	-	B	B	A	B	A	A
M. spixii (1+2)	B	B	A	C	A	A	A	B	C	A	A	A	B	B
M. surinamensis (1+2)	A	E	A	C	B	A	A	B	D	A	A	A	A	B

beck, 1992); this remained the case when the outgroup (*M. corallinus*) was deleted (nine trees, TL = 2948; g1 = −1.2094; $P < 0.01$). Two further iterations, involving first the deletion of three ingroup taxa (*M. altirostris, M. pyrrhocryptus,* and *M. spixii*; 690 trees, TL = 2636), and then two more (*M. ibiboboca* and *M. lemniscatus carvalhoi,* 52 trees, TL = 2528) still revealed significantly shorter trees (g1 = −1.4453 and g1 = −0.8790, respectively; $P < 0.01$ in both tests).

We repeated this test for the mtDNA sequences, beginning first with all taxa, then deleted the outgroup (*M. corallinus*), and then successively deleted ingroup taxa as described above. Four equally parsimonious trees were recovered from the analysis of all taxa ($n = 21$), and the tree length (550) was significantly skewed relative to the distribution of the random trees (g1 = −0.6012, $P < 0.01$). The following taxa were deleted in subsequent iterations: (1) *M. corallinus* (nine trees, TL = 476; g1 = −0.7607); (2) *M. altirostris*3, *M. baliocoryphus, M. brasiliensis*1, *M. lemniscatus carvalhoi,* and *M. lemniscatus*2 (four trees, TL = 413; g1 = −0.7641); (3) *M. altirostris*2, *M. frontalis*2, *M. hemprichii,* and *M. ibiboboca* (one tree, TL = 340; g1 = −0.9717); and (4) *M. altirostris*1, *M. brasiliensis*2, and *M. lemniscatus carvalhoi*2 (three trees, TL = 279; g1 = −0.6684); in all tests $P < 0.01$. We therefore conclude that significant phylogenetic signal is distributed throughout the internal structure of the tree topologies in both data sets.

MP Analyses

Appendix I shows that, within named taxonomic entities, most individuals are identical in electromorph composition for virtually all loci, so for the analyses of the allozyme partition alone, we combined genetically identical samples to collapse the total number of ingroup terminals from 20 to 13. Table 5 summarizes the allozyme matrix used for the MP analysis of this reduced set of terminals, for the 14 "fixed" loci (nos. 1–14 in Appendix I). The MP analysis included these loci as well as the four polymorphic loci (nos. 15–18 in Appendix I) for which frequencies were weighted by Manhattan distances (Appendix II), and recovered nine equally parsimonious trees. Bootstrap proportions included on a strict consensus of these trees (Fig. 2) revealed that, with the exception of the (*M. l. lemnsicatus* + *M. l. carvalhoi*) clade, none of the internal nodes was strongly supported (all other bootstrap values < 50%; Fig. 2). In this tree, *M. decoratus, M. spixii,* and *M. surinamensis* were recovered in basal positions, while *M. altirostris, M. brasiliensis, M. frontalis, M.*

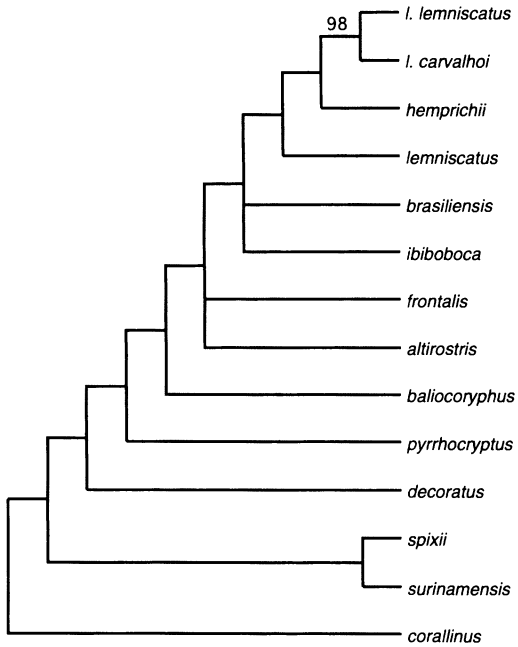

FIG. 2.—Strict consensus of nine equally parsimonious trees recovered by a weighted maximum parsimony (MP) analysis of the allozyme data (Appendix I), showing relationships for all South American triad species of *Micrurus* examined in this study. Four polymorphic loci were weighted by Manhattan distance step matrixes (Appendix II), and definitions of the condensed terminal units are given in Table 4. TL = 3751, CI = 0.840, RI = 0.769, and RC = 0.646.

hemprichii, and *M. lemniscatus*, are highly nested. The analysis failed to recover a monophyletic *M. lemniscatus*, and resolved *M. baliocoryphus* and *M. pyrrhocryptus* in intermediate positions (Fig. 2).

The mtDNA partition was then analyzed by equally weighted MP and recovered four equally parsimonious trees with strong support at many nodes (Fig. 3A presents a phylogram of one of these trees). This topology differs from the allozyme tree (Fig. 2) in the placement of several taxa (*M. hemprichii* and *M. spixii*, for example), and also provides strong support for polyphyly of *M. lemniscatus*. Reasonably strong support [using the Hillis and Bull, 1993, 70% bootstrap criteria (but see their caveats)] is also evident for a clade of all taxa except *M. surinamensis* (bootstrap = 80; see numbers above the diagonal in Fig. 3A), and a more nested clade that includes all other taxa except the (*M. hemprichii* (*M. l. lemniscatus* (*M. l. carvalhoi*1 + *M. l. carvalhoi*2))) clade, and *M. decoratus*, is strongly supported (bootstrap = 99). This clade includes many of the species recognized by Jorge da Silva and Sites (1999) in their revision of the *M. frontalis* complex, although there is no strong evidence for monophyly of this group. Within this clade, *M. baliocoryphus* and *M. pyrrhocryptus* are strongly supported as sister taxa (bootstrap = 100), and in more nested positions, there is also strong support for ((*M. frontalis*1 + *M. frontalis*2) + (*M. brasiliensis*1 + *M. brasiliensis*2)) and (*M. ibiboboca* (*M. lemniscatus*1 + *M. lemniscatus*2)) clades (bootstraps = 99 and 99, respectively), while there is moderate support for monophyly of *M. altirostris* (bootstrap = 72; Fig. 3A). Note that relationships among these groups, and *M. spixii*, are not resolved.

Because the MP analysis of the allozyme characters recovered trees with only very weakly supported internal nodes (i.e., bootstrap values were uniformly <50, with the single exception of two of the *M. lemniscatus* terminals), we judged the allozyme partition not to be in serious conflict with the mtDNA partition (Wiens and Reeder, 1997), and we combined these partitions for another weighted MP analysis. This analysis also recovered four equally parsimonious solutions with the same topologies as those recovered from MP analysis of the mtDNA alone (i.e., the phylogram in Fig. 3A). In most cases, similar levels of bootstrap support were evident (see values below the internal branches in 3A, for the combined data), and there was no additional support for the unresolved polytomies.

ML Analysis

Using the values specified in the Methods section, the ML search recovered a single tree with a −lnL score of 3800.0899 (Fig. 3B). The topology of this tree is similar to that of the MP trees (Fig. 3A) in that it (1) recovers many of the same nested monophyletic groups with moderate (>60) to strong (>90) bootstrap support;

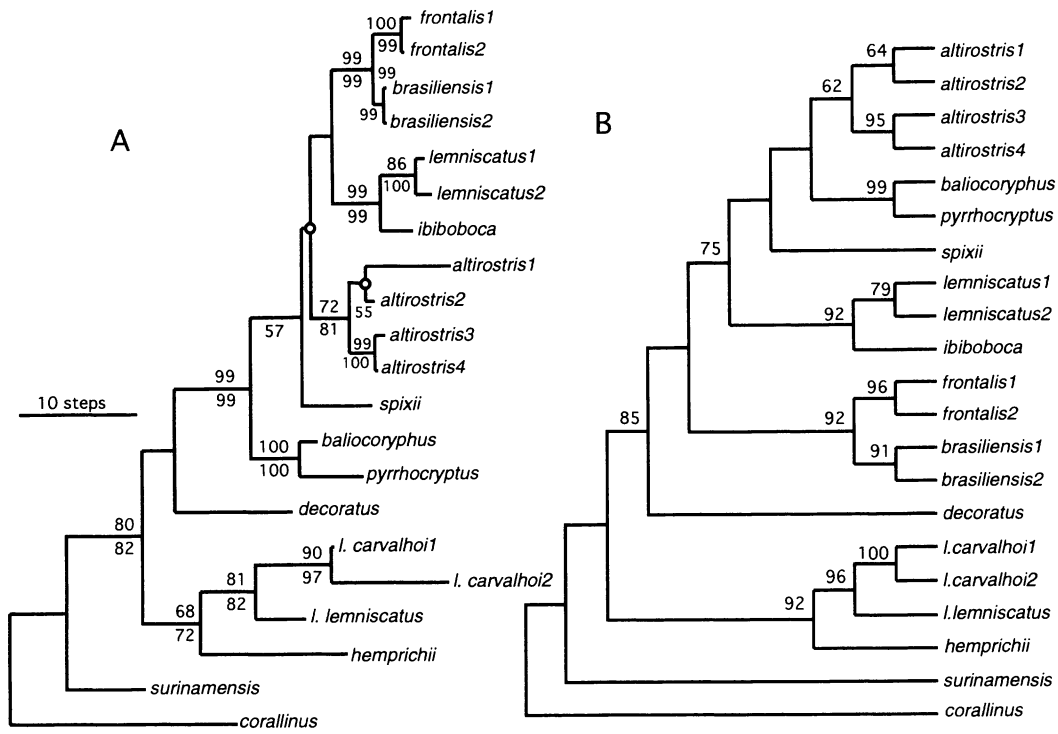

FIG. 3.—(A) Phylogram of one of four equally-parsimonious trees derived from an equally-weighted MP analysis of mtDNA sequences for all South American *Micrurus* terminals: TL = 550, CI = 0.547, RI = 0.618, and RC = 0.338; open circles identify nodes which are collapsed in the consensus tree. The numbers above the internal branches represent the bootstrap support values for this analysis. Topologies were identical for a weighted MP analysis of the mtDNA and allozyme data partitions combined, but in this case, TL = 59156, CI = 0.540, RI = 0.611, and RC = 0.330; the numbers below the internal branches are the bootstrap values for this support (shown only if >50 in both analyses). (B) Single best tree derived from a ML analysis based on the HKY85 + I + Γ model, with bootstrap values shown where these are >50 (−lLn score = 3800.0899).

(2) recovers with strong bootstrap support (85) the nested clade containing the species formerly recognized in the *M. frontalis* complex; and (3) weakly (bootstrap <50) recovers a monophyletic group exclusive of *M. surinamensis*, and with the (*M. hemprichii* (*M. "lemniscatus"*)) clade just internal to this basal terminal. The ML topology differs chiefly in recovering *M. decoratus* within (albeit in a basal position) the strongly supported clade that includes the *M. frontalis* complex, but neither the topology of the MP (Fig. 3A) nor the ML tree (Fig. 3B) are significantly more parsimonious relative to the other [by the non-parametric Templeton (1983) test; $P = 0.6698$]. We propose the strict consensus tree based on the parsimony analysis of the combined data as our working hypothesis for relationships of the taxa examined in this study (Fig. 3A; collapsed nodes identified by open circles).

DISCUSSION

Discrepancies between Data Partitions

Tree topologies differed between the allozymes and mtDNA (Figs. 2, 3), and although there was only very weak support for most internal nodes in the allozyme tree, the discrepancies between these two data partitions deserve some mention. We offer these possibilities. First, the number of parsimony informative characters (Appendix I) is small relative to the number of ingroup terminals. In a perfect data set with no homoplasy and a sequence of derivation of synapomorphic electromorphs

that are perfectly correlated each cladogenic event in the history of these taxa, there would be little more than a single character transition (n = 18 characters) for each internal node (n = 12) with diallelic loci, and a few more with multistate characters. Strong support for internal nodes with a data set as small as this one, however, would require many states per character, distributed throughout the hierarchy of the genealogy in such a manner as to provide multiple synapomorphies for each internal node, and this outcome does not appear possible with the allozyme data set alone.

Second, despite our use of multiple gel and electrophoresis buffers to detect "hidden heterogeneity" (Coyne, 1982) in the enzyme phenotypes (electromorphs), many studies have shown that often extensive effort is required to identify all alleles segregating at an enzyme locus (Aquadro and Avise, 1982; Ramshaw et al., 1979). We suspect that our allozyme data set likely contains a few to perhaps many homoplasious electromorphs, and this could in part be responsible for some of the differences between topologies based on the different data partitions. Finally, the issue of small sample sizes could also be responsible for inaccurate estimates of allele frequencies at polymorphic loci. Nine of our 13 ingroup terminals were represented by one or two individuals (Appendix I), and these small sample sizes have almost certainly introduced an unknown bias into the weighted MP analysis. For these reasons, we suggest that it will be difficult to sort phylogenetic signal from potential sources of error in this partition when it is analyzed alone, and we prefer to consider the consensus topology based on the combined data (Fig. 3A) as our working hypothesis.

Our favored phylogenetic hypothesis for the triad *Micrurus* examined in this study is a conservative choice, and while we recognize that combining data may not always lead to a better estimate of phylogeny (Bull et al., 1993), recent empirical studies show that, under a variety of conditions, combining data partitions frequently improves both character congruence and phylogenetic accuracy (Cunningham, 1997*a*,*b*; Hillis et al., 1994; Wiens, 1998*b*). In cases where significant conflict between data partitions is not improved by combination, phylogenetic accuracy may still be improved by increasing the size of the data base (Cunningham, 1997*b*; Givnish and Sytsma, 1997; Wiens, 1998*c*). Further, the concordance between the topologies derived from methods of tree reconstruction based on different optimality criteria (Fig. 3A versus 3B) is claimed by some also to provide robust support for the relationships held in common among topologies recovered by different methods (Kim, 1993), and while controversial, such a result does suggest that the stability of most of the internal nodes is not sensitive to different assumptions about character evolution (Flores-Villela et al., 2000).

Phylogenetic Relationships among South American Triads

The majority of analyses reveal several common patterns about triad relationships, and the most conspicuous include the following. First, *M. surinamensis* is basal to all of the other ingroup taxa, which are strongly supported as monophyletic to its exclusion on the basis of equally weighted MP analyses of mtDNA and weighted MP analysis of the combined data (bootstrap proportions = 80 or higher; Fig. 3A); this topology is also weakly supported by the ML analysis (Fig. 3B). *Micrurus surinamensis* is extremely differentiated from all other triads examined to date, and it is characterized by several unambiguous autopomorphic morphological character states (see Slowinski, 1995, for details), as well as autapomorphic electromorphs at five enzyme loci (nos. 2, 9, 15, 16, and 20; Appendix I). Its distinctness from other coral snakes was suggested earlier by Cadle and Sarich (1981) on the basis of immunological data, and also by Campbell and Lamar (1989). The unique morphological character states appear to be linked to its adaptation to a fully aquatic environment, and its venom characteristics appear to be highly directed to prey-specificity and a diet of knifefishes (Jorge da Silva and Aird, unpublished data). Early

studies (Bolaños et al., 1978) demonstrated the uniqueness of the venom of *M. surinamensis*, and more recent investigations have shown that this species' venom is characterized by an absence of most of the "large" enzymes (i.e., those of high molecular weight) that comprise important components of venoms of other *Micrurus* (Aird and Jorge da Silva, 1991; Aird et al, 1992; Francis et al., 1997, 1998; Jorge da Silva et al., 1991, 1992).

Second, both MP and ML analyses suggest that the *M. frontalis* complex as previously arranged (Roze, 1983, 1994, 1996; Scrocchi, 1990; including the species *M. altirostris, M. baliocoryphus, M. brasiliensis, M. frontalis,* and *M. pyrrhocryptus*) is not monophyletic (Fig. 3). Some species of the *M. frontalis* complex are strongly supported as monophyletic, but our sampling is insufficient to test adequately the monophyly of the entire complex. For example, *Micrurus frontalis* and *M. brasiliensis* are well supported as sister taxa by the mtDNA and combined MP analyses, and the ML analysis, and this relationship is corroborated by morphological data showing similarities in ventral, subcaudal and triad numbers in both sexes (Jorge da Silva and Sites, 1999). An "*M. frontalis* group" was informally recognized within the *M. frontalis* complex on the basis of overall morphological similarity, and was suggested to include *M. frontalis, M. brasiliensis,* and *M. diana* (Jorge da Silva and Sites, 1999), but the unavailability of *M. diana* for this study precludes a rigorous test of the group's monophyly.

Micrurus altirostris sampled from different parts of its range were recovered as a monophyletic group (Fig. 3), and the distinctness of this species from the (*M. frontalis* + *M. brasiliensis*) clade was also suggested by the morphological results; in *M. altirostris*, ventral scale numbers are lower and the triad number higher in both sexes, relative to *M. brasiliensis* and *M. frontalis* (Jorge da Silva and Sites, 1999). Some of the other taxa that were included in the *M. frontalis* complex (*M. baliocoryphus* and *M. pyrrhocryptus*) are consistently strongly supported as sister taxa by both the MP and ML analyses (Fig. 3). Further, recovery of a (*M. altirostris* (*M. baliocoryphus* + *M. pyrrhocryptus*)) clade, albeit with weak support, in the ML analysis (Fig. 3B), corroborates another suggestion made by Jorge da Silva and Sites (1999). On the basis of overall morphological similarity, these three species along with *M. tricolor*, comprise the "*M. pyrrhocryptus* group" (distinct from the "*M. frontalis* group"). However, the unavailability of (*M. tricolor*), and the lack of resolution in the MP analysis (Fig. 3A), requires a very tentative acceptance of monophyly of this group.

Our preferred hypothesis makes the entire *M. frontalis* complex paraphyletic with respect to the placement of *M. spixii* (although support for the position of *M. spixii* is weak), and the (*M. ibiboboca* (*M. lemniscatus*1 + *M. lemniscatus*2)) clade. Some morphological similarities between *M. isozonus* and *M. spixii* were pointed out by Campbell and Lamar (1989), and these taxa seem to be closer to *M. frontalis* (relative to other species) within the South American triad group based on hemipenial and osteological characters (Jorge da Silva, unpublished data), but additional taxa and characters will need to be included in future studies to verify the placement of *M. spixii.* Our shortest trees are not significantly better than alternatives in which all species of the *M. frontalis* complex are constrained to monophyly, under either MP or ML optimality criteria (Table 6).

Third, perhaps the most striking result of this analysis is the nonmonophyly of populations presently assigned to *M. lemniscatus.* The relationship between *M. lemniscatus, M. ibiboboca,* and *M. frontalis* have been the focus of debates beginning with the works of Schmidt (1936) and Amaral (1944), and continuing to Campbell and Lamar (1989). A close relationship between *M. ibiboboca* and coastal or Atlantic Forest samples of *M. "lemniscatus"* is recovered with a bootstrap proportion of 99 for both mtDNA and combined data (Fig. 3A). Similarly, both data sets strongly support the placement of the other three *M. "lemniscatus"* from the Amazonian Forest or other more interior localities (*M. l. carvalhoi*1, *M. l. carvalhoi*2,

TABLE 6.—Test of alternative tree topologies derived from MP and ML searches. In all comparisons, the best tree, or one of the consensus trees, is tested against alternatives in which the *M. frontalis* complex[1] (as defined by Jorge da Silva and Sites, 1999), and all samples currently recognized under the name *M. lemniscatus*[2] are constrained to be monophyletic. The nonparametric test described by Templeton (1983) is used as implemented by Larson (1994) for trees obtained by MP searches, while the test described by Kishino and Hasegawa (1989) was used to test alternative ML trees

Test category	Estimates and probabilities			
Templeton test:	TL	*n*	*z*	*P*
All data:				
Best tree	59156			
Frontalis[1]	59556	14	−1.0690	0.2850
Lemniscatus[2]	62352	61	−3.3490	0.0008
DNA only:				
Best tree	548			
Frontalis[1]	553	20	−0.8944	0.3711
Lemniscatus[2]	581	57	−3.9392	<0.0002
KH89 test (ML trees):	−ln L	diff −ln L	*T*	*P*
Best tree	3800.0899			
Frontalis[1]	3806.7298	6.6399	1.4991	0.1342
Lemniscatus[2]	3842.0309	41.9410	3.9319	<0.0001

and *M. l. lemniscatus* in Fig. 3) together with *M. hemprichii* (also from the Amazonian Forest), and the position of this entire clade is strongly supported as basal to all other triad coral snakes except *M. surinamensis* and possibly *M. decoratus*. When most parsimonious trees for either mtDNA or the combined data are tested against a tree in which the *M. lemniscatus* sequences are constrained to monophyly, the constraint topology is significantly less parsimonious ($P = 0.0008$ or lower, Table 6). This same result is also obtained under ML; the "*M. lemniscatus* monophyly" topology has a significantly lower likelihood score relative to the best ML tree ($P < 0.0001$, Table 6).

Micrurus ibiboboca is restricted to the semi-arid Caatinga region of Brazil with *M. "lemniscatus"* present in the adjacent Cerrado, and in both the Amazon and Atlantic forests. If the hypothesis presented in Fig. 3 is correct, then the *M. "lemniscatus"* from eastern coastal Brazil (*M. lemniscatus*1 and *M. lemniscatus*2) is a different species than the populations present in Amazonia (*M. l. lemniscatus*) or the drier Cerrado region (*M. l. carvalhoi*1 and *M. l. carvalhoi*2). The phylogenetic relationships suggested here between *M. ibiboboca* and eastern populations of *M. "lemniscatus"* are also supported by venom characteristics unique to these taxa (Jorge da Silva and Aird, unpublished data). The phylogram in Fig. 3A provides some perspective on the amount of character divergence (i.e., branch lengths) between the coastal Brazil versus the Amazonian plus central Brazil localities of *M. "lemniscatus"*. However, these branch lengths are conservative because some autapomorphic characters (allozyme loci 19 and 23, Appendix I) were not included in the phylogenetic analyses. The allozyme characters summarized in Appendix I reveal six markers (nos. 9, 11, 12, 16, 18, and 19) fixed for electromorphs diagnostic of the coastal samples (*M. lemniscatus*1 and *M. lemniscatus*2) to the exclusion of all other terminals under the name *M. lemniscatus*. Among the latter, another allozyme marker (no. 23 in Appendix I) separates the Amazonian sample (*M. l. lemniscatus*) from all of those in the central Cerrado region (all *M. l. carvalhoi*; see Table 1). By any of several operational species concepts (reviewed by Sites and Crandall, 1997), this is sufficient for unambiguous species recognition of the coastal populations, and possibly separating Amazonian from Cerrado populations at the species level if additional sampling confirms the pattern of

"fixed" diagnostic differences. *Micrurus hemprichii* is also an Amazonian species, and the relatively low levels of mtDNA sequence divergence among the terminal units in the (*M. hemprichii* (*M. l. lemniscatus* (*M. l. carvalhoi*1, *M. l. carvalhoi*2))) clade, coupled with paraphyly of the central Brazilian *M. "lemniscatus"* relative to the genetically distinct Amazonian specimen, suggests that this group needs extensive revisionary work.

Resumo

Este estudo apresenta novos dados moleculares (seqüências do mtDNA e alozimas) para várias espécies das serpentes corais de tríadas da América do Sul. Apresentamos uma hipótese filogenética para o grupo com base na análise de caracteres moleculares sob os critérios de otimização de máxima parsimônia (MP) e máxima verosimilhança (ML). Os resultados são concordantes com ambos os métodos, e sugerem uma posição basal para a espécie *Micrurus surinamensis,* a qual é morfologica e ecologicamente distinta. O grupo "Frontalis" apresenta-se como parafilético com respeito a várias outras espécies nas árvores de parsimônia e verosimilhança, mas a topologia restringindo esse grupo como monofilético, não pôde ser estatisticamente rejeitada quando comparada às melhores árvores de MP ou ML. Todas as análises revelam um forte suporte para considerar *M. "lemniscatus"* como polifilético, e nesse caso, a topologia alternativa de monofiletismo para o grupo é fortemente rejeitada sob ambos os critérios de otimização. Esses resultados enfatizam a necessidade de uma análise mais detalhada das populações atualmente designadas como *M. lemniscatus.*

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APPENDIX I.—Allozyme data matrix for all *Micrurus* used in this study (*M. corallinus* = outgroup). The uppercase letters denote the genotypes for each individual while missing data are identified by dashes. The parsimony informative loci (abbreviations are from Table 1) are coded as follows: 1 = Pep(Pap), 2 = βGa, 3 = Ak-A, 4 = Ck-A, 5 = Gpi-A, 6 = Iddh-A, 7 = Ldh-A, 8 = Pgk-A, 9 = M-Sod-A, 10 = Tpi-A, 11 = "Tpi-2", 12 = M-Mdh-A, 13 = Pep(Lgg), 14 = Pep(LII), 15 = M-Acoh-A, 16 = S-Acoh-A, 17 = Pnp-A, 18 = Pep(La), 19 = Mpi-A, 20 = M-Aat-A, 21 = S-Aat-A, 22 = M-Icdh-A, and 23 = Pgdh-A. Loci 15–18 are weighted by Manhattan distances (Appendix II), and the last five loci (underlined) are polymorphic among species but parsimony uninformative.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
OUTGROUP																							
M. corallinus-1*	CC	DD	AA	AA	CC	CC	AA	BB	BB	EE	AA	AA	AA	AA	EE	EE	AA	AA	AA	AA	AA	AA	AA
M corallinus-2	–	DD	AA	AA	CC	CC	AA	BB	BB	EE	AA	AA	AA	AA	EE	EE	AA	AA	AA	AA	AA	AA	AA
INGROUP TAXA																							
M. frontalis-1*	AA	AA	AA	AA	AA	–	AA	AA	–	AA	AA	AA	AA	AA	AA	AA	BB	AB	AA	AA	AA	AA	AA
M. frontalis-2*	AA	AA	AA	AA	AA	–	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AB	BB	AA	AA	AA	AA	AA
M. frontalis-3	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	BB	AA	AA	AA	AA	AA
M. frontalis-4	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AB	AA	AA	AA	AB	AA	AA	AA	AA	–
M. frontalis-5	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AB	AA	AA	AA	BB	AA	AA	AA	AA	AA
M. frontalis-6	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	–	AA	AA	AA	AB	AA	AA	AA	AA	AA
M. frontalis-7	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AB	AA	AA	AA	AB	AA	AA	AA	AA	AA
M. frontalis-8	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	BB	AA	AA	AA	AC	AA	AA	AA	AA	AA
M. frontalis-9	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	BB	AA	AA	AB	AB	AA	AA	AA	AA	AA
M. frontalis-10	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	BB	AA	AA	AA	AA	AA	AA	AA	AA	AA
M. altirostris-1*	AA	AA	AA	AA	AA	AA	AA	–	AA	AA	AA	AA	AA	BB	–	AA	–	–	–	AA	AA	AA	AA
M. altirostris-2*	AA	AA	AA	AA	AA	–	AA	AA	AA	AA	AA	AA	AA	AB	AA	AA	AB	AB	AA	AA	AA	AA	AA
M. altirostris-3*	AA	AA	–	AA	AA	AA	AA	–	AA	AA	AA	AA	AA	AB	AA	AA	AA	AB	AA	AA	AA	AA	AA
M. altirostris-4*	AA	AA	AA	AA	AA	–	AA	AA	AA	AA	AA	AA	AA	AB	AA	AA	AB	AB	AA	AA	AA	AA	AA
M. altirostris-5	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AB	AA	AA	AA	AA	AA
M. altirostris-6	AA	AA	AA	AA	AA	–	AA	AA	–	AA	AA	AA	AA	AB	AA	AA	AB	AB	AA	AA	AA	AA	–
M. altirostris-7	AA	–	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	CC	AA	AA	AA	AA	BB	–	AA	AA	AA	AA
M. altirostris-8	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AC	AB	AA	AA	AB	–	AA	AA	AA	AA	AA
M. altirostris-9	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	CC	AA	AA	AA	AA	BB	AA	AA	AA	AA	AA
M. altirostris-10	–	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	BB	AA	AA	AA	BB	AA	AA	AA	AA	AA
M. altirostris-11	–	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	BB	AA	AA	AA	BB	AA	AA	AA	AA	–
M. brasiliensis-1*	AA	AA	AA	CC	AA	AA	AA	AA	AA	–	AA	AA	AA	AA	AA	AA	AA	AB	AA	AA	AA	AA	AA
M. brasiliensis-2*	AA	AA	AA	CC	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	–	AA	AA	AA	AA	AA
*M. baliocoryphus**	AA	AA	AA	AA	BB	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	CC	AC	AA	AA	AA	AA	AA

APPENDIX I.—Continued.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
M. pyrrhocryptus-1*	–	BB	AA	AA	BB	AA	AA	AA	AA	AA	AA	AA	AC	BC	AA	AA	AB	AA	–	AA	AA	AA	AA
M. pyrrhocryptus-2	AA	BB	AA	AA	BB	AA	AA	AA	AA	AA	AA	AA	AA	BB	AA	AA	AB	BB	AA	AA	AA	AA	AA
M. pyrrhocryptus-3	–	BB	AA	AA	BB	AA	AA	AA	AA	AA	AA	AA	CC	BB	AA	AA	AA	AB	AA	AA	AA	AA	AA
M. pyrrhocryptus-4	AA	BB	AA	AA	BB	AA	AA	AA	AA	AA	AA	AA	AA	BB	AA	AA	AB	AB	AA	AA	AA	AA	AA
M. pyrrhocryptus-5	AA	BB	AA	AA	BB	AA	AA	–	AA	AA	AA	AA	AA	BB	–	AA	AA	AB	AA	AA	AA	AA	AA
*M. l. lemniscatus**	AA	–	BB	CC	BB	AA	BB	–	BB	CC	BB	AA	AA	BB	AA	BB	AA	AD	AA	AA	AA	AA	BB
M. l. carvalhoi-1*	–	–	BB	CC	BB	AA	BB	AA	BB	CC	BB	AA	AA	BB	AA	BB	AA	DD	AA	AA	AA	AA	AA
M. l. carvalhoi-2*	AA	–	BB	CC	BB	AA	BB	–	BB	CC	BB	AA	AA	BB	AA	BB	AA	DD	AA	AA	AA	AA	AA
M. l. carvalhoi-3	AA	–	BB	CC	–	AA	BB	–	BB	CC	BB	AA	AA	BB	AA	BB	AA	–	AA	AA	AA	AA	AA
M. l. carvalhoi-4	AA	–	BB	CC	BB	AA	BB	AA	BB	CC	BB	AA	AA	BB	AA	BB	AA	–	AA	AA	AA	AA	AA
M. lemniscatus-1*	AA	AA	AA	CC	AA	AA	AA	–	AA	CC	AA	BB	AA	BB	AA	AA	AA	BB	BB	AA	AA	AA	AA
M. lemniscatus-2*	AA	AA	AA	–	AA	AA	AA	–	AA	CC	AA	BB	AA	BB	AA	AA	AA	BB	BB	AA	AA	AA	AA
M. ibiboboca-1*	AA	AA	BB	CC	AA	AA	AA	AA	AA	AA	AA	AA	AA	–	AA	AA	AC	CC	AA	AA	AA	AA	AA
M. ibiboboca-2*	AA	–	BB	CC	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AC	CC	AA	AA	AA	AA	AA
M. decoratus-1*	–	BB	AA	BB	BB	–	AA	AA	CC	DD	AA	AA	–	BB	CC	AA	DD	BE	CC	AA	AA	BB	AA
M. decoratus-2	AA	BB	AA	BB	BB	–	AA	AA	CC	–	AA	AA	BB	BB	CC	AA	DD	EE	–	AA	AA	BB	AA
*M. h. hemprichii**	–	AA	AA	–	BB	BB	AA	–	BB	BB	AA	BB	AA	AA	BB	BB	AA	AA	AA	AA	BB	AA	AA
M. spixii obscurus-1*	BB	BB	AA	CC	AA	AA	AA	BB	CC	AA	AA	AA	–	BB	AA	CC	AA	FF	AA	AA	AA	AA	AA
M. spixii obscurus-2*	BB	BB	AA	CC	AA	AA	AA	BB	CC	AA	AA	AA	BB	BC	AA	CC	AA	FF	AA	AA	AA	AA	AA
M. s. surinamensis-1*	AA	EE	AA	CC	BB	AA	AA	BB	DD	AA	AA	AA	AA	BB	DD	DD	AA	AA	AA	BB	AA	AA	AA
M. s. surinamensis-2*	–	–	AA	CC	BB	AA	AA	BB	DD	AA	AA	AA	AA	BB	DD	DD	AA	AA	AA	BB	AA	AA	AA

APPENDIX II.—Manhattan distance step matrix for the loci Pep(Lgg) and Pep(LII), above and below the diagonal, respectively (panel A), and for Pnp-A and Pep(La), above and below the diagonal, respectively, in panel B. Taxa: 1 = *M. corallinus*, 2 = *M. frontalis*, 3 = *M. altirostris*, 4 = *M. brasiliensis*, 5 = *M. baliocoryphus*, 6 = *M. pyrrhocryptus*, 7 = *M. l. lemniscatus*, 8 = *M. l. carvalhoi*, 9 = *M. lemniscatus* ssp., 10 = *M. ibiboboca*, 11 = *M. decoratus*, 12 = *M. h hemprichii*, 13 = *M. spixii obscurus*, and 14 = *M. s. surinamensis*.

A

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	—	0	23	0	0	30	0	0	0	0	100	0	100	0
2	50	—	23	0	0	30	0	0	0	0	100	0	100	0
3	50	50	—	23	23	7	23	23	23	23	100	23	100	23
4	0	50	0	—	0	30	0	0	0	0	100	0	100	0
5	0	50	50	0	—	30	0	0	0	0	100	0	100	0
6	100	50	50	100	100	—	30	30	30	30	100	30	100	30
7	100	50	50	100	100	50	—	0	0	0	100	0	100	0
8	100	50	50	100	100	50	0	—	0	0	100	0	100	0
9	100	50	50	100	100	50	0	0	—	0	100	0	100	0
10	0	50	50	0	0	100	100	100	100	—	100	0	100	0
11	100	50	50	100	100	50	0	0	0	100	—	100	0	100
12	0	50	50	0	0	100	100	100	100	0	100	—	100	0
13	100	50	50	100	100	15	25	25	25	100	25	100	—	100
14	100	50	50	100	100	50	0	0	0	100	0	100	25	—

B

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	—	20	25	0	100	30	0	0	0	50	100	0	0	0
2	60	—	5	20	100	10	20	20	20	50	100	20	20	20
3	67	12	—	25	100	5	25	25	25	50	100	25	25	25
4	0	10	17	—	100	30	0	0	0	50	100	0	0	0
5	50	55	67	50	—	100	100	100	100	50	100	100	100	100
6	50	10	17	0	50	—	30	30	30	50	100	30	30	30
7	50	60	67	50	50	50	—	0	0	50	100	0	0	0
8	100	100	100	100	100	100	50	—	0	50	100	0	0	0
9	100	45	33	50	100	50	100	100	—	50	100	0	0	0
10	100	95	100	100	50	100	100	100	100	—	100	50	50	50
11	100	75	75	75	100	75	100	100	75	100	—	100	100	100
12	0	60	67	50	50	50	50	100	100	100	100	—	0	0
13	100	100	100	100	100	100	100	100	100	100	100	100	—	0
14	0	60	67	50	50	50	50	0	100	100	100	100	100	—