



High levels of mitochondrial cytochrome b divergence in annual killifishes of the genus *Cynolebias* (Cyprinodontiformes, Rivulidae)

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Phylogenetic relationships based on 324 base pairs of the mitochondrial cytochrome b gene were examined in 14 species of the genus *Cynolebias*. The monophyly of the genus relative to three outgroup taxa belonging to the family Rivulidae was supported by the sequence data. Bootstrap values corroborated the existence of intrageneric monophyletic units in both parsimony and Neighbour-joining analyses. These include a *Cynolebias bellottii*–*C. wolterstorffi* clade, a *C. adloffii-2*, *C. duraznensis*, *C. viarius* and *C. adloffii-1* group, and a *C. gymnoventris*, *C. luteoflammulatus* pair and the strongly supported assemblage that includes *C. prognathus* and *C. cheradophilus* clade. Phylogenetic relationships remain poorly supported for *C. nigripinnis*, *C. affinis* and *C. alexandri*, and unresolved among the previous ingroup clades. Cytochrome b sequences reveal an unexpectedly high level of divergence among species of the genus *Cynolebias*. Consequently, cytochrome b shows good resolution of recent cladogenetic events but limited phylogenetic information at deeper nodes. High levels of sequence divergence span a broad range within *Cynolebias*. The highest sequence divergence (c. 28%) occurred among *C. antenori* and the remaining species of the genus. The minimum divergence value (4.5%) is exhibited by sympatric species *C. cheradophilus* and *C. prognathus*.

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INTRODUCTION

The genus *Cynolebias* has been the object of systematic controversy. This genus is by far the largest of all annual South American killifishes. Adults live no more than one rainy season during which they spawn. The eggs go through three diapause stages prior to hatching in the subsequent rainy season, and survive the dry season buried in the substrate. This type of annual cycle is exhibited by a minority of the aplocheiloid species of tropical South America and Africa (Myers, 1952; Peters, 1963; Wourms, 1967, 1972a,b,c; Parenti, 1981). Parenti (1981), in the first phylogenetic and biogeographic analysis of Neotropical aplocheiloids, proposes two synapomorphies for this genus: (a) caudal fin not scaled, (b) preopercular canal closed. *Cynolebias* (*sensu* Parenti, 1981) includes additionally other genera as synonyms: *Cynopoeilus*, *Simpsonichthys*, *Campelollebias*.

In a recent reevaluation of the Rivulidae, Costa (1990, 1995) created the subfamily Cynolebiatinae, which includes eight genera: *Millerichthys*, *Terranatos*, *Maratecoara*, *Plesiolebias*, *Cynolebias*, *Leptolebias*, *Campellolebias*, *Cynopoeilus*. Costa (1990) supports the monophyly of *Cynolebias* in five generic synapomorphies and proposes additionally three autapomorphies (Costa, 1995) for this genus: (a) males with more dorsal fin rays than females; (b) females with black blotch on centre of body sides; (c) anal fin base of males enlarged. More recently, Costa (1996) transferred many *Cynolebias* species to the genus *Simpsonycthis*.

High levels of intrageneric variability are evident in the morphology (Vaz-Ferreira & Scaglia de Paulete, 1964; Vaz Ferreira & Sierra, 1972; Costa, 1988, 1990, 1995), and behaviour (Vaz Ferreira & Sierra, 1973; Vaz-Ferreira & Máspoli, 1988, 1994) of *Cynolebias*. Figure 1 shows the extremes of morphology detected in the genus: *Cynolebias prognathus* (Fig. 1A) that Costa (1995) includes in the '*C. elongatus* complex', which represents the bigger and predatory group; *C. adloffii-2* (Fig. 1B) that was included in the '*C. bellottii* complex' integrated by medium sized species (Costa, 1995). We can see in Figure 1 that *Cynolebias* shows marked sexual dimorphism like all cyprinodontiforms (Parenti, 1981). Males typically are elaborately pigmented and frequently have elongated rays in the unpaired and the pelvic fins. As all cyprinodontiforms, and particularly in aplocheiloids, *Cynolebias* exhibit sexual dimorphism in size and males (Fig. 1A, B, above) are larger than females (Parenti, 1981).

Extensive interspecific karyotypic divergence has been described in natural populations of 14 species of *Cynolebias* from Uruguay, Argentina and Rio Grande do Sul, Brazil (García & Máspoli, 1986; García *et al.*, 1988). Diploid numbers vary from 34 to 48 chromosomes, and number of chromosomes arms (NF) vary from 48 to 80. It seems that chromosomal evolution has played an important role in *Cynolebias* diversification (García *et al.*, 1993, 1995). Because *Cynolebias* is a speciose group of Neotropical fishes, it constitutes an excellent subject for speciation studies.

Mitochondrial cytochrome b (cyt b) gene sequences have proven to contain phylogenetic signal at many different taxonomic levels in numerous taxa, including fish (Kocher *et al.*, 1989; Meyer & Wilson, 1990; Martin *et al.*, 1992; Cantatore *et*

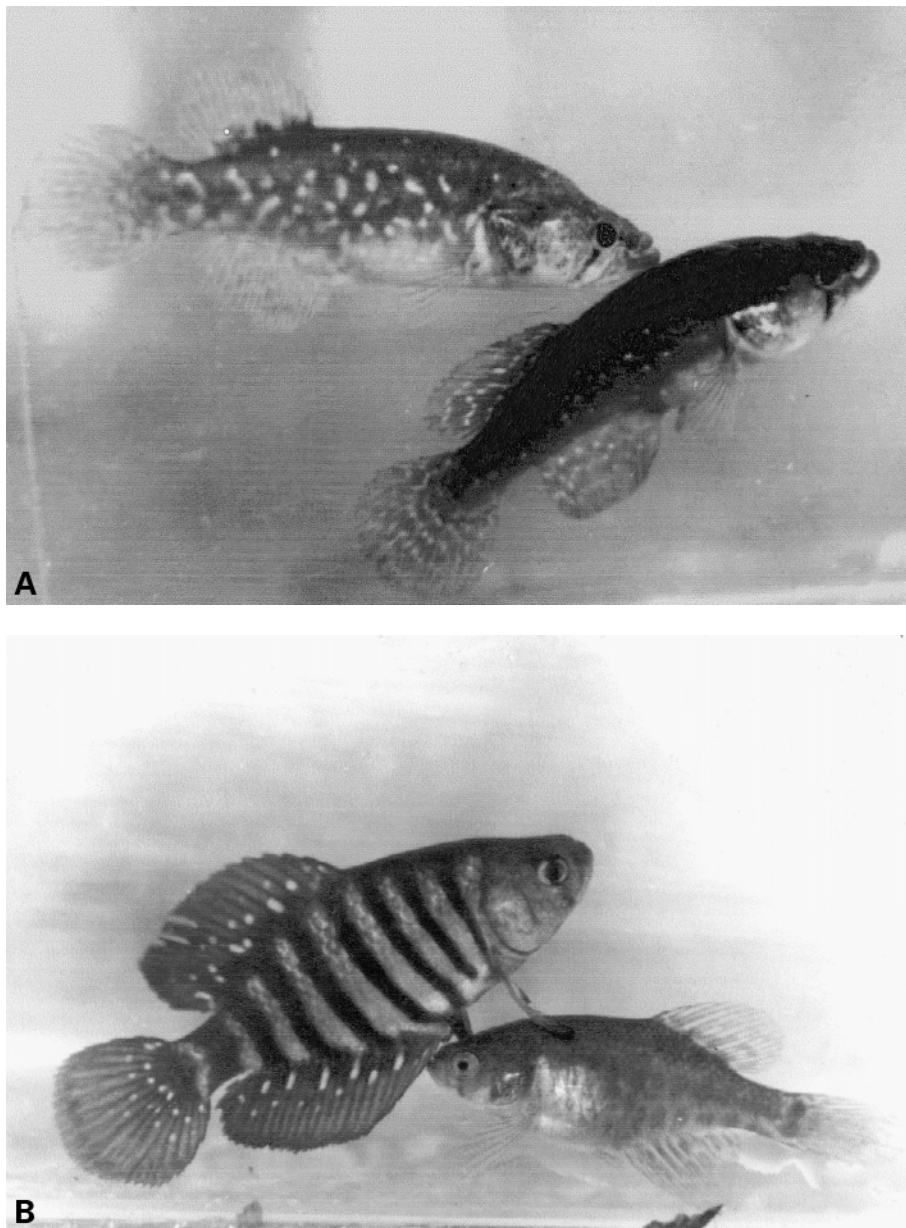


Figure 1. Two extremes of morphology within *Cynolebias*: A, *Cynolebias prognathus*, male (above) and female (below); B, *Cynolebias adloffii-2*, male (above) and female (below).

al., 1994). Cytochrome b sequences have been utilized widely as a 'molecular clock' to estimate the chronology of speciation in several taxa (Meyer *et al.*, 1990; Irwin *et al.*, 1991; Smith & Patton, 1993). At present most of the cyprinodontiform molecular phylogenetic hypotheses include higher level relationships (Meyer & Lydeard, 1993; Parker & Kornfield, 1995; Murphy & Collier, 1997; Parker, 1997) as well as intrageneric analyses (Bernardi & Powers, 1995; Lydeard *et al.*, 1995; Murphy &

Collier, 1996). Relatively little is known, however, about relationships among species of *Cynolebias*. Here, we present an initial examination of phylogenetic relationships within the genus *Cynolebias*. Coupled with recent reports on other Cyprinodontiforms (Bernardi & Powers, 1995; Murphy & Collier, 1996), our data suggest that this order has unexpectedly high level of intrageneric molecular divergence with respect to that of other fish groups (Martin & Palumbi, 1993; Cantatore *et al.*, 1994).

MATERIAL AND METHODS

Specimens examined

Partial sequences of the mitochondrial cytochrome b gene (324 bp) were obtained from specimens representing 13 different species of the genus *Cynolebias* from temporary ponds in Uruguay and Southern Brazil. Tissues and voucher specimens are deposited in the Sección Genética Evolutiva (GP), Facultad de Ciencias, Montevideo-Uruguay.

Cynolebias adloffii-1. BRAZIL: ($N=2$ males, $N=1$ female) BR (BRAZIL) 290, Rio Grande Do Sul (GP300, 313, 310); ($N=1$ male, $N=2$ females) P. Gravataí, R. G. Do Sul (GP305, 303, 311). *Cynolebias adloffii*-2. URUGUAY: ($N=1$ male, $N=2$ females) K. (kilometer) 7, R. (route) 19, Dpto. Rocha (GP331, 332, 362); ($N=1$ male, $N=1$ female) K. 489, R. 14, Dpto. Rocha (GP334, 336). *Cynolebias viarius*. URUGUAY: ($N=4$ males) R. 10 and 16, 'La Cruz', Dpto. Rocha (GP374, 376, 377, 379). *Cynolebias bellottii*. URUGUAY: ($N=1$ male) Carmelo, Dpto. Colonia; ($N=2$ females) Bañado Verocay, Dpto. Salto (GP363, 399, 401). *Cynolebias nigripinnis*. URUGUAY: ($N=3$ males) Bañado Verocay, Dpto. Salto (GP395, 396, 397). *Cynolebias duraznensis* nomen nudum. URUGUAY: ($N=2$ males, $N=1$ female) 'Estadio Ciudad Durazno', Dpto. Durazno (GP463, 462, 466). *Cynolebias luteoflammulatus*. URUGUAY: ($N=1$ male) Puerto de los Botes, Dpto. Rocha (GP413) K. 7, R. 19, Dpto. Rocha (GP437–447). *Cynolebias gymnoventris*. URUGUAY: ($N=4$ males) R. 13, Dpto. Rocha (GP478, 479, 490, 493). *Cynolebias alexandri*. URUGUAY: ($N=1$ male, $N=1$ female) Bañado Verocay, Dpto. Salto (GP475, 476). *Cynolebias cheradophilus*. URUGUAY: ($N=3$ males, $N=2$ females) R. 10, Dpto. Rocha (GP416, 417, 418, 419, 424). *Cynolebias prognathus*. URUGUAY: ($N=1$ male) K. 7, R. 19, Dpto. Rocha (GP381). *Cynolebias wolterstorffi*. URUGUAY: ($N=1$ male) R. 15, Dpto. Rocha (GP530); ($N=1$ male, $N=1$ female) R. 14, Dpto. Rocha (GP602, 603). *Cynolebias affinis*-URUGUAY ($N=2$ females) 'Tres Cruces', Dpto. Tacuarembó (GP470, 471).

A sequence from *Cynolebias antenori* (Murphy & Collier, 1997; Genbank accession U73298) was included as part of the ingroup. Three Rivulidae genera were chosen as outgroups in phylogenetic analyses: *Leptolebias citrinipinnis* (Murphy & Collier, 1997; Genbank accession U73299), *Austrofundulus limnaeus* (Murphy & Collier, 1997; Genbank accession U73299), and *Trigonectes rubromarginatus* (Murphy & Collier, 1997; Genbank accession U73299). Sequences from *Rivulus cryptocallus* (Murphy & Collier, 1996; Genbank accession U41776) and *Fundulus heteroclitus* (Parker & Kornfield, 1995; Genbank accession U063132) were integrated in divergence estimates to assess the saturation level of the sequences.

DNA extraction, amplification and sequencing

DNA was extracted from liver tissue fixed in alcohol, using proteinase K digestion, protein precipitation with sodium chloride, and ethanol precipitation of total DNA (modified from Medrano *et al.*, 1990). Amplifications of the 5' end cytochrome b were performed by the polymerase chain reaction (PCR, with 32 cycles alternating denaturation at 93°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min), using two primers: MVZ 04 (GCAGCCCCTCAGAATGATATT-TGTCCTC, 3' end located at position 14542 of *Mus*, Kocher *et al.*, 1989), and MVZ 05 (CGAAGCTTGATATGAAAAACCATCGTTG, 3' end located at position 14115 of *Mus* (Paäbo & Wilson, 1988).

All sequences were obtained by direct sequencing of PCR products using the Gibco BRL Thermal Cycle Dideoxy DNA Sequencing kit, with primers end-labelled (³³P-dATP) with polynucleotide kinase.

Sequence analysis

The cyt b sequences were aligned in ESEE version 1.09 (Cabot & Beckenbach, 1989). Pairwise estimates of sequence divergence were obtained using the two-parameter algorithm of Kimura (1980) implemented in MEGA version 1.01 (Kumar *et al.*, 1993). Percent sequence divergences, considering only transitions (Ts) for all pairwise comparisons was plotted against percent sequence divergences for transversions (Tv), to visualize Ts saturation.

Phylogenetic and biogeographical analysis

Phylogenetic analyses were performed using two different methods: (1) a Neighbour-joining (NJ) tree (Saitou & Nei, 1987) was generated using MEGA on the basis of Kimura 2-parameter distances; and (2) maximum parsimony trees were reconstructed using PAUP (version 3.1.1, Swofford, 1993). Skewness (g1 statistics) of the distribution of tree lengths as a measure of information content (Hillis & Huelsenbeck, 1992) was obtained by generating 1000 random trees using PAUP.

To circumvent problems associated with multiple island of trees (Swofford, 1993), we employed replicate heuristic searches (MULPARS option in effect, stepwise addition, Tree-bisection-reconnection TBR branch-swapping, 100 replications). The strict consensus trees were computed between rival trees for each weighting scheme (see below). The degree of confidence assigned to nodes in trees was determined by bootstrapping with 100 replicates.

In accordance with the empirical evidence for transition saturation in third codon positions discussed below, unweighted and weighted parsimony analyses were performed to examine their effect on tree topology. The simplest specification of character state change was to consider all characters as unordered and with equal weights. Alternatively a stepmatrix was employed to weight Tv (transversions) 5 times more than Ts (transitions).

Trees were rooted by the outgroup criterion. Three outgroups—*Leptolebias citrinipinnis*, *Austrofundulus limnaeus*, and *Trigonectes rubromarginatus*—were chosen among

the Rivulidae, a monophyletic clade that includes *Cynolebias* (Parenti, 1981; Costa, 1990).

For biogeographical analysis, nine endemic areas for 14 ingroup taxa of the genus *Cynolebias* and three other Rivulidae were considered as alternative character states of a single character and traced on the most parsimonious tree topology using McClade version 3.0 (Maddison & Maddison, 1992).

RESULTS AND DISCUSSION

Levels of sequence divergence and patterns of substitution

The partial sequences of mitochondrial cytochrome b show substantial variation. Among the 14 ingroup sequences, 140 of the 324 positions are variable, with 92 position being phylogenetically informative in parsimony analysis. When outgroup sequences are included, 170 positions are variable, of which 129 are phylogenetically informative. Most substitutions occur in third-base codon positions (72%) and a minority is found in first (18%) and second (10%) codon positions. There are no deletions or insertions, and the sequences translate properly to a 108 amino acid chain. Twenty amino acids are variable among the different sequences.

The base compositional biases of cyt b sequences of *Cynolebias* (Table 1) do not differ from the patterns found in other vertebrate groups (Brown *et al.*, 1982; Kocher *et al.*, 1989; Irwin *et al.*, 1991; Martin & Palumbi, 1993). Nucleotide frequencies differ greatly among codon positions. For example, the observed 6.1% G in third codon positions is comparable to the average 6.4% for other bony fish (Cantatore *et al.*, 1994) and slightly higher than the average 3.5% found in mammals (Lara *et al.*, 1996) or the 2.3% mean in sharks (Martin, 1995). Compositional constraints on base substitutions probably operate as in other animal mitochondrial sequences. They may be related to the level of sequence divergences and transitions saturation (De Salle *et al.*, 1987; Tamura, 1992; Zhu *et al.*, 1994).

Estimates of ingroup sequence divergence using Kimura's two-parameter model Ts + Tv for the cytochrome b, range from 4.5% to 25.9% considering the species from Uruguay and Southern Brazil. Among these species, the maximum divergence value occurs between *C. duraznensis* and *C. bellottii* and the minimum between *C. prognathus* and *C. cheradophilus*. The highest sequence divergence (averaging 28%) is found between *C. antenori* (from Brazil) and the remaining species of *Cynolebias*. The average degree of sequence divergence between the ingroup species and the three outgroup genera range from 33% (*Austrofundulus*) to 37% (*Trigonectes* and *Leptolebias*).

Ts outnumber Tv in all cases, in accordance with previous studies of mtDNA (Aquadro & Greenberg, 1983, Brown, 1985). However, taxa coming from Uruguay and Southern Brazil show different levels of saturation. Thus Tv:Ts ratios range from 1:5 between similar sequences (*C. bellottii* vs. *C. wolterstorffi*) to 1:2.6 between the most divergent ones (*C. bellottii* vs. *C. duraznensis*). Pairwise comparisons of sequences show an observed average Tv:Ts ratio of 1:3.

With respect to transversions, transitions increase rapidly at low levels of intrageneric divergence (between 5 and 15%) but more moderately at greater values. Ts reach 20–25% sequence divergence between *Cynolebias* and other Rivulidae. When the distantly related *Fundulus heteroclitus* is included, Ts reach 30–35% sequence divergence level (Fig. 2).

TABLE 1. Base composition at first, second and third positions of codons in 13 *Cynolebias* species and one of the genus *Cnesterodon*, for 324-bp of cyt b sequence. (All values are percentages, except totals)

	A	T	C	G	Total	A1	T1	C1	G1	Total	A2	T2	C2	G2	Total2	A3	T3	C3	G3	Total3
<i>C. ballotii</i>	26.7	34.9	22.2	16.2	315	28.6	29.5	18.1	23.8	105	21.9	36.2	23.8	18.1	105	29.5	39.0	24.8	6.7	105
<i>C. lateoformulatus</i>	26.0	34.6	22.2	17.1	315	25.7	30.5	19.0	24.8	105	22.9	37.1	21.0	19.0	105	29.5	36.2	26.7	7.6	105
<i>C. adloff-2</i>	24.0	35.6	24.0	16.5	267	24.7	28.1	21.3	25.8	89	22.5	36.0	23.6	18.0	89	24.7	42.7	27.0	5.6	89
<i>C. varius</i>	24.8	34.0	25.1	16.2	315	26.7	32.4	17.1	23.8	105	21.9	37.1	22.9	18.1	105	25.7	32.4	35.2	6.7	105
<i>C. pygmaeus</i>	26.6	33.7	23.2	16.5	267	28.1	27.0	18.0	27.0	89	22.5	38.2	23.6	15.7	89	29.2	36.0	28.1	6.7	89
<i>C. affinis</i>	25.4	36.6	21.5	16.5	279	22.6	31.2	18.3	28.0	93	22.6	36.6	22.6	18.3	93	31.2	41.9	23.7	3.2	93
<i>C. alexandri</i>	24.1	38.8	21.1	16.0	294	25.5	32.7	16.3	25.5	98	22.4	38.8	22.4	16.3	98	24.5	44.9	24.5	6.1	98
<i>C. cheradophilus</i>	24.8	32.4	26.2	16.7	210	24.3	32.9	17.1	25.7	70	27.1	32.9	21.4	18.6	70	22.9	31.4	40.0	5.7	70
<i>C. daraziensis</i>	24.4	32.8	26.9	15.9	201	25.4	26.9	20.9	26.9	67	25.4	31.3	25.4	17.9	67	22.4	40.3	34.3	3.0	67
<i>C. nigripinnis</i>	25.8	36.2	21.1	17.0	318	26.4	34.0	14.2	25.5	106	20.8	36.8	24.5	17.9	106	30.2	37.7	24.5	7.5	106
<i>C. adloff-1</i>	24.7	34.4	23.4	17.5	291	25.8	28.9	19.6	25.8	97	20.6	39.2	21.6	18.6	97	27.8	35.1	28.9	8.2	97
<i>C. wolterstorffi</i>	26.0	34.9	22.9	16.1	192	25.0	31.3	17.2	26.6	64	29.7	28.1	23.4	18.8	64	23.4	45.3	28.1	3.1	64
<i>C. gymnacentris</i>	25.5	34.3	22.3	17.9	318	25.5	31.1	17.0	26.4	106	21.7	35.8	24.5	17.9	106	29.2	35.8	25.5	9.4	106
<i>C. antenori</i>	24.2	37.0	22.9	15.8	297	27.3	35.4	15.2	22.2	99	21.2	38.4	21.2	19.2	99	24.2	37.4	32.3	6.1	99
<i>T. rubromarginatus</i>	26.9	36.7	19.9	16.5	297	25.3	31.3	19.2	24.2	99	22.2	40.4	18.2	19.2	99	33.3	38.4	22.2	6.1	99
<i>L. citrinipinnis</i>	26.3	36.4	22.6	14.8	297	26.3	27.3	23.2	23.2	99	21.2	39.4	22.2	17.2	99	31.3	42.4	22.2	4.0	99
<i>A. limnaceus</i>	25.6	39.7	19.5	15.2	297	28.3	31.3	18.2	22.2	99	21.2	41.4	17.2	20.2	99	27.3	46.5	23.2	3.0	99
	25.4	35.6	22.6	16.4	4770	26.0	30.8	18.2	25.0	1590	22.5	37.0	22.3	18.2	1590	27.7	38.9	27.4	6.0	1590

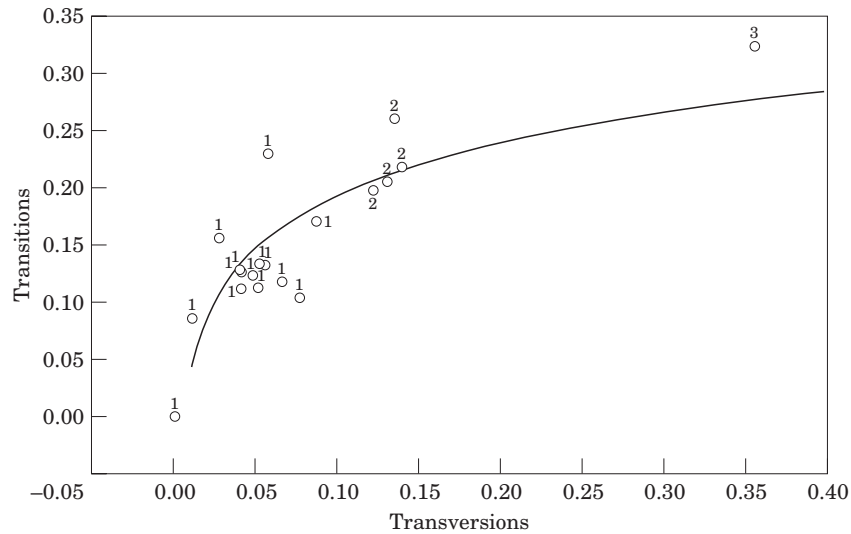


Figure 2. Percent sequence divergence in transitions and transversions estimated on the basis of Kimura two-parameter distances in 14 taxa of the genus *Cynolebias* (1), four Rivulidae of the genera *Leptolebias*, *Austrofundulus*, *Trigonectes* and *Rivulus* (2) and the distantly related genus *Fundulus* (Fundulidae) (3).

The high levels of nucleotide divergence found in *Cynolebias* are comparable to those reported in *Rivulus* (Murphy & Collier, 1996), but stand in sharp contrast with findings in other fish groups. For instance, *cyt b* analyses in sharks (Martin *et al.*, 1992) and many Perciformes (Cantatore *et al.*, 1994) suggests that the average substitution rates are 3–5 fold lower than in mammals.

Most nucleotide changes (72%) are substitutions at third codon position. As in other vertebrate, transitions accumulate at a greater rate than transversions in all positions (Brown *et al.*, 1982; Martin & Palumbi, 1993). According to Martin (1995) there are strong constraints against Tv at first codon positions and against both Ts and Tv at second codon positions. The observed average bias Tv:Ts (1:3) within *Cynolebias* and *Rivulus* (Murphy & Collier, 1996) are similar to those found in the genus *Fundulus* (Bernardi & Powers, 1995). Comparable values have been found in distantly related genera of fishes, such as Melanotaeniidae (Zhu *et al.*, 1994) and cottoid fish of Lake Baikal (Slobodyanyuk *et al.*, 1995). These findings differ considerably from a typical 1:10 bias toward Ts in mammalian mt DNA (Brown *et al.*, 1982, Irwin *et al.*, 1991; Lara *et al.*, 1996) and from those described in *Oncorhynchus* (1:9) and Pacific trout and salmon (1:5, Thomas & Beckenbach, 1989).

The high levels of intragenetic sequence divergence (up to 25.9%) seen for *cyt b* in *Cynolebias* are similar to those found in the genus *Rivulus* (22.1%–25.6%) between the Antillean endemics and mainland taxa (Murphy & Collier, 1996). However in Cyprinodontiformes of the genus *Fundulus* the average nucleotide sequence divergence between species is 17.2% and between species within subgenera 10.3% (Bernardi & Powers, 1995), whereas lower values (9.1%) are found in the genus *Gambusia* (Lydeard *et al.*, 1995). The ranges of sequence divergence observed in Atheriniformes is >17% among different genera of the family Melanotaeniidae (Zhu *et al.*, 1994). These findings are very different from early *cyt b* studies in cichlids in which a

maximum corrected sequence divergence within *Tropheus* lineages of Lake Tanganyika was 5%, and less than 2% in Malawi (Sturmbauer & Meyer, 1992).

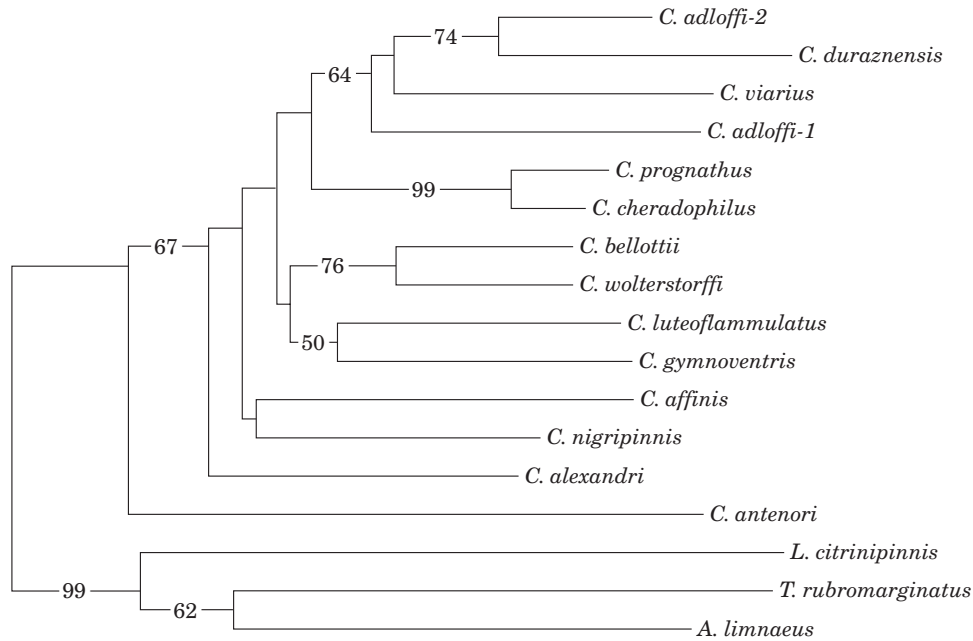
In principle, high levels of sequence divergence in *Cynolebias* may be explained by invoking either a relatively old radiation of the genus or high rates of cyt b evolution. Martin & Palumbi (1993) have pointed out that variation in the nucleotide substitution rate may be due to a series of physiological and life-history variables. These include generation time, life span, age at first reproduction, rate of population increase and metabolic rate. Because *Cynolebias* have annual life cycles, any of these variables could be correlated with the high levels of substitution detected. In sum, it seems at least possible that *Cynolebias* may have particularly high rates of cyt b evolution among fish. This issue is discussed below by examining observed nucleotide divergences in relation to the fossil record.

Molecular clock for Cynolebias cyt b sequences

To estimate the time of divergence among the cyt b sequences analysed here, Kimura two-parameter pairwise distances using only Tv at third codon position were calculated. The maximum divergence value was 27% between *C. duraznensis*–*C. luteoflammulatus* and the minimum 1.4% between *C. prognathus*–*C. cheradophilus*.

We estimated times of divergence with different rates of change proposed for this gene in the literature. These estimates are based solely on third position transversions because they are less likely to be saturated than transitions across the entire range of observed divergences. The first rate of 0.5% per Myr was estimated by Irwin *et al.* (1991) from a data set on mammals (primarily ungulates). This rate suggests that basal cladogenetic events in *Cynolebias* occurred about 54 MA (during the Eocene) and more recent ones of about 2.8 MA (Pliocene). For fishes, Cantatore *et al.* (1994) considered a rate 3–5-fold lower than in mammals to date the split of Perciformes and Acipenseriformes. With the lowest rate in that range, the basal radiation of *Cynolebias* is estimated to be very ancient (270 MA). According to Parenti (1981), the Cyprinodontiformes fossil record begins in the Oligocene in Europe and in the Miocene in the New World, providing minimum ages for the order. Based on distribution patterns, Parenti (1981) estimated the Cyprinodontiformes to be at least as old as Pangea, the ancient supercontinent that started to break up approximately 180 MA. She also pointed out that *Cynolebias* represents a derived taxon within Neotropical aplocheiloids. In sum, the rates suggested by both Irwin *et al.* (1991) and Cantatore *et al.* (1994) yield unlikely ancient times of divergence for *Cynolebias*. Alternatively, we considered a rate of 2.3% calculated for sigmodontine rodents by Smith and Patton (1993). With this rate, basal cladogenetic events now are estimated at about 11 MA (Miocene) and the most recent ones about 600 000 years ago (Pleistocene). This calibration is broadly in accordance with the fossil record, as cyprinodontiforms are first documented in South America in the Eocene (Arratia & Cione, 1996).

These computations are subjected to considerable uncertainty related to limited data for both the fossil record and mitochondrial DNA sequences. However, the low rates of cyt b evolution usually ascribed to fish, or even the rates estimated for ungulates, are hard to reconcile with the fossil evidence. It thus seems much more reasonable to accept much faster, rodent-like rates of cyt b evolution in *Cynolebias*.



Scale: each – is approximately equal to the distance of 0.003204

Figure 3. Phylogenetic tree constructed with the Neighbour-joining method from Kimura two-parameter distances of 14 ingroup taxa of the genus *Cynolebias* and three Rivalidae outgroups (*Leptolebias*, *Austrofundulus*, *Trigonetes*). Bootstrap values above 50% are shown on the relevant branches.

Phylogenetic analysis

The Neighbour-joining tree topology (Fig. 3) suggests the existence of distinct monophyletic units within *Cynolebias*. One monophyletic group includes *C. adloffii-2*–*C. duraznensis* (74% bootstrap support) and *C. viarius*. The taxon *C. adloffii-1* is the sister taxon of this clade (64% support). A second monophyletic clade consisting of *C. cheradophilus*–*C. prognathus* is supported by 99% of bootstrap replicates. A third group was integrated by the following clades: *C. bellottii*–*C. wolterstorffi* (76%) and *C. luteoflammulatus*–*C. gymnoventris* (50%). The sister taxon of this is *C. nigripinnis*–*C. affinis* and then *C. alexandri*. *Cynolebias antenori* appears as the most distinct species of the genus.

The distribution of lengths of 1000 random trees is significantly skewed ($g1 = -0.706722$, $P < 0.01$) in the unweighted analysis, as well as in the weighted parsimony analysis ($g1 = -0.895165$, $P < 0.01$) (Hillis & Huelsenbeck 1992). The most parsimonious consensus tree topologies are similar between unweighted (Fig. 4A) and weighted analyses (Fig. 4B). Only one branch appears with robust support (100%) in all analysis, this being the *C. cheradophilus*–*C. prognathus* clade. *Cynolebias antenori* from NE Brazil is always sister to all other taxa in the ingroup. In unweighted parsimony, the consensus between two shortest trees of 516 steps length is shown in Figure 4A. Four ingroup clades are similar to those found by neighbour-joining analysis (Fig. 3). Nevertheless, both analyses differ in the relative position of certain clades. One major group consists of *C. bellottii*–*C. wolterstorffi* linked to a clade formed

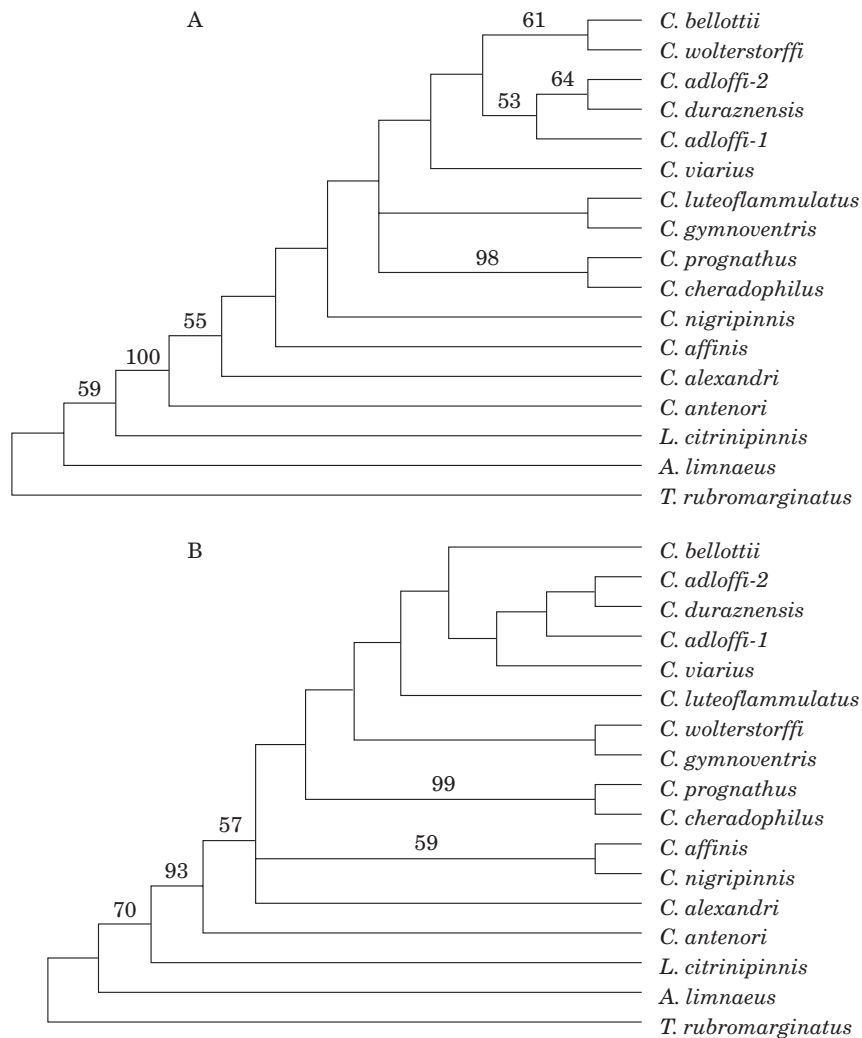


Figure 4. Phylogenetic relationships among 14 species of the genus *Cynolebias* and three Rivulidae outgroups (*Leptolebias*, *Austrofundulus*, *Trigonectes*), as inferred by parsimony heuristic searches with different weighting schemes. A, unweighted analysis: a most parsimonious consensus of two trees, 516 steps. B, weighted analysis: consensus of two shorter trees of 1234 steps. Bootstrap values above 50% are shown on the corresponding branches.

by *C. adloffii-2*, *C. duraznensis* and *C. adloffii-1*. *C. viarius* in turn, is the sister taxon of this large clade. This is not in agreement with the neighbour-joining topology. The pairs *Cynolebias luteoflammulatus*–*C. gymnoventris* and *C. cheradophilus*–*C. prognathus* are supported but join the large clade above in a polytomy. *C. nigripinnis*, *C. affinis*, *C. alexandri* appear outside this polytomy.

The consensus between two shortest trees of 1234 steps length was obtained with the weighting scheme (Fig. 4B). This analysis shows a tree topology very similar to that of unweighted parsimony, but in this case *C. affinis* and *C. nigripinnis* constitute a monophyletic clade (with 59% bootstrapping support). Bootstrap analysis shows

all remaining taxa of the ingroup except *C. antenori*, collapsing independently to a large polytomy which has 57% bootstrap support.

The pattern of *cyt b* sequence divergence observed in *Cynolebias* has consequences for phylogeny reconstruction. Because silent substitutions have been found to evolve rapidly in mitochondrial DNA (Brown *et al.*, 1982) it would not be phylogenetically informative for deep divergences. Thus this gene has the reputation of providing phylogenetic utility only for taxa with relatively recent divergences up to approximately 50 MA (Wilson *et al.*, 1985; Moritz *et al.*, 1987).

The monophyly of the genus is supported by the sequence data based on comparisons to other three Rivulidae outgroups. The expected divergence of mitochondrial *cyt b* sequence between ingroup species and other outgroup Rivulidae genera is concordant with phylogenetic hypothesis proposed by Parenti (1981) and Costa (1990). *Austrofundulus* (33% at the molecular level) is considered as the sister-group of *Cynolebias* (Cynolebiatinae *sensu* Costa, 1990) and *Trigonectes* (37%) appears as a basal group in Parenti's analysis (1981). *Leptolebias* (37%) is considered to be the sister group of *Cynolebias* within the family Cynolebiatinae (Costa, 1990).

Cynolebias antenori from NE Brazil is highly divergent (*c.* 28%) from the remaining *Cynolebias* (Figs 3, 4A, B). This result is in agreement with the phylogenetic morphological cladogram proposed by Costa (1995) in which this taxon appears as a distantly related intrageneric entity. More recently Costa (1996) relocated this taxon to the sister genus *Simpsonichthys*.

All phylogenetic analyses of *Cynolebias* are in agreement with the existence of different intrageneric lineages among species from Uruguay and Southern Brazil. However, they differ on the relationships of certain of these groups. Bootstrap values corroborated the existence of intrageneric monophyletic units in both parsimony (Fig. 4A, B) and Neighbour-joining (Fig. 3) analyses. These units are not in complete accordance with those proposed by Costa (1995) from a morphological cladistic analysis in this genus. The following associations are suggested by our analyses.

(1) The clade *C. bellottii*–*C. wolterstorffi*. These taxa are allopatric (Fig. 4) and show substantial morphological differences (Amato, 1986; Costa, 1995). Thus, they were grouped in two different complexes by Costa (1995). *Cynolebias wolterstorffi* was included in his analysis in the '*C. elongatus* complex', a monophyletic clade integrated by *C. prognathus* and *C. cheradophilus* that share one synapomorphy (anal and dorsal fins gently pointed or rounded in male) and corresponding to large predatory annual killifishes. On the other hand, *C. bellottii* integrates the '*C. bellottii* complex', a monophyletic assemblage that shares three synapomorphies (deep urohyal; bony part of basihyal reduced; upper pectoral radial very reduced or absent). However, cytogenetic studies reveal that both species share a plesiomorphic karyotype within *Cynolebias* (García *et al.*; manuscript in preparation) showing diploid numbers of $2n=48$ and $2n=46$, respectively, and predominantly subtelocentric-acrocentric chromosome types (García *et al.*, 1988, 1993).

(2) *C. adloffii-2*–*C. duraznensis*, *C. adloffii-1* and *C. viarius* with the possible addition of *C. bellottii* which is the sister-group of this clade (Fig. 5). The monophyly of this group is supported by morphological (Amato, 1986; Costa, 1995) and chromosomal studies (García *et al.*, 1988, 1993, 1995). The relative relationships of *C. viarius*, *C. adloffii-2* (from Uruguay) and *C. adloffii-1* (from southern Brazil) are matters of taxonomic controversy (Vaz-Ferreira & Melgarejo, 1984). Recent cytogenetic analysis of interspecific laboratory hybrids raises the possibility that *C. adloffii-2* might be the

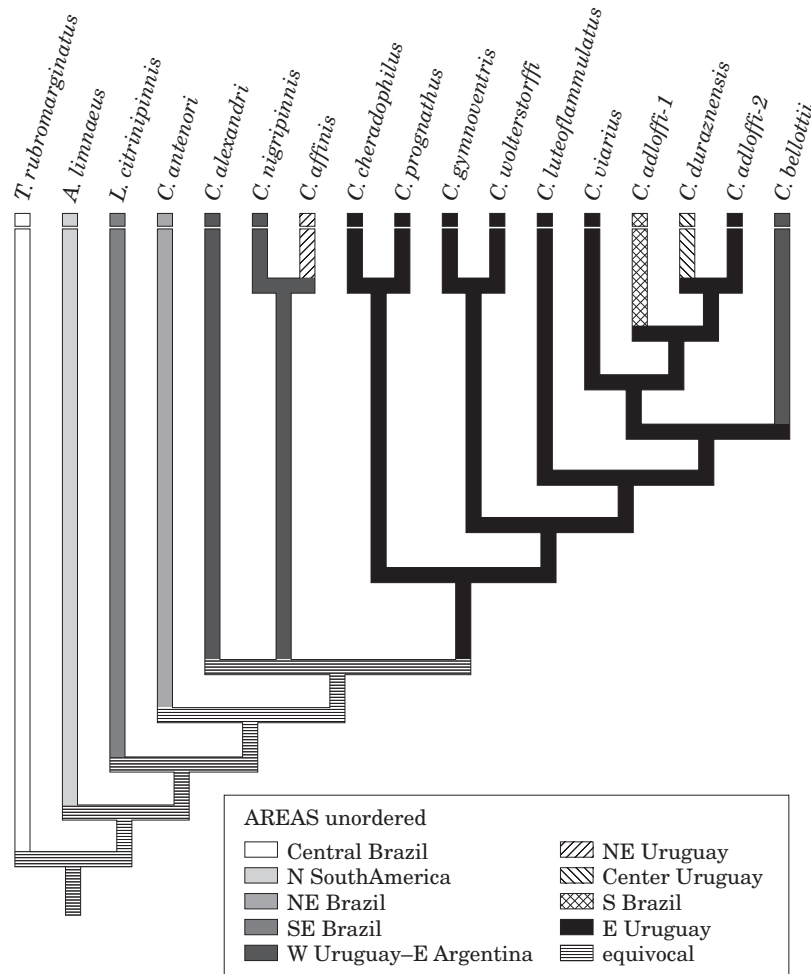


Figure 5. Biogeographical patterns of differentiation. Nine endemic areas were traced over the tree topology from weighted parsimony (Fig. 4B) for species of *Cynolebias* and other Rivulidae.

result of intragradation between *C. adloffii-1* and *C. viarius* (García, 1996). Further studies are necessary to clarify this issue.

(3) *C. luteoflammulatus* and *C. gymnoventris*. Both species have different chromosomal numbers ($2n=34$ and $2n=48$, respectively) and they could be implicated in an *in situ* cladogenetic event since they are sympatric and restricted in distribution (García *et al.*, 1995; García, 1996). This clade has certain morphological affinities with the *nigripinnis* species group (Amato, 1986). However, cyt b sequence analysis does not support the relationships between these clades.

(4) *C. prognathus*–*C. cheradophilus*. In agreement with other morphological data and phylogenetic relationships proposed previously by Costa (1995), this clade is well supported by our cyt b data. These taxa show low chromosomal numbers (*C. prognathus* $2n=36$, *C. cheradophilus* $2n=40$), probably involving fusions (García *et al.*, 1988, 1993). Because they are sympatric and restricted to Eastern Uruguay, their divergence could have occurred *in situ*.

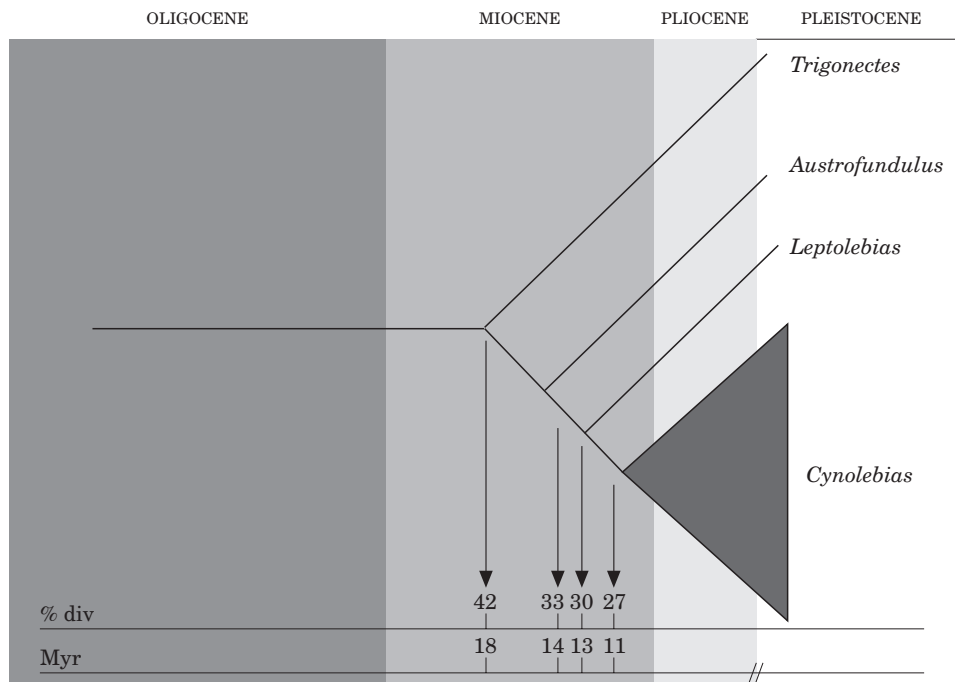


Figure 6. Chronology of the cladogenetic events in *Cynolebias* and sister genera of Rivulidae on the basis of the cytochrome b molecular clock (see text for discussion).

(5) *C. affinis*–*C. nigripinnis* could have diverged by an allopatric event (Fig. 5). Their close relationships with each other and with *C. alexandri* have been recognized in morphological and cytogenetic studies (Amato, 1986; García *et al.*, 1995; García, 1996).

Our mitochondrial cyt b analyses allow us to distinguish, within the ‘bellottii complex’ (Costa, 1995), different intrageneric lineages (associations of one, two, three and five species above) belonging to Uruguay and Southern Brazil. Phylogenetic relationships among the previous clades remain uncertain as our data lack phylogenetic signals at deep levels. At this point, it is unclear whether this lack of resolution of deep clades reflects rapid diversification or insufficient phylogenetic information in the dataset.

Evolutionary timescale and biogeography

The estimates for calibration of a molecular clock in *Cynolebias* using sigmodontine rodent rate (Fig. 6) suggest that the recent differentiation of monophyletic clades (*C. adloffii-2*–*C. duraznensis*–*C. adloffii-1*, *C. prognathus*–*C. cheradophilus*, *C. nigripinnis*–*C. affinis*) could have occurred during the Pleistocene (600 000 years ago). The earliest cladogenetic events of multiple lineages within *Cynolebias* could have occurred in the Miocene (11 MA). Costa (1995) proposed phylogenetic relationships in which *Leptolebias* is the sister-group of *Cynolebias*. Our results suggest that the differentiation of *Leptolebias* and *Cynolebias*, might have taken place some 13 MA. The divergence

times of other Rivulidae genera (Fig. 6) based on cyt b sequences are broadly in agreement with the cladistic phylogenetic hypothesis for Rivulidae (Parenti, 1981) and they could have diverged during the Miocene. *Austrofundulus*, a sister-group of Cynolebiatinae, might have split about 14 MA and *Trigonectes* some 18 MA. Figure 6 provides a summary of these time estimates.

When areas are traced as character states on the tree (Fig. 5), eastern Uruguay and southern Brazil appear as 'hot spots' of recent cladogenetic events. The split of many taxa is inferred to have occurred by allopatric differentiation (e.g. *C. bellottii* and *C. duraznensis*) while other cladogenetic events might have taken place by *in situ* divergence, perhaps associated with chromosomal rearrangements (García *et al.*, 1993, 1995; García, 1996). *Cynolebias nigripinnis* (from Western Uruguay) and *C. affinis* (from northeastern Uruguay) diverged allopatrically, perhaps by dispersal and/or extinction of intermediate populations.

In accord with Costa (1995) *Cynolebias* species range from NE, E, and S Brazil and they are absent from the Amazonian region. The basal ingroup branch corresponds to *C. antenori* from NE Brazil (Fig. 5).

Many issues remain to be examined regarding the phylogeny and biogeography of *Cynolebias*. Whereas additional mitochondrial DNA data will undoubtedly assist in this endeavour, it is likely that nuclear loci will be needed as well to clarify the uncertain arrangements related to some of the inferred rapid speciation events in this group.

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