Cytochrome *b* sequence variation and a molecular phylogeny of the live-bearing fish genus *Gambusia* (Cyprinodontiformes: Poeciliidae)

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Abstract: Nucleotide sequences for a 402-base segment of the mitochondrial cytochrome *b* gene were determined from 25 species of live-bearing fishes. A total of 34 sequences representing 24 species of the genus *Gambusia* and 1 species of *Belonesox* were generated via the polymerase chain reaction. The levels of overall variation were consistent with those from other genera of fishes. In total, 137 of 402 (34.1%) nucleotides exhibited variation within or among the species. Observed differences at 24 (17.9%) of the 134 cytochrome *b* codons would result in amino acid replacements. Phylogenetic analyses employing various weighting schemes resulted in several clades representing traditionally recognized taxonomic groups. However, precise relationships among species-groups remained uncertain. Randomization tests indicated that these topologies contained significant nonrandom phylogenetic information. As with other fishes, the overall rate of divergence appeared to be slower than that of other vertebrates and the overall replacement/substitution pattern was suggestive of nonrandom evolutionary input.

Résumé : Les séquences de nucléotides d'un segment de 402 bases du gène du cytochrome *b* mitochondrial ont été déterminées chez 25 espèces de poissons vivipares. Au total, 34 séquences représentant 24 espèces du genre *Gambusia* et 1 espèce de *Belonesox* ont été produites par l'amplification en chaîne par polymérase. De façon générale, l'importance de la variation était semblable à celle qui prévaut chez d'autres genres de poissons. Des variations ont été enregistrées dans 137 des 402 (34,1%) nucléotides, aussi bien au sein d'une seule espèce que d'une espèce à l'autre. Les différences observées dans 24 (17,9%) des 134 codons du cytochrome *b* sont susceptibles d'entraîner le remplacement des acides aminés. Des analyses phylogénétiques basées sur des stratégies variées de pondération ont généré plusieurs clades représentant les groupes taxonomiques ordinairement reconnus. Cependant, les relations précises entre les groupes d'espèces restent obscures. Des tests de randomisation ont indiqué que ces topologies contiennent une quantité sigificative d'information phylogénétique non aléatoire. Comme chez les autres poissons, la vitesse de la divergence semble avoir été plus lente que celle qui a prévalu chez les autres vertébrés et le pattern de remplacement/substitution paraît indiquer l'existence d'un apport évolutif non aléatoire.
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Introduction

Increased use of nucleotide sequences for testing systematics and biogeographically based hypotheses has made available large data bases that are well suited for comparative studies of DNA sequence evolution. Segments of the mitochondrial genome have proved to be particularly useful for these types of analyses, sequences from numerous taxa now having been reported (e.g., Brown et al. 1982; Moritz et al. 1987; Smith and Patton 1991; Meyer et al. 1990; Sturmbauer and Meyer 1992). Because of the availability of "universal" amplification primer sequences (Kocher et al. 1989), the ease of alignment, and its well-documented biochemistry (Howell 1989), the mitochondrial cytochrome *b* gene has been one of the most frequently utilized of these mitochondrial regions (Irwin et al. 1991; Meyer 1993, 1994). In the present study, we generated nucleotide sequence data from this region in order to evaluate patterns of genetic variation within and among species of live-bearing fishes of the genus *Gambusia*.

Gambusia spp. are small, surface-dwelling live-bearers that are ubiquitous members of aquatic ecosystems throughout the southern United States, Mexico, Central America, and many Caribbean islands (Rosen and Bailey 1963). The genus *Gambusia* (Cyprinodontiformes: Poeciliidae) is com-

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Table 1. The 24 species of *Gambusia* included in this study, classified after Rauchenberger (1989) as the initial taxonomic hypothesis.

Subgenus *Heterophallina*
vittata
panuco species-group
marshi
panuco
rachowi species-group
rachowi
Subgenus *Arthrophallus*
affinis species-group
affinis
holbrooki
nobilis species-group
heterochir
sexradiata
eurystoma
senilis species-group
hurtadoi
geiseri
Subgenus *Gambusia*
nicaraguensis species-group
nicaraguensis
wrayi
melapleura
puncticulata species-group
yucatana
hispaniolae
hubbsi
manni
puncticulata
oligosticta
caymanensis
punctata species-group
luma
punctata
rhizophorae

posed of approximately 45 species presently assigned to three subgenera and eight species groups based on Rauchenberger's (1989) recently proposed classification. Species of this genus have demonstrated remarkable flexibility under a variety of environmental conditions (Stearns 1983; Zimmerman et al. 1988) and have therefore been recognized as useful models for investigating evolutionary and population level processes (e.g., McClenaghan et al. 1985; Smith et al. 1989).

Nearly all systematic studies of poeciliids, including most major taxonomic treatments of *Gambusia*, have relied on morphological characters that are almost exclusively from a single morphological character complex, the gonopodium (intromittent organ of males) and its associated support structures. These characters, while taxonomically informative, may lack developmental independence and may be functionally linked, making it difficult to determine whether shared traits are due to a shared common ancestry or convergence or parallelism (e.g., Constantz 1989).

To overcome these potential limitations, we examined an alternative data set based on nucleotide level characters that do not appear to have a recognizable morphological effect. Mitochondrial DNA sequences are powerful data for examining both intraspecific and interspecific relationships and biogeographic patterns (reviewed by Wilson et al. 1985; Avise 1986; Avise et al. 1987; Moritz et al. 1987). Furthermore, the availability of protocols for the application of the polymerase chain reaction (PCR) in molecular systematic work (Kocher et al. 1989; Meyer et al. 1990) permits the rapid acquisition of DNA sequences necessary for these types of studies. Actual mtDNA sequences permit examination of more distantly related taxa than was previously possible with restriction enzyme data (Kocher et al. 1989; Meyer and Wilson 1990; Irwin et al. 1991).

As argued by Tamura (1992), a true understanding of the causal influences of observed patterns of nucleotide evolution can only be realized once data from both divergent and closely related taxa are accumulated. The purpose of the present study was to generate such an analysis for closely related taxa of *Gambusia* and to use this information to gain insight into the process of mitochondrial DNA evolution and systematics of the genus.

Materials and methods

Biological material

A total of 34 specimens representing 24 species of *Gambusia* (Table 1) plus 1 specimen of *Belonesox belizanus* were collected from the wild or obtained from aquarium stocks. The localities and (or) source of specimens examined in this study were as follows: *G. affinis*: *affinis* 1, Village Creek, Hardin County, Texas; *affinis* 2, Cow Creek, Travis County, Texas; *affinis* 3, Big Bend, Brewster County, Texas (provided by C. Hubbs); *G. caymanensis*: Palmetto Point, Grand Cayman Island; *G. eurystoma*: Arroyo del Azufre at Banos de Azufre, 10 km west of Teapa, Tabasco, Mexico; *G. geiseri*: San Marcos, Hays County, Texas (aquarium stock provided by C. Hubbs); *G. heterochir*: Clear Creek, Menard County, Texas (aquarium stock provided by C. Hubbs); *G. hispaniolae*: Dominican Republic (provided by C. Rodriguez); *G. holbrooki*: *holbrooki* 1, Highway A1A, Key West, Florida; *holbrooki* 2, Orlando, Florida (provided by F.F. Snelson, Jr.); *G. hubbsi*: Adelaid Beach, New Providence, Bahamas; *G. hurtadoi*: Chihuahua, Mexico (aquarium stock provided by C. Hubbs); *G. luma*: Guanacaste Park, 3 km north of Belmopan, Belize; *G. manni*: Lake Cunningham, New Providence, Bahamas; *G. marshi*: 1.5 km south of Hermanas, Highway 57, Coahuila, Mexico; *G. melapleura*: Bluefields River at Old Rest House, Jamaica; *G. nicaraguensis*: Rio Perezosa, near Cahuita, Costa Rica; *G. oligosticta*: Port Royal Causeway, 5.5 km west of Kingston airport, Jamaica; *G. panuco*: Rio Panuco, 6 km south of Cuidad Valles, San Luis Potosi, Mexico; *G. punctata*: Cuba (provided by B. McKeand); *G. puncticulata*: Cuba (provided by B. McKeand); *G. rachowi*: Rio Jactepec, Rio Coatzacoalcos near Jesus Carranza, Veracruz, Mexico; *G. rhizophorae*: *rhizophorae* 1, Key West, Highway A1AS, Florida; *rhizophorae* 2, La Ceiba, east of Havana, Cuba; *rhizophorae* 3, Bacuranao Canal, east of Havana, Cuba; *G. sexradiata*: Rio Papaloapan, Highway 175 near Tuxtepec, Oaxaca, Mexico; *G. vittata*: Rio Tamesi at bridge 25 km from Highway 80, Tamaulipas, Mexico; *G. wrayi*: Black River near Middle

Quarters, Route 1-2, Jamaica; *G. yucatana — yucatana* 1, Rio Coatzacoalcos 4.4 mi northeast of intersection of Highway 180 and Highway 180 bypass, Veracruz, Mexico; *yucatana* 2, Lake Coban, Quintana Roo, Mexico (provided by B. McKeand); *Belonesox belizanus*: Florida Everglades (provided by B. Loftus). Intraspecific variation was assessed by examining several specimens from a single species. In particular, *G. holbrooki*, one of the most widely distributed species in this group, was sampled from localities from the extreme points of its range, as was *G. rhizophorae*. In addition, three specimens of *G. oligosticta* from a single locality and two specimens of *G. luma* from the location above plus Dominic Isla were sequenced. Except where identified above as separate, all duplicated sequences were identical.

DNA extraction, amplification, and sequencing

Whole genomic DNA was extracted from frozen or 75% ethanol-preserved muscle tissue (<0.2 g) following Kocher et al. (1989). Symmetric and asymmetric amplifications of a 425 base pair (bp) segment of the mitochondrial cytochrome *b* gene were generated via PCR (Saiki et al. 1988) following the protocol of Kocher et al. (1989). The initial primers used were H15149 (Kocher et al. 1989) and L14724 (Meyer et al. 1990; Pääbo 1990). Symmetric amplifications were performed in 25 μL of Tris (67 mM, pH 8.8) containing 2 mM $MgCl_2$, 1 mM of each dNTP, 1 μM of each primer, *Taq* polymerase (1.25 units, Perkin–Elmer–Cetus), and DNA (50–500 ng). The amplification regime consisted of 30 cycles of denaturation at 92°C for 40 s, annealing at 52°C for 60 s, and extension at 72°C for 90 s. Single-stranded DNA was produced for sequencing via asymmetric PCR (Gyllensten and Erlich 1988) using agarose electrophoresis purified double-stranded PCR products as templates (Kocher et al. 1989) and L14724 as the limited primer. Reaction conditions for asymmetric PCR were as above with the exceptions that one primer was held limiting, the final volume of reaction cocktail was increased to 50 μL, and the total number of cycles increased to 35. Thermal cycling was performed in a programmable heating block (Perkin–Elmer–Cetus) with negative (-DNA) controls included with each reaction set.

Following purification by centrifugal filtration, single-stranded DNA was sequenced by dideoxy chain termination using *Sequenase* Version 2.0 (United States Biochemical) and instructions supplied by the manufacturer. The limiting primer in the asymmetric PCR reaction (L14724) and an internal primer designed from actual sequences of *Gambusia* (L14952, 5′-TCYTCYGTYRCCCAYAT-3′) were used as the sequencing primers, with ^{35}S included to permit autoradiographic visualization. The dideoxy-terminated chains were separated by electrophoresis on 6% polyacrylamide gels using a combination of wedge and normal gels, run from 2 to 6 h. Following electrophoresis, all gels were vacuum-dried and exposed to X-ray film for 12–48 h.

Interspecies alignments and sequence manipulations utilized the PC-based program ESEE (Cabot and Beckenbach 1989). Nucleotide analyses, amino acid predictions, and transition/transversion patterns were generated using the program MacVector (Version 3.04; International Biotechnologies). Additional sequence comparisons were performed using GENBANK (Pearson and Lipman 1988) via electronic mail. Initial sequence conformation and codon position determinations were made using alignments to known mammalian cytochrome *b* sequences (Bibb et al. 1981; Smith and Patton 1991). Statistical comparisons were made using both parametric and nonparametric procedures, the parametric results being reported when the outcomes were identical. All sequences generated have been submitted to GENBANK (Accession Nos. U18107, U18115, U18206–U18228).

Methods of phylogenetic reconstruction

Phylogenetic analyses were performed using the "phylogenetic analysis using parsimony" (PAUP, 3.1) software package (Swofford 1993). Given the number of taxa involved, a heuristic algorithm was employed to search for the most parsimonious trees, using the following options: keep minimum trees only, collapse zero-length branches, random stepwise addition of taxa with 10 replications, tree bisection and reconnection. The monotypic genus *Belonesox* was used as an out-group (Rosen and Bailey 1963; Rauchenberger 1989; A. Meyer, unpublished data). Bootstrap measures of stability (Felsenstein 1985) were estimated with 200 iterations using PAUP. We employed several strategies in the parsimony analyses: all substitutions given equal weight; substitutions in the first and second codon positions weighted two and four times, respectively; and transversions weighted four times transitions. The various weighting schemes were used in an attempt to minimize potential noise created from character homoplasy, particularly in the third codon position. The data set was tested for nonrandom information structure with the randomization procedure described by Archie (1989*a*, 1989*b*), using the programs Randomiz and SumPAUP (J. Archie, personal communication). In addition, a skewness test statistic, $g1$, was calculated on the basis of the distribution of tree lengths of a random sample of 10 000 topologies. Data matrices with strong phylogenetic signals are predicted to produce tree-length distributions that are strongly skewed to the left (Hillis and Huelsenbeck 1992).

Tests for the presence of a molecular clock were generated using the DNAML and DNAMLK programs from PHYLIP (Felsenstein 1993). Log-likelihood estimates for pruned parsimony topologies were produced via the USER TREE option. Absolute differences (×2) between the log-likelihood estimates generated for each topology with and without the assumption of a molecular clock were tested for significance using the χ^2 distribution (Felsenstein 1993).

Results and discussion

Nucleotide sequence variation

Nucleotide sequences of up to 402 bases were obtained for the cytochrome *b* region from 33 specimens representing 24 species of *Gambusia* plus one specimen of *Belonesox belizanus* (Table 1, Fig. 1). Of the 402 sites, 137 (34.1%) were variable. Of these sites, 23 (16.7%) were in the first codon position, 7 (5.1%) were in the second, and 107 (78.1%) were in the third. Consistent with reports for other species and other mitochondrial segments (Brown et al. 1982; Kocher *et al.* 1989; Thomas and Beckenbach 1989), the third position of each codon was most variable (79.8% of all third positions), the first and second positions being substantially less (17.2 and 5.2%, respectively).

Mean pairwise percent sequence differences (uncorrected

Fig. 1. Nucleotide sequence for a 402-base segment from the mitochondrial DNA cytochrome *b* gene of poeciliid fishes. Twenty-nine sequences are reported from representatives of 24 species of *Gambusia* and 1 species of *Belonesox*. The letters above each triplet are amino acid designations.

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                                                  25 ↓                                                  50 ↓
                    *   A   N   L   R   K   T   H   P   L   L   K   V   A   N   D   A
affinis 1         ATG GCC AAC CTA CGA AAA ACC CAC CCC CTC CTA AAA GTC GCA AAC GAC GCA
affinis 2         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
caymanensis       ... ... ... ... ... ... ..G ... ... ... ... ... ... ... ... ..T ...
eurystoma         ... ... ... ... ... ... ... ... ... ... ... ... A.T ... ... ..T ...
geiseri           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T AT.
heterochir        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.T ...
hispaniolae       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ..T ...
holbrooki 1       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
holbrooki 2       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
hubbsi            ... ... ... ... ... ... ..G ... ... ... ..T ... ... ... ... ..T ...
hurtadoi          ... ... ... ... ... ... ... ... ... ..T ... ... ..T ... ... A.T AT.
luma              ... ... ... ... ... ... ... ... ... ... ... ... A.T ... ... ..T ...
manni             ... ... ... ... ... ... ..G ... ... ... ... ... ... ... ... ..T ...
marshi            ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.T ...
melapleura        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ...
nicaraguensis     ... ... ... ... ... ... ..G ... ... ... ... ... ... ... ... ..T ...
oligosticta       ... ... ... ... ... ... ..G ... ... ... ... ... ... ... ... ..T ...
panuco            ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ...
punctata          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ...
puncticulata      NNN N.. ... ... ... ... ..G ... ... ... ... ... ... ... ... ..T ...
rachowi           ... ... ... ... ... ... ... ... G.. ... ... ... ..T .T. ... ..A ...
rhizophorae 1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ...
rhizophorae 2     ... ... ... ... ... ... ..G ... ... ... ... ... ... ... ... ..T ...
sexradiata        ... ... ... ... ... ... ... ... ... ... ... ... A.T ... ... ..T ...
vittata           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T AT.
wrayi             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ...
yucatana 1        ... ... ... ... ... ... ..G ... ... ... ... ... ... ... ... ..T ...
yucatana 2        ... ... ... ... ... ... ..G ... ... ... ... ... ... ... ... ..T ...
Belonesox         ... .N. ... ... ... ... ... ... ..T ... ... ... A.T ... ..T A.T ...

                                                  75 ↓                                                  100 ↓
                    L   V   D   L   P   A   P   V   N   I   S   A   W   W   N   F   G
affinis 1         CTA GTG GAT CTT CCC GCT CCT GTC AAC ATC TCA GCC TGA TGA AAC TTT GGT
affinis 2         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
caymanensis       ... ... ... ... ... ..C ..C ... ... ... ... ... ... ... ..T ... ..C
eurystoma         ... ... ... ... ... ..C ... ..T ... ..T ... ... ... ... ..T ... ..C
geiseri           ... ... ... ... ... ... ..C ... ... ... ... ... ... ... ... ... ..A
heterochir        ... ..A ... ... ... ... ..C ... ... ... ... ... ... ... ... ... ..G
hispaniolae       ... ..C ..C ... ..T ..C ..C ... ... ... ... ... ... ..G ..T ... ..C
holbrooki 1       ... ... ... ... ... ..C ..C ... ... ... ... ... ..G ... ... ... ..A
holbrooki 2       ... ..A ... ... ... ... ..C ... ... ... ... ... ..G ... ... ... ..A
hubbsi            ... ..T ... ... ... ..C ..C ... ... ... ... ... ... ... ..T ... ..A
hurtadoi          ... ..A ... ... ... ..C ..C ... ..T ... ... ... ... ... ..T ... ...
luma              ... ..A ..C ..C ... ..C ..C A.. ... ... ... ... ... ... ... ... ..G
manni             ... ..T ... ... ... ..C ..C ... ... ... ... ... ... ... ..T ... ..G
marshi            ... ..A ... ..C ..A ..C ..C ... ... ... ... ... ... ... ..T ... ..C
melapleura        ... ... ... ... ... ..C ..C ... ... ... ..G ... ... ..G ..T ... ..A
nicaraguensis     ... ... ..C ... ... ..C ..C ... ... ... ... ... ..G ... ..T ... ..G
oligosticta       ... ... ... ... ... ..C ..C ... ... ... ... ... ... ... ..T ... ..C
panuco            ... ..A ... ... ... ..C ..C ... ... ... ... ... ... ... ..T ... ..A
punctata          ... ..A ... ..C ... ..C ..C ... ... ... ... ... ... ... ..T ... ...
puncticulata      ... ..A ..C ... ... ..C ..C ... ... ... ... ... ... ... ..T ... ..C
rachowi           G.. ..A ..C ... ..A .TC ... A.T ..T ... ... ... ... ... ... ... ..G
rhizophorae 1     ... ..A ... ..C ... ..C ..C ... ... ... ... ... ... ... ... ... ..G
rhizophorae 2     ... ..A ... ..C ... ..C ..C ... ... ... ... ... ... ... ... ... ..G
sexradiata        ... ... ... ... ... ..C ... ..T ... ..T ... ... ... ... ..T ... ..C
vittata           ... ..A ... ... ... ..C ... ... ... ... ..T ... ... ... ..T ... ..C
wrayi             ... ... ... ... ... ..C ..C ... ... ... ..G A.. ... ... ..T ... ..A
yucatana 1        ... ... ... ... ... ..C ..C ... ... ... ... ... ... ... ..T ... ..C
yucatana 2        ... ... ... ... ... ..C ..C ... ... ... ... ... ... ... ..T ... ..C
Belonesox         ... ..A ..C ... ..T ..C ..C AC. ..T ... ..C A.. ... ... ... ... ..C
```

Fig. 1 (*continued*).

							125 ↓									150 ↓		
	S	L	L	G	L	C	L	L	A	Q	I	L	T	G	L	F	L
affinis 1	TCC	CTT	CTA	GGA	CTT	TGC	CTT	ATT	ACT	CAG	ATC	CTG	ACC	GGC	CTT	TTC	CTA
affinis 2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
caymanensis	...	...	...	..G	...	...	...	...	G..	..A	...	..A	..A	...	...	...	...
eurystoma	...	...	...	..T	...	...	...	..A	G..	..A	...	..A	...	..G	...	...	..G
geiseri	...	...	...	..G	...	...	..A	..C	...	..A	...	..A	...	...	...	...	...
heterochir	...	...	...	..G	...	...	...	...	...	..A	..T	..A	...	...	...	...	...
hispaniolae	...	...	...	...	...	...	...	..C	...	..A	...	...	...	...	...	...	..G
holbrooki 1	...	...	...	...	...	...	...	...	...	...	...	..A	...	...	...	...	...
holbrooki 2	...	...	...	...	...	...	...	...	...	...	...	..A	...	...	...	...	...
hubbsi	...	...	...	...	...	...	...	...	G..	..A	...	...	...	..T	...	...	..G
hurtadoi	..A	...	...	..G	...	...	...	...	G..	..A	...	T.A	...	...	...	..T	...
luma	...	..A	T..	...	...	...	...	C..	G..	..A	...	..C	..A	...	...	...	...
manni	...	...	...	...	...	...	...	...	G..	..A	...	...	...	..T	...	...	..G
marshi	...	...	...	..G	...	...	...	...	G..	..A	...	T.A	...	..T	...	...	...
melapleura	...	..C	T..	..G	...	...	...	...	...	..A	...	..A	...	...	...	...	...
nicaraguensis	...	...	...	..G	...	...	...	...	G..	..A	...	...	..A	..T	...	...	...
oligosticta	...	...	...	..G	...	...	...	...	G..	..A	...	..A	..A	...	...	...	...
panuco	...	..C	...	..G	..C	...	...	...	G..	..A	...	T.A	...	..T	...	...	...
punctata	...	...	...	..G	...	...	...	...	G.C	..A	...	...	...	...	...	...	..T
puncticulata	...	...	...	...	...	...	...	...	G..	..A	...	..A	..A	..T	...	...	...
rachowi	...	..C	..T	..G	..C	...	...	C..	...	..A	...	..A	..A	...	...	...	..G
rhizophorae 1	...	...	...	..G	...	...	...	...	G..	..A	...	...	...	...	...	...	..T
rhizophorae 2	...	...	...	..G	...	...	...	...	G..	..A	...	...	...	...	...	...	..T
sexradiata	...	..C	...	..T	...	...	...	..A	G..	..A	...	...	...	..G	...	...	..G
vittata	...	...	...	..G	...	...	...	..C	...	..A	...	T.A	...	...	...	..T	...
wrayi	...	..C	...	...	...	...	...	...	G..	..A	...	..A	...	...	...	...	...
yucatana 1	...	...	...	...	...	...	...	...	G..	..A	...	..A	...	..T	...	...	...
yucatana 2	...	...	...	...	...	...	...	...	G..	..A	...	..A	...	..T	...	...	...
Belonesox	...	..A	...	..C	T.A	...	...	...	.TC	...	..T	T.A	..T	...	..C	...	...

							175 ↓								200 ↓		
	A	M	H	Y	T	S	D	I	S	T	A	F	S	S	V	A	H
affinis 1	GCA	ATG	CAC	TAC	ACC	TCT	GAT	ATC	TCT	ACA	GCA	TTC	TCA	TCT	GTC	GCC	CAT
affinis 2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..T	...	...
caymanensis	...	..A	...	...	...	...	...	...	...	...	...	...	...	...	..A	...	..C
eurystoma	...	..A	...	...	...	...	...	..T	..C	...	..C	...	..C	...	..T	...	..C
geiseri	...	...	...	...	...	...	...	...	...	...	...	..T	...	...	..T	...	...
heterochir	...	...	...	..T	...	...	...	...	...	...	...	..T	...	...	..A	...	...
hispaniolae	...	...	...	...	...	...	...	...	..A	...	...	...	...	...	...	...	...
holbrooki 1	...	...	...	...	...	...	...	..T	..C	...	...	...	...	...	...	...	...
holbrooki 2	...	...	...	...	...	...	...	..T	..C	...	...	...	...	...	...	...	...
hubbsi	...	..A	...	...	...	...	...	...	G.C	...	...	...	...	...	..A	...	...
hurtadoi	...	...	...	...	...	..C	...	..T	...	...	...	..T	...	...	..A	...	...
luma	...	..A	...	...	...	...	...	..T	...	...	..T	...	...	..C	..G	...	..C
manni	...	..A	...	...	...	...	...	...	G.C	...	...	...	...	...	..A	...	...
marshi	...	..A	...	...	...	...	..C	...	..C	...	...	...	..G	..C	..G	...	...
melapleura	...	...	...	...	...	...	...	..T	..C	...	..T	...	...	...	..G	...	...
nicaraguensis	...	..A	...	...	...	..C	...	...	...	...	...	..T	...	...	...	...	...
oligosticta	...	..A	...	...	...	...	...	...	...	...	...	...	...	...	..A	...	..C
panuco	...	..A	...	..T	...	...	..C	...	..C	...	...	...	...	..C	..G	...	..C
punctata	...	..A	...	...	...	...	...	..T	..C	...	...	...	...	...	..T	...	...
puncticulata	...	..A	...	...	...	...	...	...	...	...	...	...	...	...	..A	...	..C
rachowi	...	..A	...	..T	...	...	...	..T	A.C	...	...	...	...	...	..A	A.G	..C
rhizophorae 1	...	..A	...	...	...	...	...	..T	..C	...	...	...	...	...	..T	A..	...
rhizophorae 2	...	..A	...	...	...	...	...	..T	..C	...	...	...	...	...	..T	A..	...
sexradiata	...	..A	...	...	...	...	...	..T	..C	...	..C	...	..C	...	..T	...	..C
vittata	...	...	...	...	...	...	...	...	..C	...	...	...	...	...	..G	...	...
wrayi	...	...	...	...	...	...	...	..T	..C	...	..T	...	...	...	..A	A.T	...
yucatana 1	...	..A	...	...	...	...	...	...	...	...	...	...	...	...	..A	...	..C
yucatana 2	...	..A	...	...	...	...	...	...	...	...	...	...	...	...	..A	...	..C
Belonesox	...	..A	..T	..T	...	..C	..C	...	...	...	...	...	...	..C	..A	...	...

Fig. 1 (*continued*).

							225 ↓								250 ↓		
	I	C	R	D	V	N	Y	G	W	L	I	R	N	M	H	A	N
affinis 1	ATT	TGC	CGA	GAC	GTT	AAC	TAT	GGC	TGA	CTC	ATC	CGC	AAC	ATA	CAC	GCC	AAC
affinis 2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
caymanensis	...	...	...	...	...	...	..C	..A	...	...	...	..A	...	...	...	...	...
eurystoma	...	...	...	...	...	...	..C	..A	..G	..T	...	..A	...	...	...	...	..T
geiseri	...	...	...	...	...	...	..C	..A	...	...	...	..G	...	...	...	...	...
heterochir	...	...	...	...	..C	...	..C	..A	...	...	...	..G	...	...	...	...	...
hispaniolae	...	...	...	...	..C	...	C.C	..A	...	...	...	..A	..T	...	...	...	...
holbrooki 1	...	...	...	...	..C	...	...	..A	...	...	...	...	...	...	...	...	...
holbrooki 2	...	...	...	...	..C	...	...	..G	...	...	...	...	...	...	...	...	...
hubbsi	...	...	...	...	...	...	..C	..G	...	T..	...	..A	...	...	...	...	...
hurtadoi	...	...	...	...	...	...	..C	..G	...	...	C..	..A	...	...	...	...	..T
luma	...	...	...	..T	..A	...	..C	..A	...	...	...	..A	...	..G	...	...	..T
manni	...	...	...	...	...	...	..C	..A	...	T..	...	..A	...	...	...	...	...
marshi	...	..T	...	...	...	...	..C	..G	...	...	...	..A	..T	...	...	...	...
melapleura	...	...	...	...	...	...	..C	..G	...	...	...	..G	...	..G	...	...	...
nicaraguensis	...	...	...	...	...	...	..C	..A	...	..T	...	..G	...	...	...	...	...
oligosticta	...	...	...	...	...	...	..C	..A	...	...	...	..A	...	...	...	...	...
panuco	...	...	...	...	...	...	..C	..A	...	...	...	..A	...	...	...	...	...
punctata	...	...	...	...	...	...	..C	..G	...	...	..T	..A	...	...	..T	..T	...
puncticulata	...	...	...	...	...	...	..C	..A	...	...	...	..A	...	...	...	...	...
rachowi	...	...	..T	..T	..A	...	.TC	..G	..G	..T	...	..A	..T	...	...	...	...
rhizophorae 1	...	...	...	...	...	...	..C	..A	...	...	...	..A	..T	...	..T	..T	...
rhizophorae 2	...	...	...	...	...	...	..C	..A	...	...	...	..A	..T	...	...	..T	...
sexradiata	...	...	...	...	...	...	..C	..A	..G	...	...	..A	...	...	...	...	..T
vittata	...	...	...	...	...	...	..C	..A	...	...	...	..A	...	...	...	...	..T
wrayi	...	...	...	...	...	...	..C	..A	...	...	...	..A	...	...	...	...	...
yucatana 1	...	...	...	...	...	...	..C	..A	..G	...	...	..A	...	..G	...	...	...
yucatana 2	...	...	...	...	...	...	..C	..A	..G	...	...	..A	...	..G	...	...	...
Belonesox	..C	...	...	..T	..A	...	..C	..G	...	...	...	..A	...	...	...	...	...

							275 ↓								300 ↓		
	G	A	S	F	F	F	I	C	I	Y	L	H	I	G	R	G	L
affinis 1	GGG	GCC	TCT	TTC	TTT	TTT	ATT	TGT	ATC	TAC	CTA	CAC	ATC	GGC	CGA	GGA	CTA
affinis 2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
caymanensis	...	...	..C	...	..C	...	..C	...	..T	..T	A..	...	...	...	...	..C	T..
eurystoma	..A	...	...	...	...	...	..C	...	..T	..T	...	...	..T	...	...	..G	T..
geiseri	...	...	...	...	...	...	..C	...	..T	..T	...	..T	...	...	...	...	...
heterochir	...	...	...	...	...	...	..C	...	..T	..T	T..	..T	...	..T	...	..G	...
hispaniolae	...	...	...	...	...	..C	..C	...	...	..T	T..	...	...	...	...	..C	T.G
holbrooki 1	..A	...	...	...	...	..C	...	...	...	...	...	...	...	...	...	...	...
holbrooki 2	..A	...	...	...	...	..C	...	...	...	...	...	...	...	...	...	..G	...
hubbsi	...	...	..C	...	..C	...	..C	...	..T	..T	...	..T	...	...	...	..C	T..
hurtadoi	..A	...	...	...	...	...	..C	...	..T	...	...	...	...	...	...	..T	..G
luma	...	...	..C	...	...	...	..C	..C	C..	...	..T	...	...	...	...	..G	T..
manni	...	...	..C	...	..C	...	..C	...	..T	..T	...	..T	...	...	...	..C	T..
marshi	...	...	..C	...	...	...	...	...	..T	..T	A..	...	...	...	...	..C	...
melapleura	...	...	...	...	...	..C	...	...	...	...	..G	...	...	..T	...	..C	T..
nicaraguensis	...	...	..C	...	..C	...	..C	...	..T	...	...	...	...	...	...	..C	T..
oligosticta	...	...	..C	...	..C	..C	..C	...	..T	..T	A..	...	...	...	...	..C	T..
panuco	...	...	...	...	...	...	...	...	..T	..T	T..	...	...	...	...	..C	...
punctata	...	..T	..C	...	...	...	..C	...	..T	...	...	...	...	...	...	..C	T..
puncticulata	...	...	..C	...	..C	...	..C	...	..T	..T	A..	...	...	...	...	..C	T..
rachowi	...	...	...	...	...	...	..C	...	...	...	...	...	...	...	...	...	T..
rhizophorae 1	...	...	...	...	...	...	..C	...	...	...	...	...	..T	...	...	..C	...
rhizophorae 2	...	...	...	...	...	...	..C	...	...	...	...	...	..T	...	...	..C	T..
sexradiata	...	...	...	...	...	...	..C	..C	..T	..T	...	...	..T	...	...	...	T..
vittata	..A	...	...	...	...	...	..C	..C	...	...	...	...	...	...	...	..C	...
wrayi	...	...	...	...	...	..C	..C	...	...	...	..G	...	...	..T	...	..C	T..
yucatana 1	...	...	..C	...	..C	...	..C	...	..T	..T	A..	...	...	...	...	..C	T..
yucatana 2	...	...	..C	...	..C	...	..C	...	...	..T	A..	...	...	...	...	..C	T..
Belonesox	..A	...	..C	...	..C	..C	...	...	..T	..T	..C	...	..T	..T	...	..C	T..

Fig. 1 (*concluded*).

						325 ↓									350 ↓		
	Y	Y	G	S	Y	L	F	K	E	T	W	N	T	G	V	I	L
affinis 1	TAC	TAC	GGC	TCC	TAC	CTA	TTT	AAA	GAG	ACA	TGA	AAC	ACT	GGT	GTA	ATC	CTT
affinis 2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
caymanensis	...	...	..A	...	...	..C	..C	...	..A	...	...	..T	...	...	..C	..T	..C
eurystoma	...	...	...	...	.T.	...	..C	...	...	...	...	...	...	...	..T	...	...
geiseri	...	...	..G	...	...	..G	..C	...	...	...	...	...	..C	..C	..T	...	...
heterochir	...	...	...	...	...	..C	..C	...	...	...	...	...	..C	...	..T	...	..A
hispaniolae	...	...	...	...	...	..C	..C	...	...	..C	...	...	..C	...	..T	G..	..C
holbrooki 1	...	...	...	...	..T	...	...	...	...	...	...	...	...	...	...	...	...
holbrooki 2	...	...	...	...	..T	...	...	...	...	...	...	...	...	...	...	...	...
hubbsi	...	...	..G	...	...	..C	..C	...	...	...	...	...	...	...	..T	..T	...
hurtadoi	...	...	...	...	...	...	...	...	..A	...	..G	..T	...	...	..T	...	..C
luma	...	...	...	...	..T	..C	..C	...	..A	..C	...	...	CTG	...	..T	...	..G
manni	...	...	..G	...	...	..C	..C	...	...	...	...	...	...	...	..T	..T	..C
marshi	...	..T	...	...	...	..C	..C	...	...	...	...	...	...	...	..C	...	...
melapleura	...	...	...	...	...	..C	..C	...	...	...	...	...	..C	...	..T	...	..C
nicaraguensis	...	...	..A	...	...	..C	..C	...	..A	..G	..G	...	..C	...	..T	...	..C
oligosticta	...	...	..A	...	...	..C	..C	...	..A	...	...	..T	...	...	..C	..T	..C
panuco	...	...	...	...	...	..C	..C	...	...	...	...	...	...	...	..T	...	...
punctata	..T	...	...	...	...	...	..C	...	...	..T	...	..T	..C	...	..C	...	..C
puncticulata	...	...	..A	...	...	..C	..C	...	..A	...	...	..T	...	...	..C	..T	..C
rachowi	...	...	...	...	...	..T	..C	...	...	...	...	...	...	...	...	...	...
rhizophorae 1	..T	...	...	...	...	..G	..C	...	..A	..T	...	..T	..C	...	..C	...	...
rhizophorae 2	..T	...	...	...	...	..G	..C	...	..A	..T	...	..T	..C	...	..C	...	...
sexradiata	...	...	...	...	.T.	..C	..C	...	...	...	...	...	...	...	..T	...	...
vittata	...	...	...	...	...	..C	..C	...	...	...	...	...	...	...	..C	...	...
wrayi	...	...	...	...	...	..C	..C	...	...	...	...	...	..C	...	..T	...	..C
yucatana 1	...	...	..A	...	...	..C	..C	...	..A	...	...	...	...	...	..T	..T	..C
yucatana 2	...	...	..G	...	...	..C	..C	...	..A	...	...	...	...	...	..T	..T	..C
Belonesox	..T	...	...	...	..T	..T	..C	...	..A	...	...	...	...	...	..T	..A	T.A

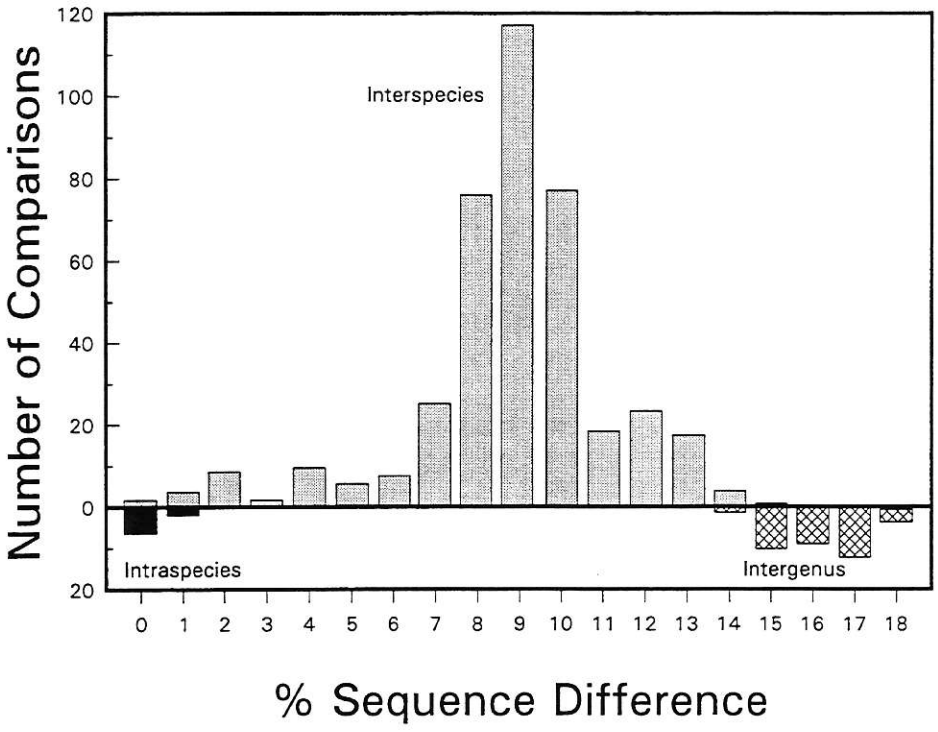
						375 ↓								400 ↓	
	L	L	L	V	I	I	T	A	F	V	G	Y	V	L	P
affinis 1	CTT	CTT	CTA	GTC	ATA	ATA	ACC	GCC	TTC	GTA	GGT	TAT	GTC	CTA	CCC
affinis 2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
caymanensis	..C	..C	...	..T	...	...	...	...	...	...	...	..C	.NN	NNN	NNN
eurystoma	..C	...	...	..T	...	...	...	...	..T	...	..A	...	...	..C	...
geiseri	...	...	...	..T	...	...	...	...	...	...	..C	...	..T	...	...
heterochir	...	...	...	..T	...	...	...	...	...	...	...	...	..T	...	...
hispaniolae	..C	..C	..G	...	...	...	..A	...	..T	...	..C	...	...	...	...
holbrooki 1	...	..C	...	..T	...	...	..T	...	...	...	...	...	...	...	...
holbrooki 2	...	..C	...	..T	...	...	..T	...	...	...	...	...	...	...	...
hubbsi	...	..C	...	..T	...	..G	..A	...	..T	...	...	..C	...	..G	...
hurtadoi	..C	..C	..G	..T	...	...	...	...	...	...	..G	...	..T	...	...
luma	...	..C	..G	..T	...	...	...	...	...	...	...	...	...	..C	...
manni	...	..C	...	..T	...	..G	..A	...	..T	...	...	..C	...	...	...
marshi	..C	...	...	..T	...	...	...	...	...	...	..G	...	..T	...	...
melapleura	..C	..C	...	..T	...	...	..G	...	..N	...	..C	..N	NNN	NNN	NNN
nicaraguensis	..C	..C	...	..T	...	..G	...	...	...	..G	...	..C	...	..T	...
oligosticta	..C	..C	...	..T	...	...	...	...	...	...	...	..C	...	...	...
panuco	..C	...	...	...	...	...	...	...	..N	NNG	..A	...	..T	...	...
punctata	..C	..C	..G	..A	...	...	...	...	...	...	..C	...	...	...	...
puncticulata	..C	..C	...	..T	...	...	...	...	...	...	...	..C	...	...	...
rachowi	..C	...	..G	...	...	...	...	...	...	...	..C	...	...	...	...
rhizophorae 1	...	...	..N	N.N	...	..G	...	...	...	...	..C	...	...	...	...
rhizophorae 2	...	...	..G	..G	...	..G	...	...	...	...	..C	...	...	...	...
sexradiata	..C	...	...	..T	...	...	...	...	..T	...	...	...	...	..C	...
vittata	..C	...	..G	...	...	...	...	...	...	...	..G	...	...	...	...
wrayi	..C	..C	...	..T	...	...	...	...	..T	...	..C	...	...	..C	...
yucatana 1	..C	..C	...	..T	...	...	...	...	...	...	..C	..C	...	...	...
yucatana 2	..C	..C	...	..T	...	...	...	...	...	...	..C	..G	C..	...	...
Belonesox	...	..C	..C	...	..G	..G	...	...	..T	...	..A	...	...	..C	...

Table 2. Nucleotide differences for cytochrome *b* among 25 species of poeciliid fishes.

Species	1	2	3	4	5	6	7	8	9	10	11	12
1 *affinis* 1	—	26/10	31/11	23/6	24/7	32/8	13/2	26/12	34/8	31/19	26/11	29/9
2 *caymanensis*	2.6	—	23/14	26/8	25/7	30/9	29/10	17/7	30/8	27/16	15/6	24/5
3 *eurystoma*	2.8	1.6	—	31/11	33/12	33/14	32/11	23/18	36/11	30/20	23/17	30/12
4 *geiseri*	3.8	3.2	2.8	—	14/5	32/9	29/4	29/9	31/8	38/17	29/8	29/9
5 *heterochir*	3.4	3.6	2.8	2.8	—	31/8	29/5	31/8	32/7	37/12	28/7	28/6
6 *hispaniolae*	4.0	3.3	2.4	3.6	3.9	—	31/9	32/8	42/9	35/18	29/7	36/8
7 *holbrooki* 2	6.5	1.7	2.9	7.3	5.8	3.4	—	30/11	35/8	32/17	30/10	33/9
8 *hubbsi*	2.2	2.5	1.3	3.2	3.9	4.0	2.7	—	40/11	30/20	4/1	27/10
9 *hurtadoi*	4.3	4.8	3.3	3.9	4.6	4.7	4.4	3.6	—	36/19	39/10	36/5
10 *luma*	3.4	1.7	1.6	2.2	3.1	1.9	1.9	1.7	1.9	—	31/19	35/18
11 *manni*	2.4	2.5	1.4	3.6	4.0	4.1	3.0	4.0	3.9	1.6	—	28/9
12 *marshi*	3.2	4.8	2.5	3.2	4.7	4.5	3.7	2.7	7.2	1.9	3.1	—
13 *melapleura*	3.4	3.9	2.8	3.7	7.3	4.5	3.3	5.2	5.1	2.4	6.4	5.2
14 *nicaraguensis*	3.1	3.0	2.6	5.5	4.4	3.8	4.5	3.4	3.4	2.1	5.2	3.8
15 *oligosticta*	2.7	1.0	1.6	3.5	3.9	3.2	2.8	2.7	4.0	1.6	3.5	5.2
16 *panuco*	3.3	4.2	3.4	4.8	8.7	5.0	5.3	4.3	9.5	2.5	2.7	5.3
17 *punctata*	4.1	2.4	2.4	4.6	2.9	5.2	4.7	2.2	4.3	1.7	2.3	3.4
18 *puncticulata*	2.8	4.0	2.3	3.9	4.7	3.2	3.1	2.4	4.1	1.5	2.3	5.0
19 *rachowi*	1.9	2.2	1.1	2.4	2.9	1.8	2.5	2.3	2.2	1.7	1.1	2.4
20 *rhizophorae*	3.7	2.9	2.5	5.8	5.0	5.7	6.4	3.0	5.3	1.8	3.0	3.4
21 *sexradiata*	2.4	2.3	1.7	2.6	3.1	3.1	2.8	1.5	3.2	1.4	1.6	2.8
22 *vittata*	2.9	4.7	2.4	2.7	6.0	4.1	3.3	3.8	6.8	1.9	4.1	9.0
23 *wrayi*	3.0	4.2	2.7	4.1	7.8	3.1	3.6	3.4	5.1	2.7	3.7	5.8
24 *yucatana* 1	3.1	7.0	1.5	4.0	4.8	3.6	3.4	2.8	5.0	1.4	2.8	6.8
25 *Belonesox*	3.1	3.4	2.2	3.3	4.1	3.2	2.9	2.6	4.3	2.9	2.8	4.2

NOTE: The numbers of transitions/transversions are listed above the diagonal with the corresponding ratios below the diagonal.

Fig. 2. Sequence differences among poeciliids at three taxonomic levels: intraspecies, interspecies, and intergenus.



Species												
13	14	15	16	17	18	19	20	21	22	23	24	25
27/8	31/10	27/10	26/8	29/7	28/10	33/17	26/7	29/12	23/8	27/9	28/9	52/17
27/7	18/3	1/0	25/6	22/9	4/0	37/17	26/9	25/11	28/6	25/6	7/1	47/14
31/11	34/13	24/15	37/11	29/12	35/15	26/24	30/12	5/3	26/11	27/10	21/14	53/16
26/7	33/6	28/8	29/6	32/7	31/8	40/17	29/5	31/12	22/8	29/7	28/7	56/17
29/4	31/7	27/7	26/3	32/8	33/7	40/14	30/6	34/11	30/5	31/4	29/6	49/12
27/6	34/9	29/9	35/7	31/6	29/9	35/20	34/6	34/11	29/7	25/8	29/8	51/16
23/7	36/8	28/10	32/6	33/7	31/10	38/15	32/5	34/12	26/8	25/7	31/9	49/17
31/6	24/7	19/7	30/7	26/12	17/7	42/18	30/10	23/15	34/9	27/8	17/6	47/18
36/7	34/10	32/8	28/4	30/7	33/8	41/19	37/7	38/12	27/4	36/7	35/7	55/13
31/13	32/15	28/17	37/15	30/18	25/17	34/20	29/16	27/19	32/17	32/12	26/18	47/16
32/5	31/6	21/6	16/6	21/11	14/6	19/17	27/9	23/14	33/8	26/7	14/5	48/17
31/6	34/9	26/5	16/3	27/8	25/5	39/16	27/8	31/11	27/3	35/6	27/4	51/12
—	31/6	26/7	31/3	24/8	31/7	37/16	32/6	32/8	28/5	12/1	26/6	52/13
5.2	—	19/4	35/6	29/9	18/4	44/17	32/7	36/10	37/8	33/5	19/5	53/15
3.7	4.8	—	27/6	23/9	5/0	38/17	27/9	26/12	29/6	24/7	8/1	46/15
10.3	5.8	4.5	—	31/7	26/6	34/15	33/5	27/10	28/2	32/3	26/5	48/11
3.0	3.2	2.6	4.4	—	24/9	31/20	15/1	30/11	27/7	24/8	27/8	53/16
4.4	4.5	5.0	4.3	2.7	—	38/17	32/9	27/12	30/6	27/7	8/1	46/15
2.3	2.6	2.2	2.3	1.6	2.2	—	29/18	28/23	32/17	35/16	37/18	48/24
5.3	4.6	3.0	6.6	15.0	3.6	1.6	—	30/11	26/7	28/6	31/8	53/15
4.0	3.6	2.2	2.7	2.7	2.2	1.2	2.7	—	27/10	28/7	24/11	57/15
5.6	4.6	4.8	14.0	3.9	5.0	1.9	3.7	2.7	—	29/5	32/5	59/9
12.0	6.6	3.4	10.7	3.0	3.9	2.2	4.7	4.0	5.8	—	24/6	51/12
4.3	3.8	8.0	5.2	3.4	8.0	2.1	3.9	2.2	6.4	4.0	—	49/14
4.0	3.5	3.1	4.4	3.3	3.1	2.0	3.5	3.8	6.6	4.3	3.5	—

for multiple hits) ranged from 0.0 to 1.0% within a species, with the highest value observed in the comparison of *G. holbrooki* from Orlando, Florida, with *G. holbrooki* from Key West, Florida (Table 2). Interspecies values averaged 9.1% with a range from 1.0 to 15.2% (Fig. 2). The lowest values observed were for comparisons between *G. oligosticta*, *G. caymanensis*, *G. puncticulata*, and *G. yucatana* of the *puncticulata* species-group (range 1.0–2.8%), *G. hubbsi* and *G. manni* of the *puncticulata* species-group (1.2%), and *G. sexradiata* and *G. eurystoma* (2.0%). The highest average interspecies differences were found for *G. luma* and *G. rachowi* versus all other species of *Gambusia* (range 10.6–15.2%). Intergeneric percent differences between the out-group, *Belonesox*, and *Gambusia* ranged from 14.8 to 18.2%. Overall, with the exception of a few extreme values, intraspecies, interspecies, and intergenus percent sequence differences clustered into distinct groups that exhibited minor overlap (Fig. 2).

Base compositional bias

Differences among codon positions were also noted when base composition and bias were considered (Fig. 3). For *Gambusia*, as with other vertebrates (Irwin et al. 1991), the greatest departure from equal base utilization occurred in the third position, where G was found very infrequently (average 5.9%), while C was most abundant (average 44.6%). Second-position nucleotides exhibited a less deviant but similar pattern, G again being the least frequent base (15.7%) but T now being most frequent (39.7%). Only a slight departure from equal representation was noted in the first positions, with all nucleotides averaging within 2% of expected. Estimates of compositional bias (Fig. 3) reflected this pattern, with averages increasing from near zero for first positions to 0.293 in third positions. Meyer (1993) observed that anti-G bias is common in fish cytochrome *b* sequences, especially in third positions.

Transitions and transversions

The number of transitions and transversions for all pairwise comparisons are shown in Table 2. The mean number of transitions among species of *Gambusia* was 28.5 (range 1–44) and transversions 9.3 (range 0–24). The mean number of transitional differences between *Belonesox* and all species of *Gambusia* was 48.8, while transversions averaged 14.5. The mean transition/transversion ratio for *Gambusia* was 3.7 (range 1.0–15.0), with a lower value, 3.6 (range 2.0–6.6), observed among the *Belonesox* with *Gambusia* comparisons (Table 2).

Several investigators (Cracraft and Helm-Bychowski 1991; Smith and Patton 1991; Meyer 1993,1994) have noted that the disparity in substitution rates between third positions and first/second positions can affect overall measures of sequence difference. The general prediction from these studies is that, owing to a greater rate of evolution, third positions will become saturated at low overall levels of sequence divergence, so they become phylogenetically noninformative. For our data, percent sequence differences at the third position were well below the 30–40% range reported among cichlid

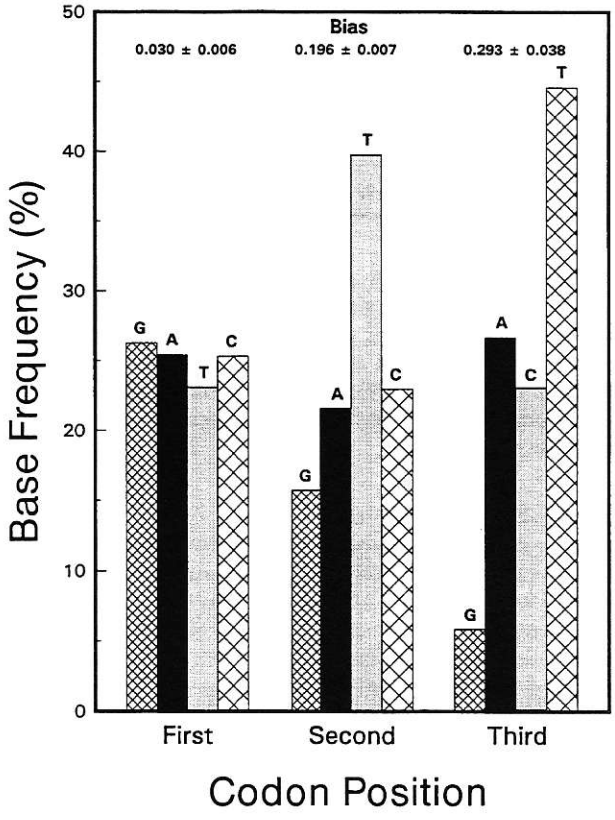
Table 3. Total silent and replacement differences from comparisons between a nucleotide sequence from *Gambusia affinis* and each of 27 additional sequences.

	Codon position			
	First	Second	Third	Total[a]
Transitions				
Silent	26 (2.8)	0	632 (67.4)	658 (70.2)
Replacement	39 (4.2)	6 (0.6)	0	45 (4.8)
Transversions				
Silent	0	0	216 (23.0)	216 (23.0)
Replacement	16 (1.7)	3 (0.3)	0	19 (2.0)

NOTE: Numbers in parentheses are percentages.

[a]Cell and row total percentages were calculated independently as proportions of the total count (938) × 100.

Fig. 3. Percent frequencies of the four nucleotides from a 402-base segment of the mitochondrial cytochrome *b* gene separated as to their codon positions. Values represent averages across 28 sequences from *Gambusia*. Standard deviations were all less than 3.0%. Base composition bias was calculated using the formula of Prager and Wilson (1988).



fishes where saturation was thought to occur (Meyer 1993). Nonetheless, these results suggest that even when considering taxa with relatively low levels of sequence divergence, attention must be given to the dynamics of sequence evolution prior to attempts at phylogenetic reconstruction.

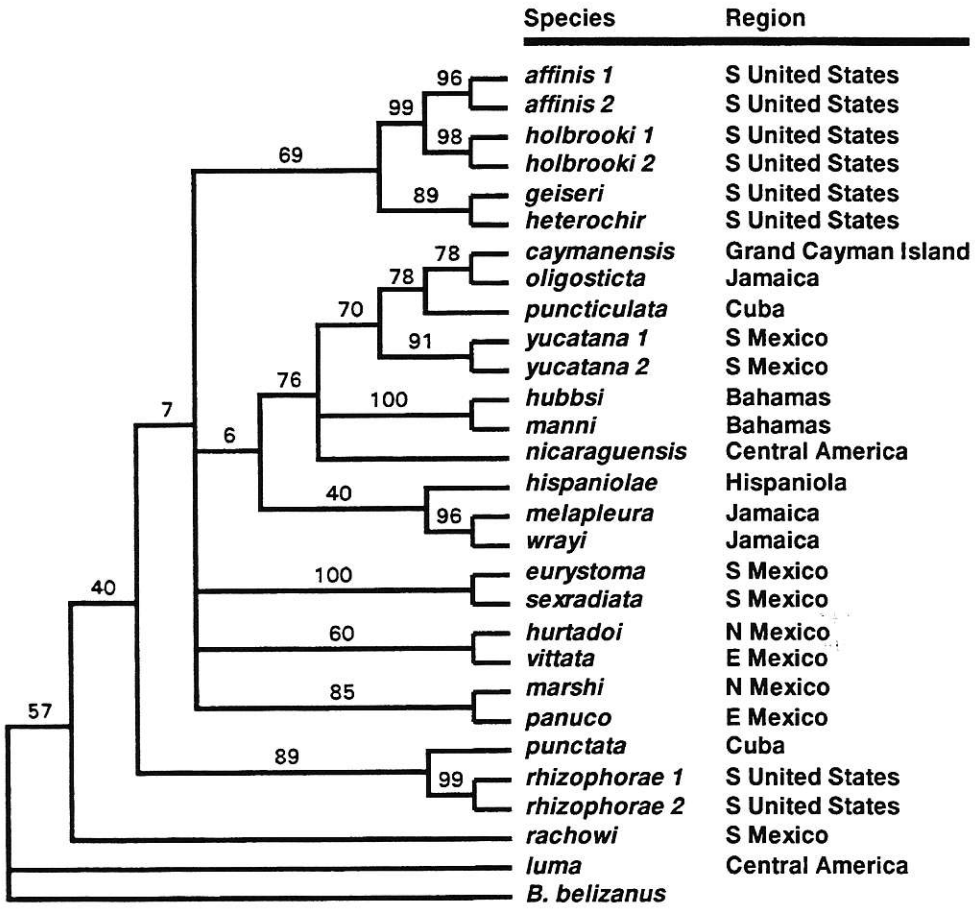
Amino acid differences

Based on comparisons between the sequence from *G. affinis* 1 and each of the remaining 27 *Gambusia* sequences (Fig. 1), the inferred number of amino acid replacements that occurred in each codon position along the cytochrome *b* sequence were determined. The 402-base fragment was translated into an amino acid sequence 134 residues in length. Of the 134 residues, 20 were variable (12 phylogenetically informative) among the taxa. Most changes in first- and all in second-codon positions are predicted to result in replacement substitutions, while all changes in the third positions represent silent substitutions (Table 3). All transversions observed in both the first and second positions would result in amino acid replacements. Fourteen of the amino acid replacements were due to either a transition or a transversion in the first-codon position. Three replacements were from transitions in the second-codon position. Three replacements were from a change in both the first- and second-codon positions. Interestingly, 32% (Table 3) of the total substitutions in the first-codon position would not result in amino acid changes. These silent substitutions were leucine/leucine.

Systematics and phylogenetic analyses of *Gambusia*

The first analytical approach, in which all base substitutions were weighted equally, produced three equally parsimonious trees of 426 steps with a consistency index (CI) of 0.45 or 0.40, excluding uninformative characters. A strict consensus tree of the three equally parsimonious trees is shown in Fig. 4, with bootstrap values (Felsenstein 1985) provided for each monophyletic group. To test whether our cytochrome *b* data set contained nonrandom and, thus, informative phylogenetic information, a comparison between the number of steps required to produce our observed and a distribution of minimum steps from 200 randomized-data trees was made (Archie 1989*a*, 1989*b*). For each randomized data set, minimum-length trees were estimated using PAUP (with options HOLD = 10, MULPARS and SWAP = GLOBAL). The minimum tree length of 426 for the three equally parsimonious topologies generated from the observed data was significantly less (one-tailed $t = 564.8$, df = 199, $P < 0.001$) than the mean length (561.8) from the 200 randomized data trees. In addition, the random topology distributions for the equal-weight trees and the two weighted trees all demonstrated significant skewness ($g1 \geq -0.534$; $P < 0.01$ for all three analyses). While interpretations of results from resampling algorithms in general are controversial, we do believe that the results from these analyses clearly indicate that the sequencing of the cytochrome *b* gene region yielded signifi-

Fig. 4. A strict consensus tree for three equally parsimonious topologies for *Gambusia* derived from up to 402 bases of a segment of the mitochondrial cytochrome *b* gene. Bootstrap values (percentages of 200 replicates) are given for each node. "Region" identifies general geographic areas from which specimens were obtained.



cant nonrandom phylogenetic information suitable for estimating a hypothetical evolutionary topology for these 25 taxa.

The second analysis, weighting first and second codon position substitutions two and four times, respectively, resulted in four equally parsimonious trees of total length 499 and a CI of 0.48 or 0.41, excluding uninformative characters. A strict consensus tree of the four equally parsimonious trees is shown in Fig. 5. The final analysis, weighting transversions four times transitions, yielded one tree of total length 695 (Fig. 6). An identical tree was obtained when transversions were weighted five through eight times.

All three analytical approaches yielded several phylogenetic patterns in common. *Gambusia luma* was consistently depicted as the most basal clade. This result was unexpected, but not unlikely given previous difficulty in assigning this species to a particular species-group. For instance, *G. luma* has been placed in the *punctata* species-group (Rosen and Bailey 1963; Rauchenberger 1989) and the *nobilis* species-group (Rivas 1963). The next most basal clade contained *G. rachowi*. Although they were placed in the genus *Gambusia* by Rosen and Bailey (1963) and Rauchenberger (1989), other investigators have considered them to be in a separate but closely related genus called *Heterophallus* (Regan 1914; Hubbs 1926). Our data support *G. rachowi* being a distinct, basal member of the genus, but placing it in a separate genus would render *Gambusia* paraphyletic. We believe that *G. rachowi* should remain in the genus *Gambusia* until a phylogeny of the entire family Poeciliidae is available.

In addition to the above patterns, all three phylogenetic analyses resulted in trees that contained the following monophyletic groups, many of which correspond to traditional species-group assignments (Table 1): *G. affinis* + *G. holbrooki* of the *affinis* species-group + *G. geiseri* of the *senilis* species-group + *G. heterochir* of the *nobilis* species-group; *G. caymanensis* + *G. oligosticta* + *G. puncticulata* + *G. yucatana* + *G. hubbsi* + *G. manni* of the *puncticulata* species-group + *G. nicaraguensis* of the *nicaraguensis* species-group; *G. melaplura* + *G. wrayi*, Antillean members of the *nicaraguensis* species-group; *G. eurystoma* + *G. sexradiata* of the *nobilis* species-group; *G. punctata* + *G. rhizophorae* of the *punctata* species-group; *G. hurtadoi* of the *senilis* species-group + *G. vittata*; and *G. marshi* + *G. panuco* of the *panuco* species-group.

Perhaps the most unusual finding was the sister relationship between *G. hurtadoi* of the *senilis* species-group and *G. vittata* of the subgenus Heterophallus (Rauchenberger 1989). *Gambusia vittata* has been considered by some to be

Fig. 5. A strict consensus tree for four equally parsimonious topologies for *Gambusia* derived from up to 402 bases of a segment of the mitochondrial cytochrome *b* gene. For this analysis, first- and second-codon positions were weighted two and four times, respectively. Bootstrap values (percentages of 200 replicates) are given for each node.

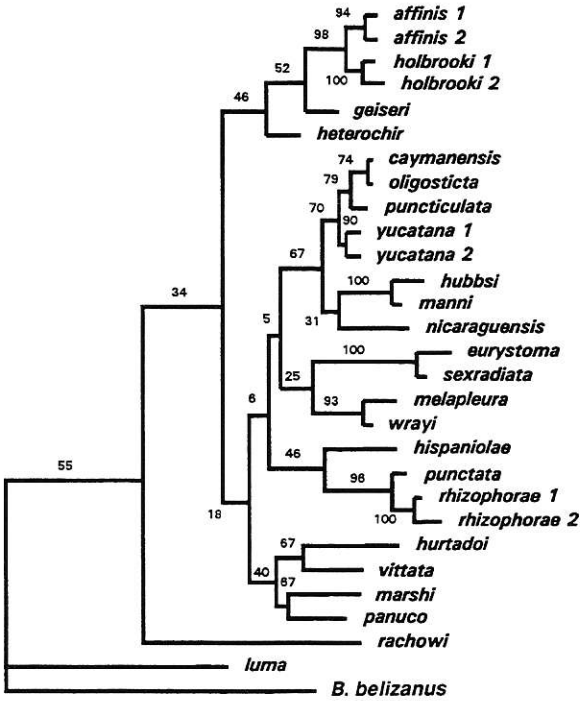
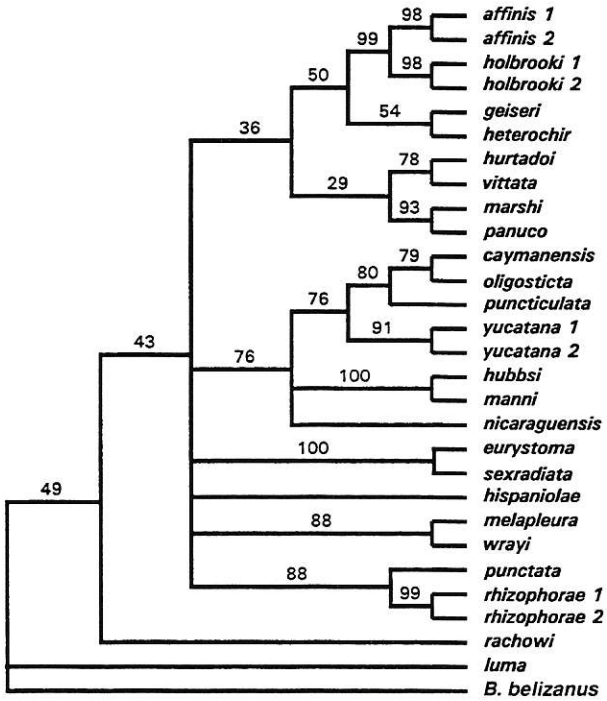


Fig. 6. The single minimum-length topology for *Gambusia* derived from up to 402 bases of a segment of the mitochondrial cytochrome *b* gene when transversions were weighted four times transitions. Bootstrap values (percentages of 200 replicates) are given for each node.



closely related to *G. marshi* and *G. panuco* of the *panuco* species-group (Rauchenberger 1989), while others have placed it in its own genus, *Flexipenis* (Rivas 1963). Our data suggest that *G. vittata* is not only a member of the genus *Gambusia*, but together with *G. hurtadoi* is sister to the *panuco* species-group. This pattern was found in one of the three equally parsimonious trees with substitutions equally weighted and in the trees from the other two analyses.

The *puncticulata* species-group was examined in detail by Fink (1971*a*). He suggested that the nominal forms, as used herein, represented a single species; he synonymized the eight forms in the group under the name *G. puncticulata* as follows: *G. puncticulata puncticulata* (= *G. puncticulata*, *G. oligosticta*, *G. caymanensis*, *G. hubbsi*, and *G. howelli*); *G. p. manni*, *G. p. bucheri*, *G. p. baracoana*, and *G. p. yucatana*. The DNA-based trees support his view in part. *Gambusia puncticulata* s.str. appears to be closely related to *G. oligosticta* and *G. caymanensis*; however, *G. hubbsi* did not group with *G. p. puncticulata* but with *G. manni*, as Greenfield and Wildrick (1984) and Rauchenberger (1989) hypothesized. Based on examinations of morphological characters of the *puncticulata* species-group and allozyme data from *G. yucatana*, *G. hubbsi*, and *G. caymanensis*, Greenfield and Wildrick (1984) hypothesized that the *puncticulata* clade could be divided into three distinct groups: the *yucatana* complex containing *G. yucatana*, which they believed warranted species-level status; the *puncticulata* forms (= *G. oligosticta*, *G. puncticulata*, *G. howelli*, and *G. caymanensis*); and the *hubbsi* forms (*G. bucheri*, *G. baracoana*, *G. monticola*, *G. manni*, and *G. hubbsi*). Our phylogeny supports their hypothesis with the exception of their placement of *G. yucatana* as the sister-species to all Antillian *Gambusia*; our data suggest that it is sister to only the *puncticulata* forms. In addition, unlike previous investigators, we found that *G. nicaraguensis* is not sister to Antillean members of the hypothesized *nicaraguensis* group (*G. wrayi* and *G. melaplura*; Table 1), but is either the most basal member of the *puncticulata* species-group or sister to *G. manni* and *G. hubbsi* of the *puncticulata* species-group.

Differences among the phylogenies obtained from the three different weighting schemes appear to be associated with more weakly supported basal nodes and the placement of particular problematic taxa. For instance, *G. hispaniolae* is sister to *G. wrayi* + *G. melaplura* when substitutions are given equal weighting; however, when first and second positions are weighted differently, *G. hispaniolae* is either sister to *G. wrayi* + *G. melaplura* (one of four equally parsimonious trees) or is the next most basal clade following the clades of *G. luma* and *G. rachowi* (three of four equally parsimonious trees). Alternatively, when tranversions are weighted four times transitions, *G. hispaniolae* is sister to Antillean members of the *punctata* species-group. All bootstrap values for these alternative patterns are below 50%. These problems are not, however, unique to this study. Rauchenberger (1989) hypothesized *G. hispaniolae* to be a member of the *nicaraguensis* species-group, while Fink (1971*b*) placed *G. hispaniolae* with Antillean members of the *nicaraguensis* species-group. We believe problems such as these can be resolved by examining sequence data from other genes that

exhibit greater variability in the more conservative first and second positions and (or) by looking at more conservative noncoding regions.

Based on a cladistic analysis of the entire genus, Rauchenberger (1989) recognized three subgenera of *Gambusia*: Heterophallina, Arthrophallus, and Gambusia (see Table 1). Heterophallina was defined by two synapomorphies, distinctively shaped hooks on rays 4p and 5a of the gonopodium and a unique morphology of the medial teeth of the tooth plate of the third intrapharyngobranchial (IPB3). Originally (Hubbs 1926), Heterophallina included *G. vittata*, *G. panuco*, and *G. regani* (*G. marshi* was not described yet). However, Rauchenberger (1989) chose to include *G. rachowi* and *G. echeagayari*. The DNA-based phylogeny supports the close relationships of members of the *panuco* species-group and *G. vittata*, but does not support placement of *rachowi* species group members in the subgenus. It is likely that the distinctively shaped hooks of the gonopodium evolved independently, and the IBP3 teeth with medial serrate pad character is found in only three of the six species assigned to the subgenus.

The subgenus *Arthrophallus* is defined by the presence of a distinct break in the infraorbital section of the cephalic sensory canal system, leaving two discontinuous grooves. Rauchenberger (1989) stated that it is found "in all members" (p. 37); however, in actuality it is missing in three species (*G. krumholzi*, *G. eurystoma*, and *G. sexradiata*). Interestingly, the trees based on the DNA sequence data suggest the character may be a synapomorphy of the clade that contains *G. affinis*, *G. holbrooki*, *G. heterochir*, and *G. geiseri*, as shown in Figs. 4 and 5, with the independent evolution of it in *G. hurtadoi*.

Seven synapomorphies define the subgenus *Gambusia*, which contains the *puncticulata*, *punctata*, and *nicaraguensis* species-groups. One of the seven characters was, however, variable between left and right sides of the same individual and may not be reliable (Rauchenberger 1989). Our trees, based on transversions being weighted four times transitions, support a close relationship among members of the *puncticulata* and *nicaraguensis* species-groups, but not the *punctata* species-group.

The data reported here are an important addition to our understanding of the systematics of *Gambusia*, because the cytochrome *b* sequence was informative about the affinities of key taxa whose relationships, based on morphological data, are in dispute. However, we believe important knowledge and insight can be gained from further analysis of additional morphological and molecular data from these taxa.

Test of the molecular clock hypothesis

One of the most enduring controversies in molecular evolution has been whether rate constancy exists at the molecular level and, if so, whether it is a local or global phenomenon (Li and Graur 1991)? Since these questions, in the form of a molecular clock hypothesis, are central to neutral theory (Kimura 1987), and because mitochondrial DNA variation has provided valuable data for testing this hypothesis (Adachi et al. 1993), we evaluated our cytochrome *b* sequences for evidence of rate constancy. Our analysis followed the procedure described by Felsenstein (1993). Using the three equal-weighted trees as the best hypothetical constructs, we compared the log-likelihood estimates from the programs DNAML and DNAMLK of PHYLIP for each of three topologies. To alleviate numerical inflation due to near zero branch lengths, five branches representing replicate or nearly identical taxa were pruned from the trees. These branches included one *G. holbrooki*, one *G. affinis*, one *G. yucatana*, one *G. rhizophorae*, and one *G. caymanensis*. Analyses that included these sequences as replacements for their respective counterparts resulted in identical overall conclusions.

The same single tree was identified by both DNAML and DNAMLK as having the highest likelihood estimate, given the data and topologies provided. This topology is represented by Fig. 4 with the exceptions that *G. hurtadoi*, *G. vittata*, *G. marshi*, and *G. panuco* were placed as a sister-clade to *G. affinis*, *G. holbrooki*, *G. geiseri*, and *G. heterochir*. The log-likelihood estimate for this tree restrained by the assumption of a molecular clock (DNAMLK) was -1918.7, which was significantly worse than the value derived without this constraint (DNAML = -1888.8; $\chi^2 = 59.8$, df = 22, $P < 0.001$; topologies and copies of analyses are available from the authors). Our conclusion from this analysis is that for the parsimony-based representation of the evolutionary history of these taxa, there exists evidence for rejecting the hypothesis of a global molecular clock. Rejection of rate constancy is certainly not unique to this study but it does represent the first logical step in identifying the major factors responsible for forming the boundaries within which this genus evolved.

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