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Palm-Pitviper (*Bothriechis*) Phylogeny, mtDNA, and Consilience

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The phylogeny of the neotropical palm-pitviper genus *Bothriechis* has been previously inferred from morphology and allozymes. These nuclear-based data sets were found to be congruent and also consilient with the geologic history of the region. We present mtDNA sequence data as an additional data set in the inference of *Bothriechis* phylogeny and analyze it separately and combined with previous data. The mtDNA phylogeny is incongruent with the nuclear data sets. Based on a number of factors, we hypothesize that the incongruence is due to both mtDNA introgression and lineage sorting. We argue that mtDNA represents extrinsic data and as such should be used as a consilient data set.

INTRODUCTION

The genus *Bothriechis* is a clade of seven (possibly eight; Solórzano *et al.*, 1998) species of arboreal venomous snakes that occur from southern Mexico through Central America and into northwestern South America (Fig. 1). The interspecific relationships of *Bothriechis* were first discussed in a defensible phylogenetic context by Werman (1992). In a cladistic analysis of morphology and allozymes, Werman recovered a monophyletic *Bothriechis* (but he included only three taxa) with *Ophryachus* as the sister lineage. Crother *et al.* (1992) inferred the phylogeny of *Bothriechis* with allozymes and morphology and found the two data sets to be congruent (Fig. 2). They (Crother *et al.*, 1992) also detailed the historical biogeography of *Bothriechis* and the models of the vicariant history of Middle America and determined these to be entirely consilient with their phylogenetic hypotheses of *Bothriechis* relationships. Werman (1997) found that lactate dehydrogenase phenotypes corroborated his hypothesis of a monophyletic *Bothriechis*. Gutberlet (1998) employed morphological data in a phylogenetic study of Middle American pitvipers and his phylogeny corroborated Werman's (1992, 1997) hypothesis, with the placement of *Ophryachus* as sister to a monophyletic *Bothriechis*. Most recently, Parkinson (1999) used 12S and 16S mtDNA in a broad-scale (45 taxa) study of pitviper relationships. Contrary to the previous work, the mtDNA data did infer neither a monophyletic *Bothriechis* nor a sister relationship between *Ophryacus* and *Bothriechis*. However, this study included only three species (*schlegelii, nigroviridis*, and *lateralis*) of *Bothriechis*.

Consilient tests (*sensu* Frost and Kluge, 1994; Siddall and Kluge, 1997) provide a robust means in which to evaluate support for phylogenetic hypotheses. The test consists of discovering a phylogeny based solely on

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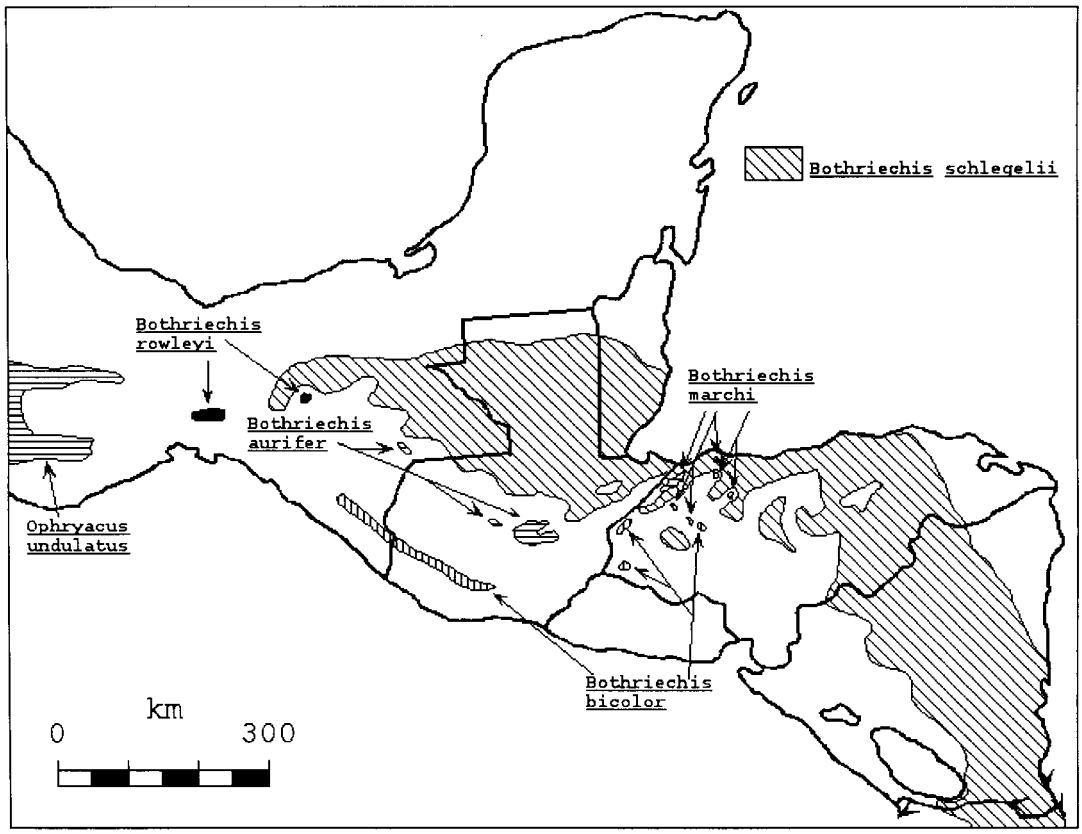

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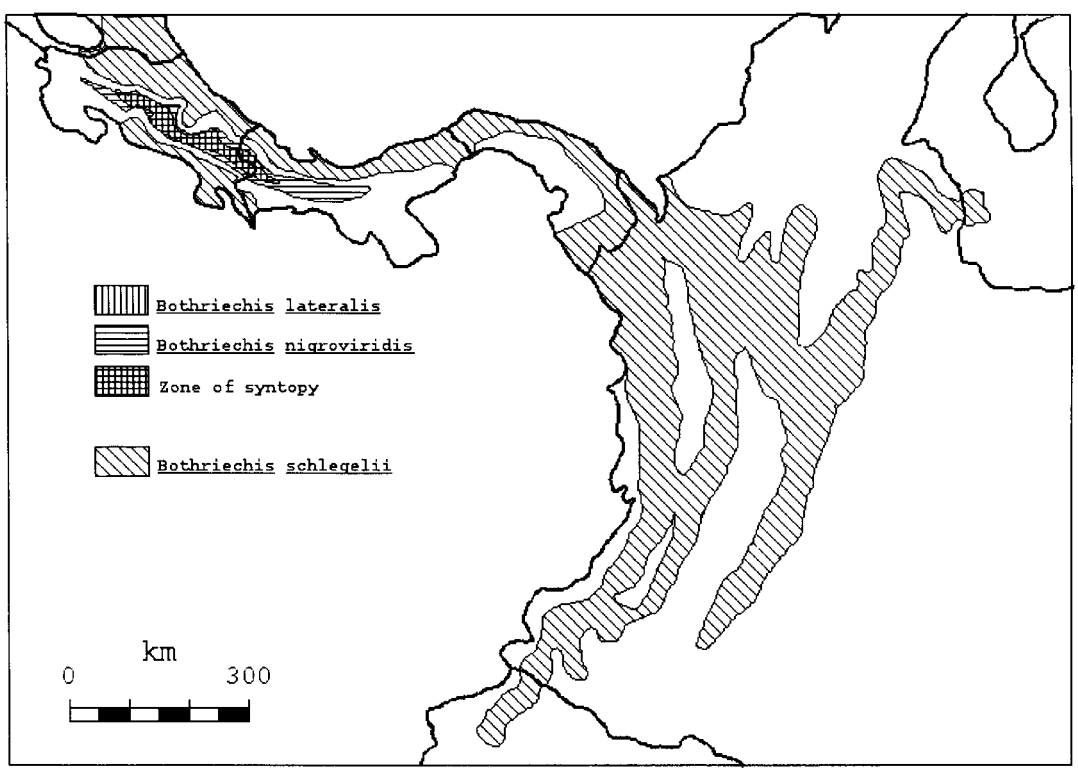

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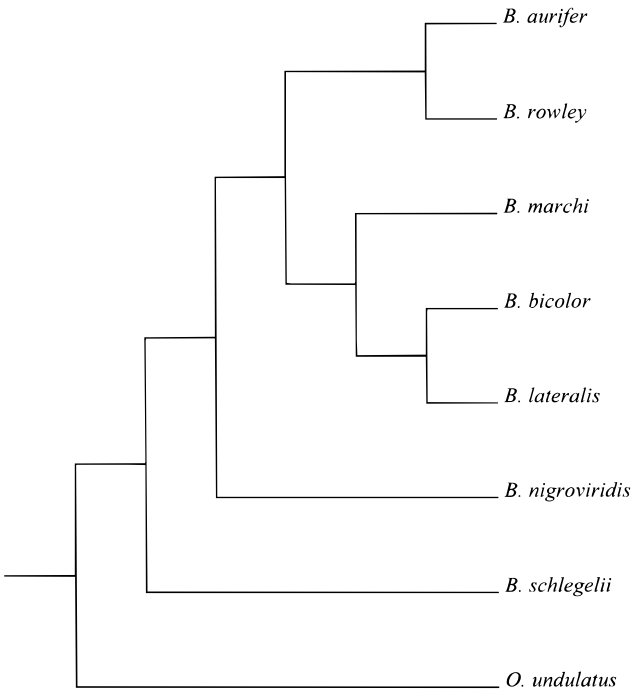

FIG. 2. The morphology- and allozyme-based phylogeny inferred by Crother *et al.* (1992).

data extrinsic to the organismal clade being tested. Examples of utilizing extrinsic data include the comparison of the phylogenies of symbiotic taxa (coevolution) or of taxa that occupy similar areas (vicariance biogeography). While consilient tests can provide strong support for a phylogenetic hypothesis, only intrinsic tests can reject one. The mitochondrial genome (mtDNA) originated as an endosymbiont and is independently replicated and inherited from the coevolving intrinsic nuclear genome, the systematic unit of interest (Frost and Kluge, 1994). All independent characters can have their own origin, function, and fate; however, if intrinsic, they also share the same history. Extrinsic data do not *necessarily* share the same history. Sharing the same history is the implicit defining fact of intrinsic data and clearly separates it from extrinsic information. The extrinsic nature of mtDNA, coupled with the relative ease in which phylogenetically informative nucleotide sequences can be obtained, makes mtDNA an ideal choice in which to frame a consilient test (T. W. Taggart *et al.*, submitted for publication). The purpose of this study was to infer a mtDNA phylogeny of *Bothriechis* and to employ it in a consilient fashion to test the previous nuclear-based phylogeny.

METHODS

Whole genomic DNA, including the mtDNA, was extracted from muscle and liver using a standard phenol–chloroform protocol. One specimen each of *Bothriechis bicolor* (Bb), *B. marchi* (Bm), *B. rowleyi* (Br), and *B. aurifer* (Ba); two specimens each of *B. lateralis* (Bl) and *Ophryacus undulatus* (Ou); and three specimens of *B. schlegelii* (Bs) were used. Sources of tissues and/or DNAs are given in the Appendix. The 12S sequence for *B. nigroviridis* (Bn) was from Parkinson (1999) and was included in the alignment and subsequent analysis.

Primer pairs 12SL 5′-aaactgggattagataccccactat-3′ and 12SH 5′-ctacacttaccttgttacgactt-3′ were selected to amplify a region of the 12S rDNA from the mtDNA genome. The PCR amplification conditions began with

TABLE 1
Morphology and Allozyme Data Set from Crother *et al.* (1992), Used in the Combined Analysis

Ophryacus	ABADACABBCBCAAEBAB00000000000000000000000000000000
Bothriechis bicolor	AABABABCAAAAAAAAAA01101111111112111110111111111
B. lateralis	A?BABBBBAAABBCAA?A01001111001112111111001111111
B. marchi	AACAB?ABAAACBBDA?A01001111111112111110101111111
B. nigroviridis	BBDBBBABBBAE?ACAAC00001111110000000000001111111
B. rowleyi	BA?BBDACABAD?AAABA10001111011111002000101111111
B. schlegelii	BBACBCAACAACCBABCC01110000000000000000001111111
B. aurifer	BACAAAACAAAFABCABB10001111001111002000001111111

FIG. 1. Distribution maps of the taxa addressed in this study. (A) East of the Isthmus of Tehuantepec to northern Costa Rica. (B) Southern Nicaragua to northwestern South America.

TABLE 2
Alligned Sequence Data for 382 bp of 12S Ribosomal mtDNA for All 12 OTUs

Bs1	GG-CGGTGTG	T-ACGCACTT	CATTGCGTTG	TGTTCAGTTA	GGTG-TTTTA	TCCCT-ATCT
Ou1	GGGCGGTGTG	T-ACGCACTT	CATTGCGTTG	TGTTCAGTTA	GGTG-TTTTA	TTCCT-ATCT
Bs2	-GGC-GTTTT	T-AC-CAC-T	CATT-CGTTT	TGTTCAGTTA	-GTG-BKKTA	TCCCT-ATCT
Bm	GGGCGGTGTG	T-ACACACTT	CATCGCGTTA	TGTTCAGTTA	GGT-ATTTTA	TTCCT-ATCT
Bs3	GGGCGGTGTG	T-ACGCACTT	CATTGCGTTG	TGTTCAGTTA	GGTG-TTTTA	TCCCT-ATCT
Bb	GGNCGGTGTG	T-ACACACTT	CATTGCGTTA	TGTTCAGTTA	GAT-ATWWTA	TACCTAATCT
Bl1	GGGCGGTGTG	T-ACACACTT	CATTGCGTTG	TGTTCAGTCA	GGT-ATTCTA	TTCCT-GTCT
Bl2	GGKCGGTGTG	T-ACACACTT	CATTGCGTTG	TGTTCAGTCA	GGT-ATTCTA	TTCCT-GTCT
Br	GGNCGGTGTG	T-ACACACTT	CATTGCGTTA	TGANCAATTA	GAT-AAWAWA	TTCCT-ATCT
Ou2	GGGCGGTGTG	T-ACGCACTT	CATTGCGTTG	TGTTCAGTTA	GGT-GTGTTA	TTCCT-ATCT
Bn	GGGCGGCGTG	TNACGCACTT	CATTGCGTTG	TGTTCAGTTG	GGT-ATTCTA	TTCCT-GTCT
Ba	GGGCGGTGTG	T-ACACACTT	CATTGCGTTA	TGTTCAGTTA	GGT-ATTTTA	TTCCT-ATCT
Bs1	TACTGCTAAG	TCCGCCTTTA	AGGAGTAA-T	TTCATAGTGC	TATTCGTATA	CCCGGTT-G-
Ou1	TACTGCTAAA	TCCGCCTTTA	AAGACTAA-T	TTCGTAGTGT	TGTCCGTATG	CTCGGTTGGA
Bs2	TACTGCCAAA	TCCCCCTTT-	AAGAGBAA-T	GTCATAGTGC	TAGTCGTATA	CCCGGTTGG-
Bm	TACTGCTAAA	TCCGCCTTTA	AATACTAA-G	TTCATAGTGT	TGTCCGTATG	CTCGGTT-GA
Bs3	TACTGCTAAA	TCCGCCTTTA	AGGAGTAA-T	TTCATAGTGC	TATTCGTATA	CCCGGTT-G-
Bb	TACTGCWAAA	TCCGCCWTTA	AAGACTAA-G	TTCGTAGTGT	TGTCCGTGTG	CTCGGTT-GA
Bl1	TACTGCTAAA	TCCGCCTTTG	AAGACCGC-T	TTCATGGTGT	TTTCCGTATG	CTCGGTT-GA
Bl2	TACTGCTAAA	TCCGCCTTTG	AAGACCAC-T	TTCATGGTGT	TTTCCGTATG	CTCGGTT-GA
Br	TACTGCTAAA	TCCGCCAWAA	AARACWAA-G	ATCATAGTGT	TGTCCGTATG	CTCGGTT---
Ou2	TACTGCTAAA	TCCGCCTTTA	AAGACTAA-T	TTCGTAGTGT	TGTCCGTATG	CTCGGTTGGA
Bn	TACTGCTAAA	TCCGCCTTTG	AAGACTNAST	TTCGTAGTGT	TGTCCGTATG	CTCSGTTNNN
Ba	TACTGCTAAA	TCCGCCTTTA	AAGACTAA-G	TTCGTAGTGT	TGTCCGTGTG	CTCGGTT-GA
Bs1	GG--AATGTA	GCCCATCTTA	GTCCTCTTCA	TGAGTTACAC	CTCGACCTGT	CGTTTTAGTG
Ou1	G---AATGTA	GCCCATCTTG	GTCCTCTTCA	TGAGTTACAC	CTCGACCTGT	CGTTTTAGTG
Bs2	---GAATGTA	GCCCATCTTA	GTCCTCCTCA	TGAGTTACAC	CTCGACCTG-	CGTTTTAGT-
Bm	G---AATGTA	GCCCATCTTG	GACCTCTTCA	TAAGTTACAC	CTCGACCTGT	CG---TATTA
Bs3	G--GAATGTA	GCCCATCTTA	GTCCTCTTCA	TGAGTTACAC	CTCGACCTGT	CGTTTTAGTG
Bb	---GAATGTA	GCCCATCTTG	GGCCCCWTCA	TAAGTTACAC	CTCGACCTGT	CGTGTTAGTG
Bl1	---GAATGTA	GCCCATCTTA	G-CCCCCCCA	TAAGTTACAC	CTCGACCTGT	CGTGTTAGTG
Bl2	---GAATGTA	GCCCATCTCA	GCCCCCCCCA	TAAGTTACAC	CTCGACCTGT	CGTGTTAGTG
Br	-RAAAATGTA	GCCCATCTTG	GGCCCCATCA	TAAGTTACAC	CTCGACCTGT	CGTGTTAGTG
Ou2	G---AATGTA	GCCCATCTTG	GTCCTCTTCA	TGAGTTACAC	CTCGACCTGT	CGTTTTAGTG
Bn	GAG-AATRTA	GCCCATCTTA	NTCCCCCTCA	TTAGTTACAC	CTCGACCTGT	CGTATTAGTG
Ba	GGA--ATGTA	-CCCATCTTG	GGCCCCTTCA	TAA-TTACA-	CTCGA-CTGT	CGTGTTAGTG
Bs1	--CAGTGCTA	TTTAGCTTAC	TTTATTTCTT	TTACAAGGTA	AGCTGGCGAC	GGCGGTAT-A
Ou1	--TGKTACTW	TTTAGCTCAC	TTTATCTCTT	TCACAAGGTA	AGCTGGCGAC	GGCGGTAT-A
Bs2	--CAGTGCTA	TTTAGCTTAC	TTTATTTCTT	TTACAAGGTA	AGCT-GCGAC	-GCGGTAT-A
Bm	G-TGGTGCTA	TTTGGCCTAC	TATTTTTCTT	TTACAAGGTA	GGCTGGCGAC	GGCGGTAT-A
Bs3	--CAGTGCTA	TTTAGCTTAC	TTTATTTCTT	TTACAAGGTA	AGCTGGCGAC	GGCGGTAT-A
Bb	----GTGCTG	TTTGGCCTAC	TWTTTTTCTT	TTACAAGGTA	GGCTGGCGAC	GGCGGTATAA
Bl1	---A-TGCTA	TTTGGCTTAC	TTTTTTTCTT	TTACAAGGTA	AGCTGGCGAC	GGCGGTAT-A
Bl2	---A-TGCTA	TTTGGCTTAC	TTTTTTTCTT	TTACAAGGTA	AGCTGGCGAC	GGCGGTAT-A
Br	----GTGCWA	TTTGGCCTAC	WAWTTTTCTC	TTACAAGGTA	GGCTGGCGAC	GGCGGTAT-A
Ou2	--TGGTACTA	TTTAGCTCAC	TTTATCTCTT	TCACAAGGTA	AGCTGGCGAC	GGCGGTAT-A
Bn	GNNNNTGCTA	TTTAGCTTAC	TTTTTTTCTT	TTACAAGGTA	AGCTGGCGAC	GGCGGTAT-A
Ba	----GTGCTG	TTTGGCT-AT	TTTATTTCTT	TTAC-AGGTA	GGC-GGCGA-	-GCGGTAT-A
Bs1	T-AGACTGTT	GGGCAAGAAG	GGGCTGGGTT	AATCGTGGAT	CGTCGGTTAT	T-GGACAGGC
Ou1	T-AGACTGTT	GGGCAAGAAG	GGGTTGGATT	AATCGTGGAT	TGTCGGTTAT	T-AGACAGGC
Bs2	-TAGACTGTT	GGGCAAGAAG	GGGCTGGGTT	AATCGTGGAT	CGTCGGTTAT	T-GGACAGGC
Bm	-TAGACTGTT	AGGCAAGAAG	AGGTTAGGTT	AATAGTGGGT	TGTCGGTTAT	T-AGACAGGC
Bs3	T-AGACTGTT	GGGCAAGAAG	GGGCTGGGTT	AATCSTGGAT	CGTCGGTTAT	T-GGACAGGC
Bb	TAAGACTGTT	AGGCAAGAAG	GGGTGGGGTT	AATCGTGGGT	TGTCGGTTAT	TAAGACAGGC
Bl1	T-AGACTGTT	GGGCAAGAGG	GGGTTGGGTT	AATCGTGGAT	TGTCGGTTGT	T-AGACAGGC
Bl2	T-AGACTGTT	GGGCAAGAGG	GGGTTGGGTT	AATCGTGGAT	TGTCGGTTGT	T-AGACAGGC
Br	T-AGACTGTC	AGGCAAGAAG	GGGTTGGGTT	AATCGTGGGT	TGTCGGTTGT	T-AGACAGGC

TABLE 2—*Continued*

Ou2	-TAGACTGTT	GGGCAAGAAG	GGGTTGGATT	AATCGTGGAT	TGTCGGTTAT	T-AGACAGGC
Bn	T-AGACTGTT	AKGCAAGAGG	GGGTTGGGTT	GATCRTGGAT	TATCTGTTGT	T-AGACAGGC
Ba	T--G-CTGTT	AG-C-AGA--	GGGT-GGGTT	--TCGTGGGT	TGTCGGTTAT	TAGGACAGGC
Bs1	TCCTCTAGAG	CG-TGGTGAA	GTACCGTCAA	GTCTTTTAAG	TTTTAAGTT-	TGACTCGTAG
Ou1	TCCTTTAGGN	CGNNN-TGAA	GTACCNTCAA	GTCTTTTAAG	TTTTAAGTG-	TGACTCGTAG
Bs2	TCCTCTA-GA	CG-TGGTGAA	GTACCGTCAA	GTCTTTTAAG	TTTTAAGT-T	TGACTCGTAG
Bm	TCCTCTAGGG	CG-TGGTGAA	GTACCGTCAA	GTCTTTTAAG	TTTTAAGTG-	GGACTCGTAG
Bs3	TCCTCTAGAG	CG-TGGTGAA	GTACCGTCAA	GTCTTTTAAG	TTTTAAGTT-	TGACTCGTAG
Bb	TCCTCTAGGG	CG-TGGTGAA	GTACCGTCAA	GTCTTTTAAG	TTTTAAGTG-	GGACTCGTAG
Bl1	TCCTCTAGGG	CG-TGGTGAA	GTACCGTCAA	GTCTTTTAAG	TTTTAAGTG-	GGACTCGTAG
Bl2	TCCTCTAGGG	CG-TGGTGAA	GTACCGTCAA	GTCTTTTAAG	TTTTAAGTG-	GGACTCGTAG
Br	TCCTCTAGGG	CG-TGGTGAA	GTACCGTCAA	GTCTTTTAAG	TTTTAAGTG-	GGACTCGTAG
Ou2	TCCTCTAGGG	CGGGG-TGAA	GTACCGTCAA	GTCTTTTAAG	TTTTAAGT-G	TGACTCGTAG
Bn	TCCTCTAGGG	CGNTGGTGAA	GTACCGTCAA	GTCTTYTAAG	TTTTAAGNTG	GGACTCGTAG
Ba	TC-TCTAGGG	CG-TGGTGAA	GTACCGTCAA	GTCTTTTAAG	-TTTA-GT--	GG-CTCGT-G
Bs1	TTGTTTGGCG	AGCAATTGGT	AG			
Ou1	TTNTTTNGCG	AGCAATTNGT	GA			
Bs2	TTGTTTGGCG	AGCAATTGGT	AG			
Bm	TTGTTTGGCG	AACAATTGGT	AG			
Bs3	TTGTTTGGCG	AGCAATTGGT	AG			
Bb	TTGTTTGGCG	AACAATTGGT	AG			
Bl1	TTGTTTGGCG	AACAATTGGT	AA			
Bl2	TTGTTTGGCG	A-CANT-GGT	A-			
Br	TAGTTTGGCG	AACAATTGGT	AA			
Ou2	TTGTTTGGCG	AGCAATTGGT	GA			
Bn	TTGTTTGGCG	AACAATTGGT	AG			
Ba	TTGTTTGGCG	A-CAATTGGT	AG			

a 4-min jump start at 85°C after which the *Taq* polymerase was added and cycling was resumed. The denaturing, annealing, and extension cycles were 35 s at 94°C, 40 s at 52°C, and 40 s at 73°C, respectively. To minimize the risk of contamination by the amplification of the foreign nucleic acids, proper controls, both negative and positive, were used following the cautions of Palumbi (1996). The amplification products were ligated into the vector pCR2.1, transformed into competent cells, and grown in LB and NCZYM broth following the protocols of the Invitrogen Original TA Cloning Kit. DNA plasmids were isolated from cells using the PerfectPrep system (Eppendorf 5 Prime), digested with *Eco*RI, and separated on 1% agarose gels to confirm the presence of the insert.

The insert was sequenced from within the plasmid using the SequiTherm Excel II DNA Sequencing Kit LC (Epicentre Technologies) with fluorescently labeled M13-Forward and M13-Reverse primers (LiCor). Sequences were resolved on a LiCor 4200 automated sequencing system.

The sequences generated by the automated sequencing software were aligned by eye with the help of the computer program Sequencher version 3.0 on a Power Macintosh 7600. An effort was made to maximize the number of matching bases among the sequences and minimize the number of insertions and deletions, thereby resulting in a conservative estimate of alignment. The aligned sequences were output from Sequencher in the NEXUS format and analyzed in *PAUP*: Phylogenetic Analysis Using Parsimony* (version 4.0; Swofford, 1998).

The two mtDNA sequences derived from *O. undulatus* were used as outgroups, as in Crother *et al.* (1992). A heuristic search was performed using the optimality criterion of maximum parsimony with the gaps treated as missing. Nucleotide positions were treated as unordered discrete characters and were given equal weight. Starting trees were obtained via stepwise addition, with a random addition sequence. The stepwise addition consisted of 100 replicates, with one tree held at

each step. The branch-swapping algorithm was tree-bisection-reconnection and the MULPARS option was in effect. The steepest descent option was not in effect and the initial "MaxTrees" setting was 10,400 and was set to be auto-increased by 100. Branches were collapsed if maximum branch length was zero.

An exhaustive search was performed on the mtDNA sequences derived from the eight taxa by including only one sequence for each of the taxa. The sequences excluded were Bs2, Bs3, Bl2, and Ou1.

The consistency index (CI) (Kluge and Farris, 1969), rescaled consistency index (RC) (Farris, 1989), and retention index (RI) (Farris, 1989) were calculated as descriptive metrics of the most parsimonious tree (MPT). The Bremer support index was also calculated (Bremer, 1988).

Sequence divergence was estimated using absolute distance, which is calculated by cumulatively recording dissimilarities at each nucleotide position. The χ^2 test of homogeneity of base frequencies across taxa was also performed. The transition/transversion ratio (ti/tv) was plotted against the pair-wise absolute distances. The assumption was made that absolute distance increases with time; however, no attempt was made to calibrate the distance value with time or to fashion a molecular clock (Hillis *et al.*, 1996).

A total evidence philosophy was justified by combining the data from Crother *et al.* (1992) (Table 1) and the mtDNA sequences used for all eight taxa in the exhaustive search above. This was done to study the effect on phylogenetic inference of the larger mtDNA data set (382 characters) on the smaller data set (48 characters), while recognizing that two separate histories were actually being combined. The mtDNA data were also constrained to the Crother *et al.* (1992) topology to examine the resulting differences in tree length and support indices to the actual mtDNA topology.

RESULTS

Nucleotide sequences (382 bp) of the mitochondrial 12S rRNA for the 12 individuals and eight taxa of this study are aligned in Table 2. Of the 382 nucleotide positions, 285 (74%) exhibited no variation across the specimens studied. Fifty-four characters were parsimony informative. The χ^2 test of homogeneity of base frequencies across taxa (Table 3) shows that comparisons are not statistically skewed ($\chi^2 = 9.59$, $df = 21$, $P = 0.984$). Base frequencies are shown in Table 4 and show that thymine is the most abundant nucleotide, followed by guanine, adenine, and cytosine for the 12 samples. Dinucleotide frequencies for pair-wise sequence comparisons of all 12 individuals were calculated (not shown). The ti/tv ratio is shown in Table 4. A matrix of the absolute distance for all pair-wise comparisons of mtDNA sequence from the 12 specimens is shown in Table 5. Figure 3 shows the ti/tv ratio (Table 4) against patristic distance (Table 5) for each pair-wise comparison.

The heuristic analysis of all 12 specimens yielded a single most parsimonious tree of 122 steps (CI = 0.861, RI = 0.865, RC = 0.745; Fig. 4). The MPT derived from the heuristic search, ((Ou1) (Ou1) ((((Bs1) (Bs3)) (Bs2)) ((((Bm) (Br)) ((Bb) (Ba))) (((Bl1) (Bl2)) (Bn)))), is incongruent with the topology discovered by Crother *et al.* (1992). The monophyly of those taxa with multiple

TABLE 3
χ^2 Test of Homogeneities of Base Frequencies across Taxa

Taxon	A	C	G	T
Bs1				
O	73	68	97	128
E	75.41	67.46	98.50	124.63
Bm				
O	80	65	94	126
E	75.20	67.27	98.24	124.29
Bb				
O	80	66	101	121
E	75.82	67.83	99.04	125.31
Bl1				
O	70	71	99	124
E	75.00	67.09	97.97	123.95
Br				
O	90.5	68	94	110.5
E	74.79	66.90	97.70	123.61
Ou2				
O	73	67	101	127
E	75.82	67.83	99.04	125.31
Bn				
O	70	69.5	96.5	128
E	75.00	67.09	97.97	123.95
Ba				
O	61	60	98	123
E	70.46	63.03	92.05	116.46

Note. $\chi^2 = 9.590665$ ($df = 21$), $P = 0.98375302$. This test ignores correlation due to phylogenetic structure. A, adenine; C, cytosine; G, guanine; T, thymine; O, observed; E, expected.

TABLE 4
Dinucleotide Frequencies and Transition/Transversion (ti/tv) Ratios for Pair-wise Comparisons of All 12 Samples

	ti		tv				Ident						
Taxa	AG	CT	AC	AT	CG	GT	AA	CC	GG	TT	Prop diff	ti/tv ratio	Total
Bs1 vs Ou1	13	11	0	0	1	1	65	61	81	122	0.07	12.00	355
Bs1 vs Bs2	4	2	0	0	1	5	71	68	79	116	0.03	1.00	346
Bs1 vs Bm	19	9	1	3	1	5	64	61	81	116	0.11	2.80	360
Bs1 vs Bs3	1	0	0	0	0	0	73	68	94	128	0.00	—	364
Bs1 vs Bb	19	7	1	1	1	6	61	62	84	113	0.10	2.89	355
Bs1 vs B11	16	12	1	2	1	3	61	62	85	117	0.10	4.00	360
Bs1 vs B12	13	14	1	2	1	3	61	62	85	115	0.10	3.86	357
Bs1 vs Br	19	10	0	10	1	5	63	62	80	101	0.13	1.81	351
Bs1 vs Ou2	13	10	0	0	1	2	66	62	87	120	0.07	7.67	361
Bs1 vs Bn	17	10	0	2	1	3	60	62	79	119	0.09	4.50	353
Bs1 vs Ba	16	8	0	0	1	4	51	57	83	117	0.09	4.80	337
Ou1 vs Bs2	10	13	0	0	2	5	66	61	70	111	0.09	3.29	338
Ou1 vs Bm	16	6	1	3	0	4	65	62	77	118	0.09	2.75	352
Ou1 vs Bs3	12	11	0	0	1	1	66	61	81	122	0.07	11.50	355
Ou1 vs Bb	14	6	0	2	0	5	64	63	79	113	0.08	2.86	346
Ou1 vs Bl1	16	10	1	1	0	3	61	63	80	118	0.09	5.20	353
Ou1 vs Bl2	14	12	1	1	0	3	60	63	79	116	0.09	5.20	349
Ou1 vs Br	15	8	0	10	0	4	64	63	75	102	0.11	1.64	341
Ou1 vs Ou2	0	1	0	0	0	1	72	66	89	126	0.01	1.00	355
Ou1 vs Bn	14	8	0	2	0	4	60	63	74	119	0.08	3.67	344
Ou1 vs Ba	13	5	0	0	0	4	53	58	76	119	0.07	4.50	328
Bs2 vs Bm	14	11	1	4	2	8	65	61	71	107	0.12	1.67	344
Bs2 vs Bs3	3	2	0	0	1	5	72	68	80	116	0.03	0.83	347
Bs2 vs Bb	15	7	1	3	2	9	62	62	74	106	0.11	1.47	341
Bs2 vs Bl1	12	10	1	2	2	7	63	63	75	109	0.10	1.83	344
Bs2 vs Bl2	9	12	1	2	2	7	63	63	74	107	0.10	1.75	340
Bs2 vs Br	17	11	1	7	2	7	64	62	69	96	0.13	1.65	336
Bs2 vs Ou2	11	12	0	0	2	7	67	62	75	112	0.09	2.56	348
Bs2 vs Bn	13	9	0	2	2	9	62	63	66	109	0.10	1.69	335
Bs2 vs Ba	12	10	0	1	2	8	52	57	72	106	0.10	2.00	320
Bm vs Bs3	18	9	1	3	1	5	65	61	81	116	0.10	2.70	360
Bm vs Bb	8	2	1	2	0	3	71	64	89	114	0.05	1.67	354
Bm vs Bl1	16	8	2	1	0	4	65	63	84	116	0.09	3.43	359
Bm vs Bl2	14	9	3	1	0	4	64	63	83	114	0.09	2.88	355
Bm vs Br	8	4	1	8	0	1	73	64	86	103	0.06	1.20	348
Bm vs Ou2	17	5	1	3	0	6	66	63	83	118	0.09	2.20	362
Bm vs Bn	17	6	1	3	0	5	62	63	78	116	0.09	2.56	351
Bm vs Ba	8	4	1	2	0	2	58	58	86	116	0.05	2.40	335
Bs3 vs Bb	18	7	1	1	1	6	62	62	84	113	0.10	2.78	355
Bs3 vs Bl1	15	12	1	2	1	3	62	62	86	117	0.09	3.86	361
Bs3 vs Bl2	12	14	1	2	1	3	62	62	85	115	0.09	3.71	357
Bs3 vs Br	19	10	0	10	1	5	64	62	79	101	0.13	1.81	351
Bs3 vs Ou2	12	10	0	0	1	2	67	62	87	120	0.07	7.33	361
Bs3 vs Bn	15	10	0	2	1	3	61	62	80	119	0.09	4.17	353
Bs3 vs Ba	15	8	0	0	1	4	52	57	82	117	0.08	4.60	336
Bb vs Bl1	16	4	1	1	0	3	62	65	89	115	0.07	4.00	356
Bb vs Bl2	14	5	1	1	1	3	61	65	89	113	0.07	3.17	353
Bb vs Br	7	2	0	6	0	1	70	66	92	104	0.05	1.29	348
Bb vs Ou2	16	5	0	3	0	6	64	64	85	112	0.08	2.33	355
Bb vs Bn	17	2	0	2	0	5	60	65	80	115	0.08	2.71	346
Bb vs Ba	2	2	0	2	0	0	59	60	93	114	0.02	2.00	332
Bl1 vs Bl2	1	1	0	0	0	0	67	71	97	122	0.01	—	359
Bl1 vs Br	13	5	3	7	0	2	65	65	86	105	0.09	1.50	351
Bl1 vs Ou2	17	9	1	1	0	5	62	64	85	116	0.09	3.71	360

TABLE 4—*Continued*

	ti		tv				Ident						
Taxa	AG	CT	AC	AT	CG	GT	AA	CC	GG	TT	Prop diff	ti/tv ratio	Total
Bl1 vs Bn	10	4	1	1	0	3	62	67	84	120	0.05	2.80	352
Bl1 vs Ba	15	6	1	1	0	2	50	60	84	117	0.07	5.25	336
Bl2 vs Br	12	6	3	7	1	2	63	65	86	103	0.09	1.38	348
Bl2 vs Ou2	15	11	1	1	0	5	61	64	84	114	0.09	3.71	356
Bl2 vs Bn	9	6	1	1	0	3	60	67	83	118	0.06	3.00	348
Bl2 vs Ba	13	7	1	1	1	2	50	60	83	115	0.08	4.00	333
Br vs Ou2	16	7	0	10	0	5	65	64	81	101	0.11	1.53	349
Br vs Bn	18	4	2	8	0	4	62	65	77	104	0.10	1.57	344
Br vs Ba	8	4	0	10	0	0	57	60	86	102	0.07	1.20	327
Ou2 vs Bn	15	7	0	2	0	5	61	64	81	117	0.08	3.14	352
Ou2 vs Ba	15	4	0	0	0	6	53	59	83	117	0.07	3.17	337
Bn vs Ba	17	4	0	2	0	3	49	60	78	117	0.08	4.20	330

samples, *O. undulatus*, *B. schlegelii*, and *B. lateralis*, was supported.

The single most parsimonious tree from the exhaustive search was 109 steps (CI = 0.853, RI = 0.686, RC = 0.586; Fig. 5). The MPT derived from the exhaustive search, ((Ou2)) ((((Bm) (Br)) ((Bb) (Ba))) ((Bl1) (Bn))) (Bs1))), is incongruent with the topology estimated by Crother *et al.* (1992).

The exhaustive search in the total evidence analysis resulted in five MPTs of 229 steps each (CI = 0.790, RI = 0.571, RC = 0.452). One of the five trees was identical to the phylogeny of Crother *et al.* (1992). None of the five MPTs supported the sister relationship of *lateralis* and *nigroviridis*. A strict consensus tree and a majority rule consensus tree are illustrated (Fig. 6). When the mtDNA data set was constrained to the Crother *et al.* (1992) topology, the resulting tree length was 122 (13 steps longer than the mtDNA MPT) and the support indices were CI = 0.76, RI = 0.43, RC = 0.33.

DISCUSSION

Data

For *Bothriechis*, the frequency of transitions in the mitochondrial small subunit (12S rRNA) initially increased with the corresponding increase in distance; however, after approximately 26 distance units the

TABLE 5
Patristic Distance Matrix for All 12 Samples

	1	2	3	4	5	6	7	8	9	10	11	12
1 Bs1	—											
2 Ou1	26	—										
3 Bs2	12	30	—									
4 Bm	38	30	40	—								
5 Bs3	1	25	11	37	—							
6 Bb	35	28	39	16	34	—						
7 Bl1	35	31	35	31	34	27	—					
8 Bl2	34	31	34	31	33	27	2	—				
9 Br	45	37	47	23	45	16	31	32	—			
10 Ou2	26	2	32	32	25	31	33	33	39	—		
11 Bn	33	28	36	32	31	28	19	20	36	29	—	
12 Ba	29	23	33	17	28	6	25	25	22	25	26	—

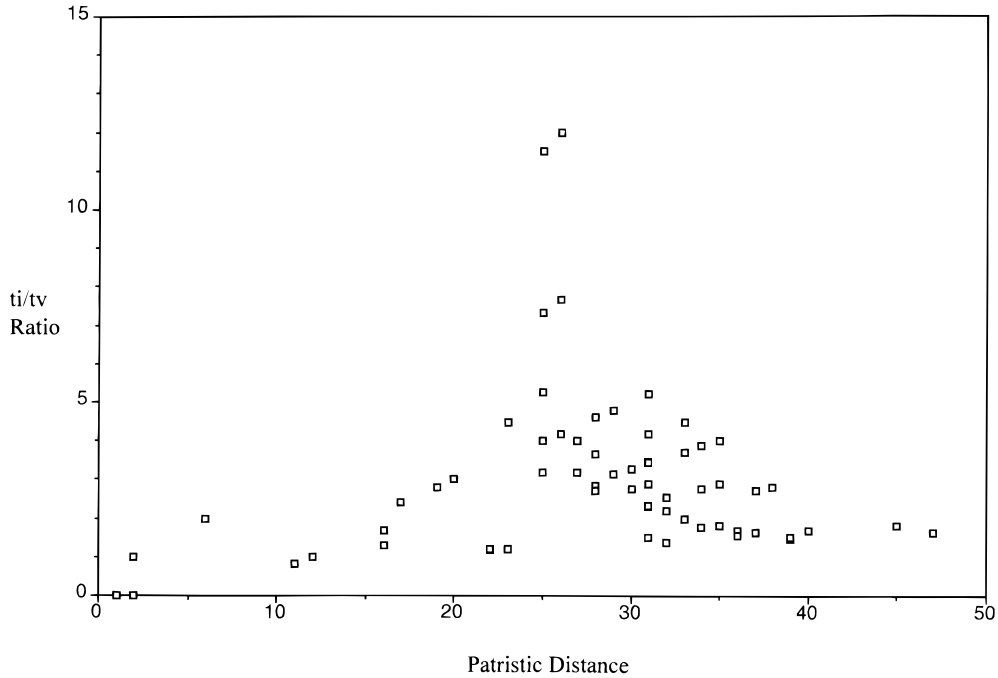

FIG. 3. Plot of transition-to-transversion ratio versus patristic distance for all pair-wise comparisons of all 12 OTUs.

trend reversed and the frequency of transitions reduced back to initial levels (Fig. 3). In contrast Mindell and Honeycutt (1990) found that the percentage of transitions in the 12S rRNA decreased with an increase in the estimated time of divergence. In a study on 12S rRNA divergence among families, genera, and species of *Hystricognath* rodents, Nedbal *et al.* (1994) also found that as the distance increased the ti/tv ratio decreased. This suggests that for *Bothriechis* transitions are saturated. A typical response to this situation would call for the elimination or at least down-weighting of transitions from the analysis. Recent work (Källersjö *et al.*, 1999) suggested that increasing saturation and the accompanying homoplasy actually increases phylogenetic structure. Even so, we examined the effect of down-weighting transitions (based on the mean empirical ti/tv ratio) in a parsimony analysis and with a maximum-likelihood analysis. In both cases, the results (not shown) were the same as those of the original equal-weighted parsimony analysis, with *lateralis* and *nigroviridis* sister taxa.

Phylogeny

The previous studies of nuclear-based data (Werman, 1992, 1997; Gutberlet, 1998; Crother *et al.*, 1992) were incongruent with the single mtDNA-based phylogeny (Parkinson, 1999). While the mtDNA indicated a sister relationship of *nigroviridis–lateralis*, none of the morphology or allozyme data sets did. However, the incongruence between these data may be because the mtDNA study included only three species (*schlegelii*, *nigroviridis*, and *lateralis*) of *Bothriechis*.

The mtDNA topologies inferred herein included all the species of *Bothriechis* and were incongruent with those from Crother *et al.* (1992), Gutberlet (1998), and Werman (1992, 1997). Although there is incongruence among the northern taxa, the critical point of departure between the two hypotheses is in the position of *B. lateralis*. Crother *et al.* (1992) inferred that *B. lateralis* was nested within the northern taxa, whereas the mtDNA indicates (here and in Parkinson, 1999) a sister relationship between *B. lateralis* and *B. nigroviridis*. As a consilient test, this result cannot reject the previous hypothesis (as explained earlier), but does necessitate explanation. Two possible explanations for gene tree/species tree incongruence are lineage sorting (Nigel and Avise, 1986) and lateral transfer by hybridization and introgression (first reported in mtDNA by Ferris *et al.*, 1983) (Fig. 7).

The incongruence of the northern allopatric montane forms (*marchi*, *rowleyi*, *bicolor*, and *aurifer*) exhibited by

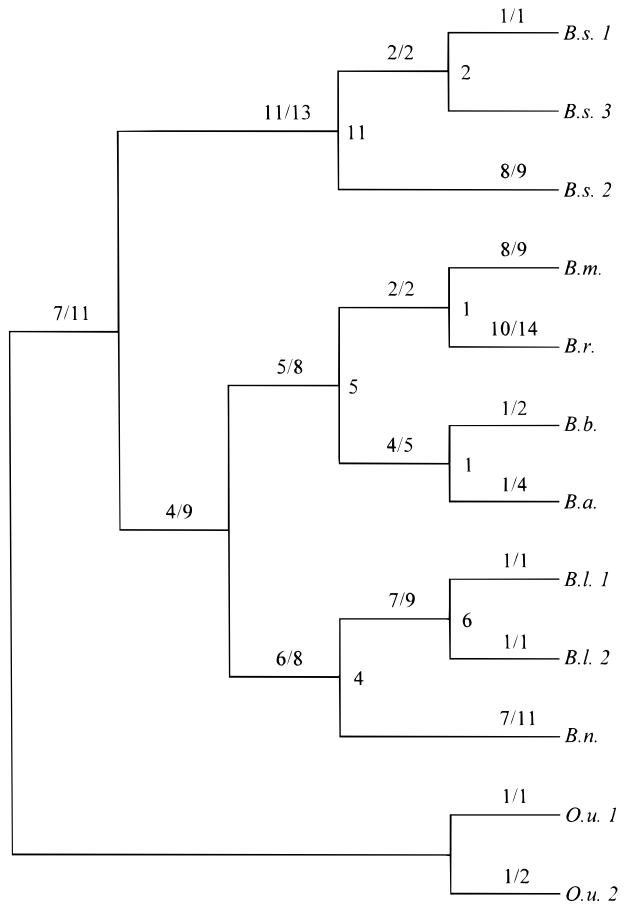

FIG. 4. Single most parsimonious cladogram inferred from a heuristic search: 122 steps, CI = 0.86, RI = 0.86, RC = 0.74.

the mtDNA tree (Fig. 8) may be attributed to lineage sorting because by the nature of their vicariant history these taxa have presumably not been in contact since speciation. This is an appropriate example of the importance of incorporating the biology of the organisms (and in this case their obligate symbionts) when examining character evolution in a phylogenetic framework.

The vicariant history of Middle America is consilient with the hypothesis of allopatric speciation presented by Crother *et al.* (1992). However, the incongruence exhibited between the mitochondrial lineages and the nuclear (morphology and allozymes) lineages for the two sympatric taxa (*lateralis* and *nigroviridis*) cannot be determined, because lineage sorting cannot be completely ruled out. However, because these taxa are sympatric, perhaps the more compelling explanation would be introgression. Of the seven possible taxa with which *lateralis* could have aligned, each had an equal a priori probability. But only *nigroviridis* is syntopic with *lateralis* and presented the potential for introgression. There are many reported instances of mtDNA crossing taxonomic boundaries (Table 6), so such a case of lateral transfer of haplotypes is not an unusual occurrence. Admittedly, because lineage sorting cannot be ruled out, invoking introgression as the cause of the incongruence is speculation. However, it is at least consistent with the evidence and serves as a hypothesis for further tests. This empirical finding casts doubt on the superiority of mtDNA in phylogenetic analysis (Moore, 1995).

The larger mtDNA data set did not completely swamp out the smaller morphology / allozyme data set in the total evidence analysis, based on the fact that

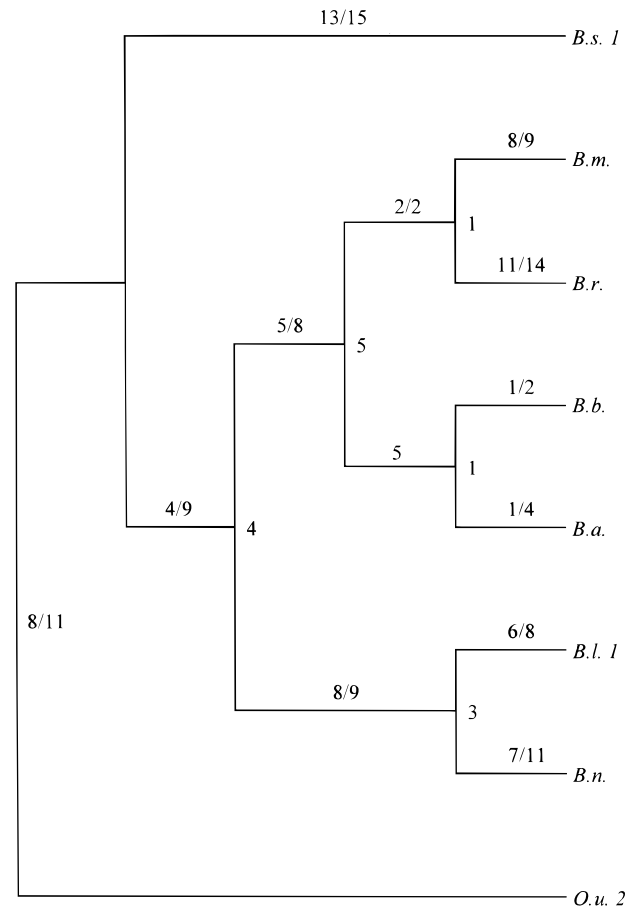

FIG. 5. Single most parsimonious cladogram inferred from an exhaustive search of eight representative OTUs: 109 steps, CI = 0.85, RI = 0.69, RC = 0.59.

lateralis and *nigroviridis* were not sister taxa (Fig. 5) as they were in the mtDNA-based topology. One of the five MPTs was identical to the tree in Crother *et al.* (1992); however, the placement of *lateralis* among the northern montane forms varied so much within the five trees that resolution was lost upon the computing of the strict consensus tree (Fig. 6). This is due to the influence of the mtDNA data set, and it illustrates the potential for erroneous results from the combined analysis of mitochondrial and nuclear-based characters. The inclusion of mtDNA in a total evidence analysis is inappropriate, not because it represents a separate class of data (*sensu* Kluge, 1989), but because it represents a separate history (T. W. Taggart *et al.*, submitted for publication).

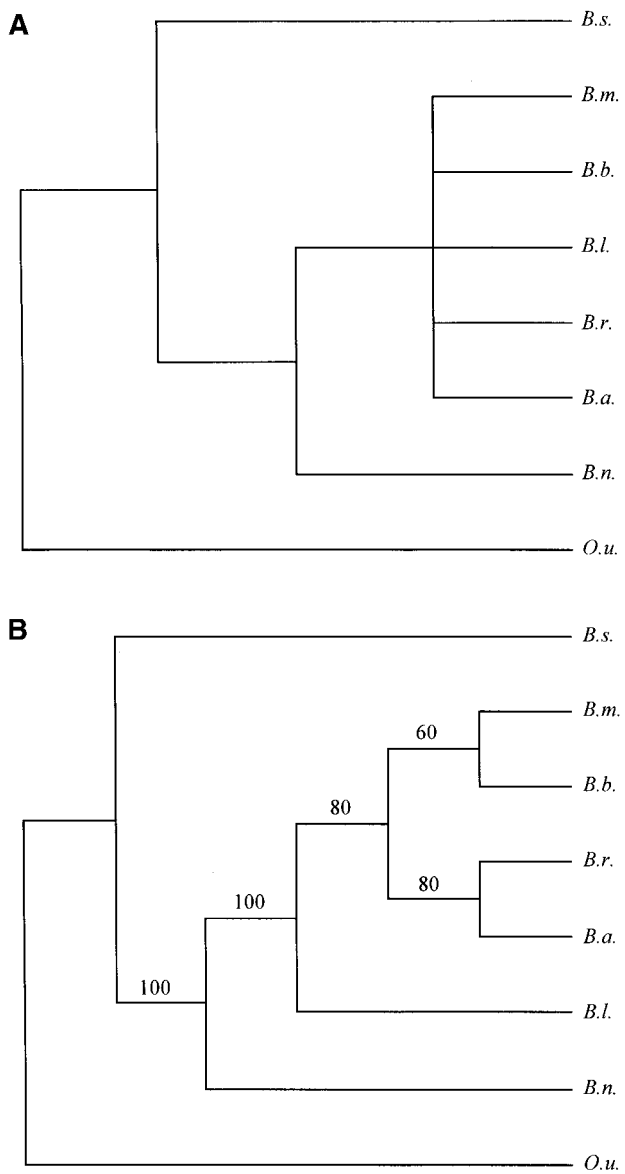

FIG. 6. Strict consensus tree (A) and a majority-rule consensus tree (B) of five equally most parsimonious trees from the combined data analysis. The morphology–allozyme tree of Crother *et al.* (1992) was one of the five most parsimonious trees. The trees were 229 steps, with CI = 0.79, RI = 0.57, RC = 0.452.

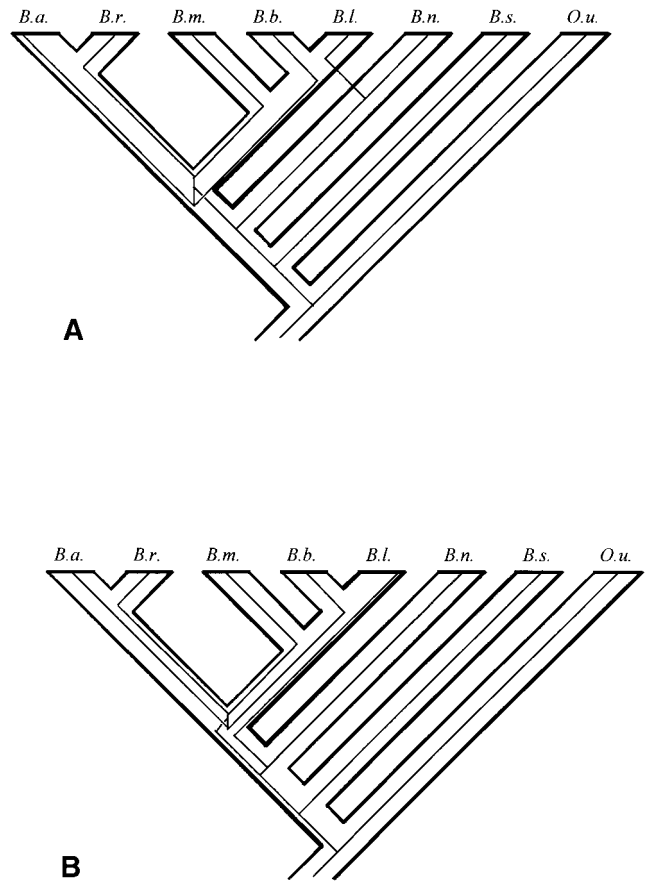

FIG. 7. Alternative hypotheses to explain the incongruence between the phylogeny of *Bothriechis* (= species tree; thick lines) and the phylogeny of their endosymbiotic mitochondrial genome (thin lines). (A) Xenologous mtDNA relationship between *B. lateralis* and *B. nigroviridis* resulting from the lateral transfer of mtDNA. (B) Paralagous mtDNA relationships of *B. lateralis* and *B. nigroviridis* resulting from lineage sorting.

Taxonomic Comment

Solórzano *et al.* (1998), based on external morphology and preliminary allozyme data, resurrected the name *Bothriechis supraciliaris* for a geographically delimited

TABLE 6
Reported Instances of mtDNA Crossing Taxonomic Boundaries

Generic: Mammalia
- *Bison bison* / *Bos taurus*, Polziehn *et al.* (1995)
- *Cystophora cristata* / *Phoca groenlandica*, Kovacs *et al.* (1997)

Species
- Insecta
  - *Bombus canariensis* / *B. terrestris*, Estoup *et al.* (1996)
  - *Caledia*, Marchant (1988), Wilson *et al.* (1985)
  - *Drosophila pseudoobscura* / *D. persimilis*, Powell (1983)
  - *Drosophila*, Solignac and Monnerot (1986)
  - *Gryllus*, Harrison *et al.* (1987)
  - *Gryllus firmus* / *G. pennsylvanicus*, Rand and Harrison (1989)
  - *Heliconius cydno* / *H. melpomene*, Brower (1996)
  - *Pissodes strobi* group, Boyce *et al.* (1994)
- Crustacea
  - *Menippe adina* / *M. mercenaria*, Bert *et al.* (1996)
- Fishes
  - *Brevoortia patronus* / *B. tyrannus*, Bowen and Avise (1990)
  - *Notropis chrysocephalus* / *N. cornutus*, Dowling *et al.* (1989)
  - *N. cornutus* / *N. chrysocephalus*, Dowling and Hoeh (1991)
  - *Pomoxis annularis* / *P. nigromaculatus* Travnichek *et al.* (1997)
  - *Salvelinus confluentus* / *S. malma*, Baxter *et al.* (1997)
  - *Tridentiger brevispinis* / *T. obscurus*, Mukai *et al.* (1997)
- Amphibia
  - *Hyla gratiosa* / *H. cinerea* (Amphibia), Lamb and Avise (1986)
  - *H. gratiosa* / *H. cinerea* (Amphibia), Lamb and Avise (1987)
  - *Rana*, Spolsky and Uzzell (1984), Spolsky and Uzzell (1986)
  - *Rana nigromaculata* / *R. porosa*, Sumida and Ishihara (1997)
  - *Xenopus*, 7 spp., Carr *et al.* (1987)
- Testudines
  - *Gopherus agassizi* / *G. berlandieri*, Lamb *et al.* (1989)
- Reptilia
  - *Bothriechis lateralis* / *B. nigroviridis*, this study
- Mammalia
  - *Canis latrans* / *C. lupus*, Lehman *et al.* (1991)
  - *Canis*, 5 spp., Vila *et al.* (1997)
  - *Clethrionomys*, Telgelstrom (1987b), Tegelstrom *et al.* (1988)
  - *Equus*, Hutchison *et al.* (1974)
  - Mice, Gyllensten and Wilson (1987)
  - *Mus musculus* / *M. domesticus*, Ferris *et al.* (1983)
  - *M. molossinus* / *M. domesticus*, Ferris *et al.* (1982)
  - *M. domesticus* / *M. musculus*, Yonekawa *et al.* (1982)
  - *Odocoileus hemionus* / *O. virginianus*, Carr *et al.* (1986)
  - *Ursus arctos* / *U. maritimus*, Cronin *et al.* (1991)
- Aves
  - *Anas platyrhynchos* / *A. rubripes*, Avise *et al.* (1990)
  - *Calidris ferruginea* / *C. melanotos*, Christidis *et al.* (1996)

"Subspecies"
- Insecta
  - *Apis mellifera*, 3 ssp., Smith *et al.* (1989)
  - *Heliconius erato*, 14 ssp., Brower (1996)
  - *H. melpomene*, 13 ssp., Brower (1996)
  - *H. cydno*, 3 ssp., Brower (1996)
- Fishes
  - Cutthroat trout, 2 ssp., Gyllensten *et al.* (1985b)
- Amphibia
  - *Rana*, 2 ssp., Sumida (1997b)
  - *Xenopus*, 4 ssp., Carr *et al.* (1987)

TABLE 6—*Continued*

"Subspecies"—*Continued*
- Mammalia
  - *Bison bison*, 2 ssp., Polziehn *et al.* (1996)
  - *Canis familiaris*, 8 ssp., Okumura *et al.* (1996)
  - *Enhydra lutris*, 2 ssp., Cronin *et al.* (1996)
  - *Perognathus amplus*, 2 ssp., McKnight (1995)
  - *Odocoileus hemionus*, 6 ssp., Cronin and Bleich (1995)
  - *Rangifer trandus*, 2 ssp., Cronin *et al.* (1995)
  - *Thomonys bottae*, 2 ssp., Ruedi *et al.* (1997)
  - *Ursus americanus*, 3 ssp., Paetkau and Strobeck (1996)

Linguistic: *Homo sapiens*, Barbujani *et al.* (1996)

population of *B. schlegelii*. Our study is the first to include *supraciliaris* in a sequence-based phylogeny so a comment is warranted. The terminal branch of *supraciliaris* is the sister to a *schlegelii* clade, but most interesting are the number of synapomorphies supporting the lineages: *schlegelii* has two characters and *supraciliaris* has nine (eight unambiguous). Although we recognize the Phylogenetic Species Concept as a useful operation, we do not claim to fully understand how to diagnose species based on DNA sequence data (e.g., is one nucleotide enough?). We would argue, however, that as an extrinsic data set, mtDNA should not be used to diagnose species, but could be used to support species diagnoses in a consilient fashion. An examination of relative character support is constructive and may be the appropriate approach. Only *rowleyi* is supported by more apomorphies that *supraciliaris* (Fig. 4), yet the other taxa are easily diagnosed morphologically (Crother *et al.*, 1992). We are led to conclude that *supraciliaris* is indeed on a unique evolutionary trajectory and should be recognized.

CONCLUSIONS

The hypothesis generated by the mtDNA lineages was not consilient with the nuclear DNA-derived hypothesis. The mtDNA data are extrinsic to the organisms under analysis, and so the lack of support does not falsify the nuclear DNA based phylogeny. Additionally, the opportunity for introgression between *lateralis* and *nigroviridis* suggests that the mtDNA results may not reflect species relationships. Given this, the

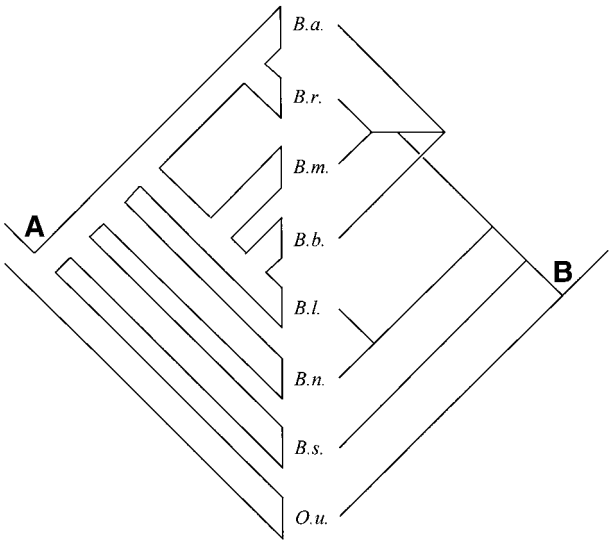

FIG. 8. Coevolution of *Bothriechis* spp. (A) and the mtDNA genes (B) collected from them. They are drawn as separate diagrams, unlike in Fig. 7, to illustrate that two separate histories exist and this is the appropriate way to compare them. The two cladograms are largely congruent, but "host switching" has occurred in the mtDNA gene collected from *B. lateralis* (B.l.). The incongruence of the clade containing the northern montane forms *aurifer* (B.a.), *bicolor* (B.b.), *marchi* (B.m.), and *rowleyi* (B.r.) has resulted from lineage sorting events of their respective mtDNA genomes.

nuclear-based phylogeny is the hypothesis that should serve in the application of subsequent examinations.

APPENDIX: SPECIMENS EXAMINED

Bothriechis aurifer, *Yellow-Blotched Palm-Pitviper*

Ba—JAC 10611: Guatemala, La Union Barrios, 1500 m.

Bothriechis bicolor, *Guatemalan Palm-Pitviper*

Bb—JAC 15698: Guatemala, Escuintla.

Bothriechis lateralis, *Side-Striped Palm-Pitviper*

Bl1—No. 8207095: Costa Rica, exact locality unknown.

Bl2—MZUCR 11155, CLP 48: Costa Rica, Acosta.

Bothriechis marchi, *March's Palm-Pitviper*

Bm—JAC 15699: Honduras, exact locality unknown.

Bothriechis nigroviridis, *Black-Speckled Palm Pitviper*

Bn—MZUCR 11151, CLP 49: Costa Rica, San Gerondo de Dota.

Bothriechis rowleyi, *Rowley's Palm-Pitviper*

Br—JAC 15701: Mexico, Cerro Baul, 1500 m.

Bothriechis schlegelli, *Eyelash Palm-Pitviper*

Bs1—Captive specimen: Exact locality unknown.

Bs2—MZUCR 11149, CLP 51: Costa Rica, Cariblanco de Sarapique.

Bs3—SLU 0001: Exact locality unknown.

Ophryacus undulatus, *Mexican Horned Pitviper*

Ou1—Mexico: Collected by JAC, Audubon Zoo No. R266.

Ou2—CLP 74: Mexico.

Abbreviations used: JAC, Dr. Jonathan A. Campbell, University of Texas, Arlington; CLP, Dr. Christopher L. Parkinson, Indiana University, Bloomington; MZUCR, Museum of the Universidad de Costa Rica; SLU, Southeastern Louisiana University frozen tissue collection.

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