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Grant, E. Christopher, M.S.

University of Nevada, Las Vegas, 1994

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THE PHYLOGENETIC AFFINITIES OF *CRENICHTHYS* AND *EMPETRICHTHYS* USING mt DNA

by

E. Christopher Grant

A thesis submitted in partial fulfillment
of the requirements for the degree of

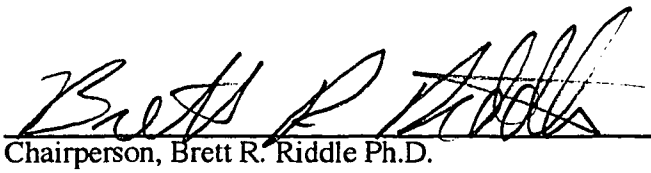
Master of Science

in

Biology

Department of Biological Sciences
University of Nevada, Las Vegas
August 1994

The Thesis of E. Christopher Grant for degree of Master of Science is approved.



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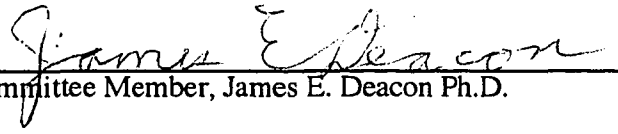

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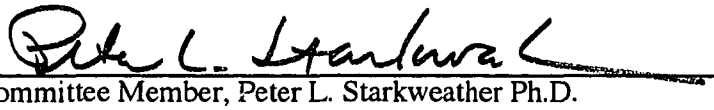

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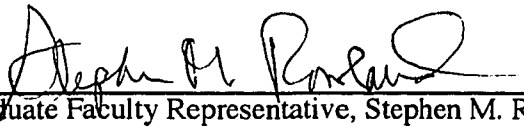

Graduate Faculty Representative, Stephen M. Rowland, Ph.D.

Dean of the Graduate College, Ronald W. Smith Ph.D.

University of Nevada, Las Vegas
August 1994

ABSTRACT

Crenichthys (Springfish) and *Empetrichthys* (Poolfish) are two relictual genera of cyprinodontiform fishes that are restricted to the state of Nevada. Of five families proposed, three, Cyprinodontidae, Goodeidae, and Empetrichthyidae, are still under consideration. The Goodeidae, fishes endemic to the Mexican Plateau, are viviparous, whereas *Crenichthys* and *Empetrichthys* are oviparous. Approximately 300 base pairs of the mitochondrial gene Cytochrome-b were sequenced in order to address the familial phylogenetic relationships of *Crenichthys* and *Empetrichthys*. All analyses concur that *Crenichthys* and *Empetrichthys* are related to members of the family Goodeidae rather than to the Cyprinodontidae. Therefore, this phylogeny supports a model of historical biogeographic affinities between fishes of the Mexican Plateau and fishes of the Mojave and Great Basin deserts.

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ACKNOWLEGEMENTS

I must first and foremost thank my advisor and mentor Brett Riddle for his patience and support. Without his guidance this body of work would not have been possible. A very special thanks to Jim Deacon whose inspiration and expertise in desert fishes are unsurpassed. I would also like to thank Peter Starkweather and Steve Rowland for serving on my committee and for their valuable comments and editing, as well as Stan Hillyard for his timely inclusion in my committee. Fran Taylor was instrumental in directing me toward this project and was remarkably capable in cutting through bureaucratic obstacles. Jim Heinrich of the Nevada Department of Wildlife was instrumental in helping me acquire needed permits. Tom and Pebbles Lee trained me in all laboratory techniques for which I am continually grateful. Thanks to Giacomo Bernardi who donated two important sequences as well as his ongoing encouragement. This project would not have been possible without the specimen donations of Bob Mckean and Dale Weber. I would like to thank Jef Jaeger for his persistent editing, requested or not, and David Orange for his caustic wit. I would also like to thank Gillian Weathers for her editing.

CHAPTER 1

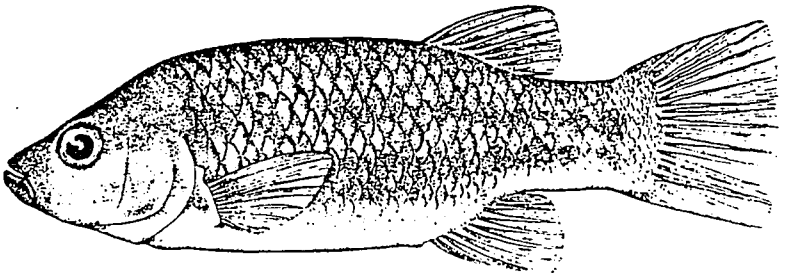
INTRODUCTION

For the past century, cyprinodontiform fishes have been studied in the American west. Cyprinodontiform fishes include killifishes, four-eyed fishes, and poeciliids (Rosen 1964). Two cyprinodontiform genera, *Empetrichthys* (poolfish) and *Crenichthys* (springfish) are relict endemics restricted to pools and streams of the Great Basin and Mojave deserts within the state of Nevada (Gilbert 1893; Hubbs 1932). *Empetrichthys latos* is listed as an endangered species and *Crenichthys nevadae* is listed as threatened. Two subspecies of *Crenichthys baileyi* are also listed as endangered. The classification of these fishes has been and is still being debated. Historically, *Crenichthys* and *Empetrichthys* have been placed in each of the following five families: Cyprinodontidae (Gilbert 1893), Poeciliidae (Gill 1894), Orestiidae (Eigenmann 1920), Empetrichthyidae (Jordan et. al. 1930), or Goodeidae (Parenti 1981). Their assignment to one of three families, Cyprinodontidae, Goodeidae, or Empetrichthyidae, is still being debated (Sigler and Sigler 1987; Mayden et al. 1992; Miller and Smith 1986). I have used mitochondrial DNA nucleotide sequencing to test the phylogenetic relationships and historical biogeographic affinities of these relict genera.

TAXONOMIC AND BIOLOGICAL BACKGROUND

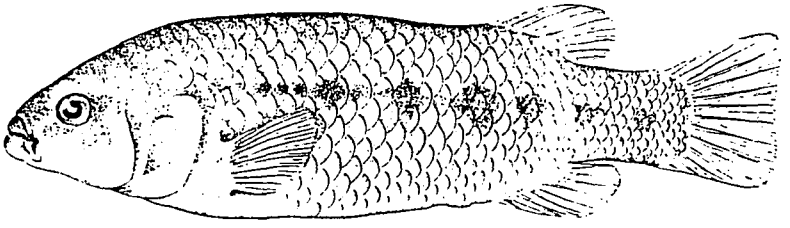
Empetrichthys presently consists of a single extant species, *Empetrichthys latos* (Figs. 1 and 2), one of two species originally described. *E. latos* was initially described as

A



Crenichthys baileyi

B



Crenichthys nevadae

C

Empetrichthys latos

Figure 1. Illustrations of A, *Crenichthys baileyi*; B, C. *nevadae*; and C, *Empetrichthys latos*. A and B drawn by Silvo Santina, C drawn by Grace Eager (La Rivers 1962)

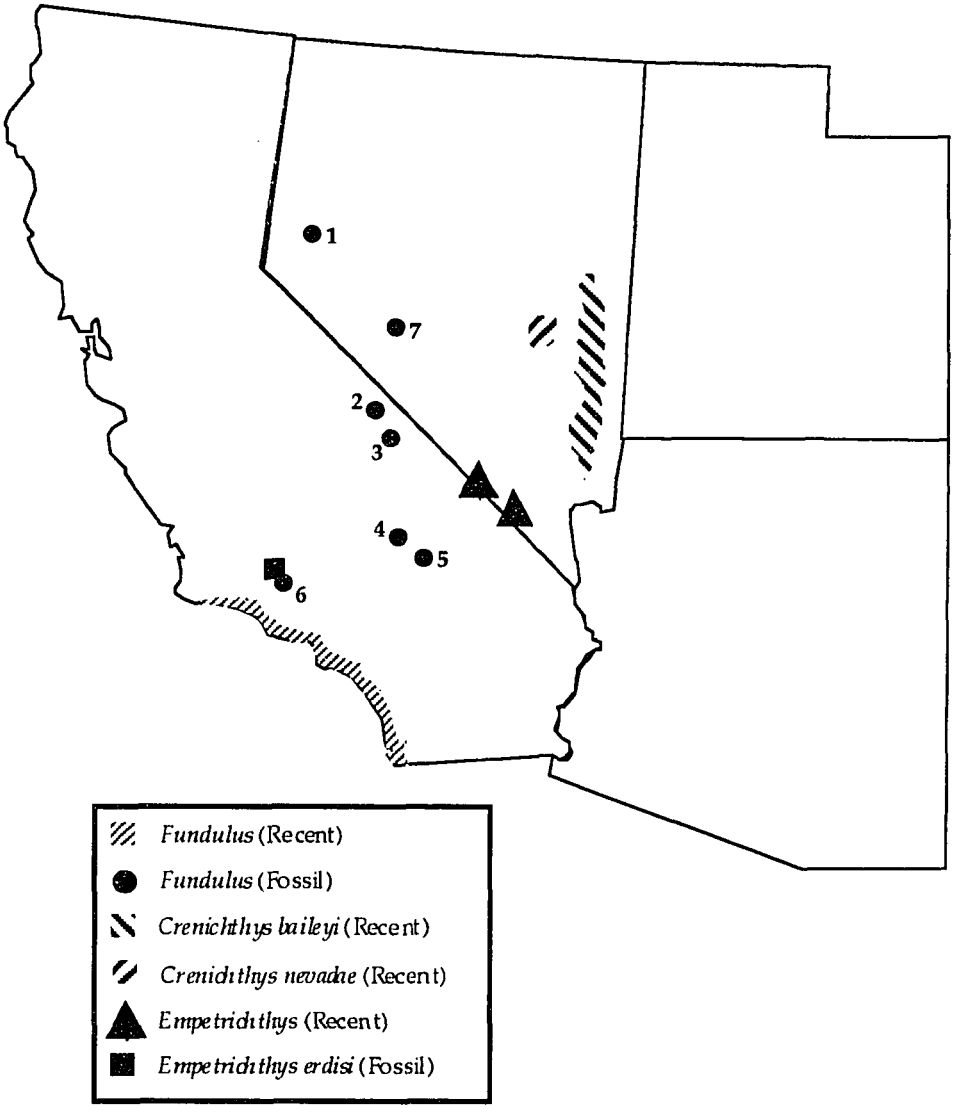


Figure 2. The southwestern United States showing the distribution of recent *Crenichthys*, *Empetrichthys*, and *Fundulus* with the fossil *Empetrichthys erdisi* and fossils of *Fundulus*: 1.*F. nevadensis*; 2. *F. eulepis*; 3. *F. curryi*; 4. *F. davidae*; 5. *Fundulus* sp.; 6. *Fundulus* sp. derived from Uyeno and Miller (1962). 7. *Fundulus lariversi* (Lugaski, 1977)

a subspecies of *Empetrichthys merriami* (Gilbert 1893), but was reclassified by Miller (1948) as *E. latos*. *Empetrichthys merriami* of Ash Meadows (Death Valley) is extinct (Minckley and Deacon 1968). *Empetrichthys latos latos*, the only surviving subspecies, was translocated from its native habitat in Manse Spring (Pahrump, Nevada) to Corn Creek, Nevada in 1971 (Deacon 1979). Two other subspecies of *Empetrichthys latos*, *E. l. pahrump* and *E. l. concavus*, became extinct due to ground water pumping (Deacon 1979).

Crenichthys baileyi is native to several springs along the pluvial White River in Nevada (Figs. 1 and 2). Five subspecies are included in *Crenichthys baileyi*: *C. b. baileyi, C. b. albivallis, C. b. thermophilus, C. b. grandis,* and *C. b. moapae* (Williams and Wilde 1981). *Crenichthys baileyi* was originally named *Cyprinodon macularius baileyi* (Gilbert 1893), and later renamed *Cyprinodon baileyi* (Jordan and Evermann 1896). In a physiological study, Sumner and Sargent (1940), on the recommendation of Hubbs, placed *Cyprinodon baileyi* into the genus *Crenichthys.* This taxonomic placement was disputed since the name *Cyprinodon macularius baileyi* resurfaced twice (Tanner 1950; Bohlke 1953). *Crenichthys nevadae* (Hubbs 1932) is the only other species in the genus *Crenichthys*. *C. nevadae* is native to seven springs in Railroad Valley Nevada (Figs. 1 and 2) and has been introduced to several additional springs (Williams and Williams 1981). Hubbs (1932) was the first to suggest that *Crenichthys nevadae* and *Cyprinodon baileyi* are sister taxa. Hubbs (1932) aligned *Crenichthys nevadae* with *Empetrichthys merriami* using the following characters: presence of protractile premaxillaries, placement of the pectoral, dorsal and anal fins, with vomerine teeth and pseudobranchiae absent. Hubbs differentiated the two using intestinal length, jaw, and tooth structure. He also indicated a similarity between *Empetrichthys* and two genera in the family Goodeidae, *Characodon* and *Zoogoneticus* but stated that they were obviously "superficial." Subsequent to the discovery of *Crenichthys nevadae* (Hubbs 1932) and the reclassification of *Cyprinodon*

baileyi to *Crenichthys baileyi* (Sumner and Sargent 1940; Hubbs and Miller 1941), the genera *Crenichthys* and *Empetrichthys* have been considered sister taxa.

The higher taxonomic placement of *Empetrichthys* is unclear. Gilbert (1893) placed *Empetrichthys* in the family Cyprinodontidae. Garmen (1895) retained this classification adding that *Empetrichthys* is allied to *Fundulus.* Jordan and Evermann (1896) and Gill (1894) lumped all Cyprinodonts in the Family Poeciliidae. Jordan changed this classification (1923) after Eigenmann (1920) placed *Empetrichthys* and *Orestias* (a fish similar in appearance of the high Andes) in the family Orestiidae. Hubbs (1924) supported Garmen's (1895) placement of *Empetrichthys* in the family Cyprinodontidae adding that the southern Mexico genus *Profundulus* was likely ancestral to *Empetrichthys.* Jordan, Evermann, and Clark (1930) placed *Empetrichthys* in the new family Empetrichthyidae. Myers (1931) put *Empetrichthys* in the subfamily Fundulinae of the family Cyprinodontidae along with *Fundulus* (Fig. 3). Hubbs (1932) supported this classification, as have most investigators since (Uyeno and Miller 1962; Rosen 1964; Sigler and Sigler 1987).

The discovery of five fossil species of *Fundulus* in the Southwestern deserts: *F. nevadensis* in the Lahonton Basin, *F. eulepis* in Death Valley, *F. curryi* in Death Valley, *F. davidae* and *F. sp.* in the Mojave Desert (Miller 1945), supported Garman and Myers' hypothesis of a funduline ancestry for *Empetrichthys* (Fig. 2). *Fundulus nevadensis* is considered early Pliocene while *F. eulepis* and *F. curryi* may be Pliocene or older (Uyeno and Miller 1962). The age of *F. davidae* is undetermined. These fishes greatly resemble *Fundulus parvipinnis*, which is found in the coastal brackish waters of southern California. This suggests a biogeographic connection between coastal California and the Amargosa (Death Valley) system. Furthermore, *Fundulus curryi* shows a strong resemblance to *Empetrichthys* (Miller 1948). A fossil Funduline species, *Fundulus lariversi* (Lugaski 1977) was found in Miocene strata near Tonopah, Nevada (Fig. 2). This fossil, which

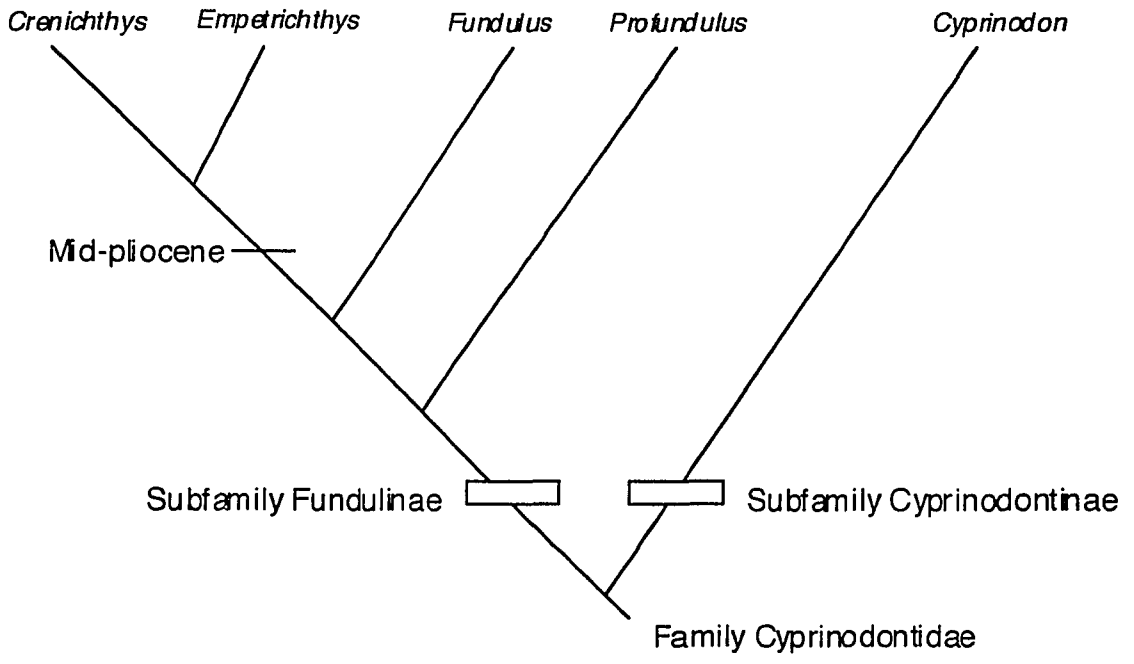


Figure 3. A cladogram of Cyprinodontiform fishes (Rosen 1964) based on Myers' (1931) division of Cyprinodontidae into two subfamilies and Uyeno and Miller's (1962) reassessment.

shows a close affinity to *Empetrichthys*, further supports the hypothesis that *Empetrichthys* and *Crenichthys* are derived from Funduline stock (Lugaski 1977).

A Miocene fossil species of *Empetrichthys, E. erdisi*, was discovered in Los Angeles County California (Uyeno and Miller 1962; Link 1982). *Fundulus* fossils and living populations of *Fundulus parvipinnis* occur in relatively close geographic proximity (Fig. 2). The fossil species *Parafundulus erdisi* (Jordan 1924), is probably a synonym of *Empetrichthys erdisi* (Uyeno and Miller 1962).

Based on derived characters involving jaw structure, caudal fin, first pluvial rib, and the pectoral girdle, Parenti (1981) provided an important revision of the Cyprinodontiform fishes. This restructuring included placing *Crenichthys* and *Empetrichthys* in the family Goodeidae. With the exception of *Crenichthys* and *Empetrichthys*, the Goodeidae includes 16 genera whose ranges are limited to central Mexico (Fig. 4). *Fundulus* was placed by Parenti in the new family Fundulidae. *Profundulus* was placed in its own family Profundulidae. The family Cyprinodontidae was left with *Cyprinodon* and a host of other genera.

Parenti's phylogenetic hypothesis (Fig. 5), postulates that Poeciliids, Goodeids, and Cyprinodontines share an ancestor that doesn't include Fundulines or *Profundulus*. Under this scheme, *Crenichthys* and *Empetrichthys* are more closely related to other Goodeids than to *Fundulus*. The biogeographic implications are interesting because, instead of *Crenichthys* and *Empetrichthys* sharing a common ancestor with the geographically adjacent genus *Fundulus*, their nearest relatives are indigenous to Central Mexico. While Parenti's phylogenetic hypothesis seems biogeographically less plausible (Fig. 4), it has been accepted by several investigators (Deacon and Williams 1984; Minckley et al. 1986; Rhinne and Minckley 1991; Wiley et al. 1991; Burr and Mayden 1992; Grudzien et al. 1992). Miller and Smith (1986) classified *Crenichthys* and *Empetrichthys* in the family Empetrichthyidae (Jordan et al. 1930); and suggested that Empetrichthyidae and Goodeidae had a Miocene connection. The acceptance of Parenti's

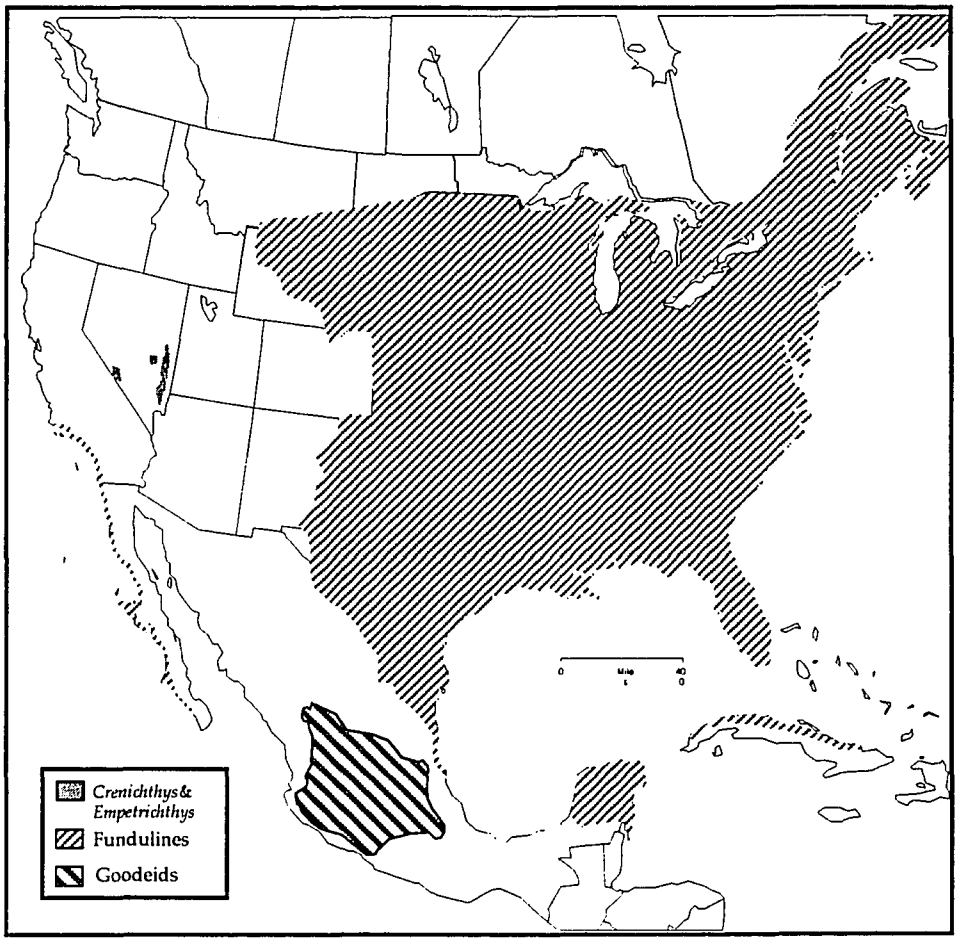


Figure 4. The distribution of *Crenichthys*, *Empetrichthys*, Funduline, and Goodeid fishes.

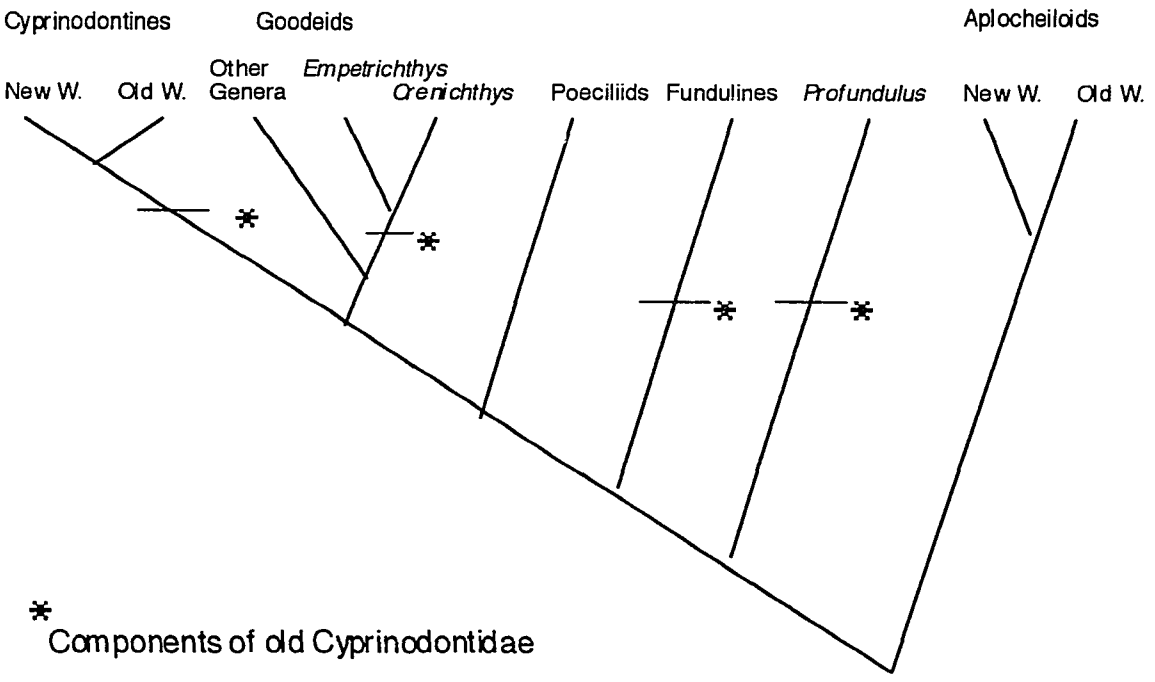


Figure 5. A cladogram of higher taxa based on Parenti's (1981) reclassification of Cyprinodontiform fishes. Derived from Fig. 9, page 366.

system is not universal. For example Sigler and Sigler (1987) continued to list *Empetrichthys* and *Crenichthys* in the family Cyprinodontidae (Table 1).

HYPOTHESES AND IMPLICATIONS

I have used nucleotide sequence data from a mitochondrial (mt) DNA gene (cytochrome-b) to test the alternative hypotheses presented by Parenti (1981) and Uyeno and Miller (1962) for the phylogenetic affinities of *Empetrichthys* and *Crenichthys* (Fig. 6).

Molecular data sets offer an analysis independent of morphologically based phylogenetic hypotheses. With DNA sequence data it is possible to obtain many more characters than morphological data could provide. Unlike nuclear DNA, mitochondrial DNA is a non-recombined molecule and is relatively easy to isolate and assay (Avise et al. 1987). Since mtDNA is passed on maternally, intra-population lineage-sorting is likely to rapidly result in a homozygous population of mtDNA genotypes. Therefore, for analysis at the taxonomic level of species or higher taxa, mtDNA techniques require only one or two specimens from each taxon studied (Hillis 1987). Recently, for example, valuable information was obtained on the intra-generic relationships of *Cyprinodon* (Echelle and Dowling 1992) using mtDNA restriction site data, thereby indicating the potential value in this approach for understanding phylogeny and biogeography of North American fishes.

A *Crenichthys* and *Empetrichthys* sister-taxon relationship with fundulines would support a model of historic hydrological connections between coastal southern California and the Amargosa Valley. Alternatively *Crenichthys* and *Empetrichthys* are monphyletic with goodeids, then either there was hydrological connectivity between the Mojave and Great Basin deserts of Nevada and the Mexican Plateau, or they are of sufficient antiquity to have become disjunct by tectonic processes.

Table 1

Taxonomic History of Crenichthys and Empetrichthys

Investigator	Family	Sister Taxon
Gilbert (1893)	Cyprinodontidae	
Garmen (1895)	Cyprinodontidae	*Fundulus*
Gill (1894); Jordan and Evermann (1896)	Poeciliidae	
Eigenmann (1920); Jordan (1923)	Orstiidae	*Orestias*
Hubbs (1924)	Cyprinodontidae	
Jordan, Evermann, and Clark (1930)	Empetrichthyidae	
Myers (1931) Supported by Hubbs (1932); Uyeno and Miller (1962); Rosen (1964)	Cyprinodontidae [Fundulininae]	
Parenti (1981)	Goodeidae	
Miller and Smith (1986)	Empetrichthyidae	Goodeidae

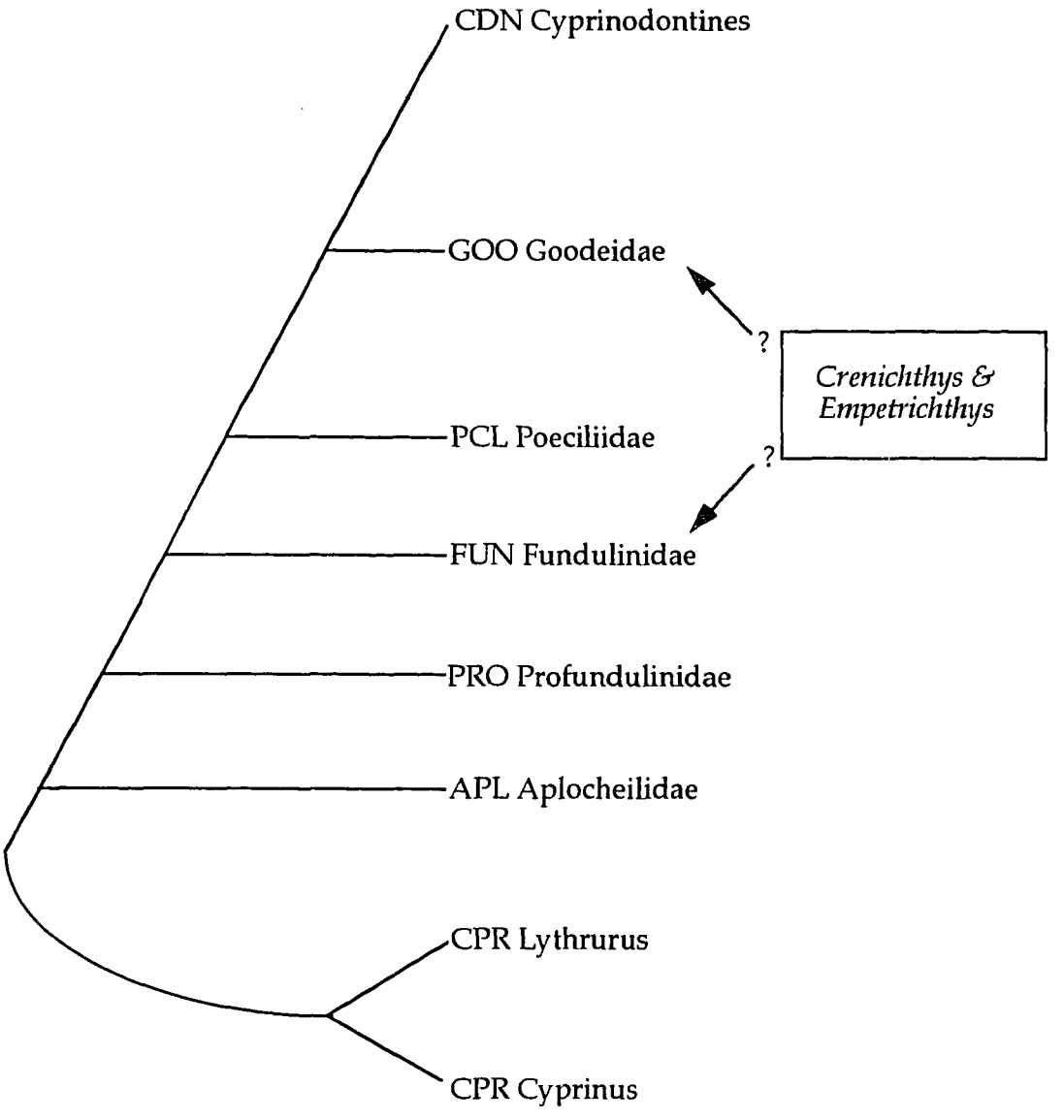
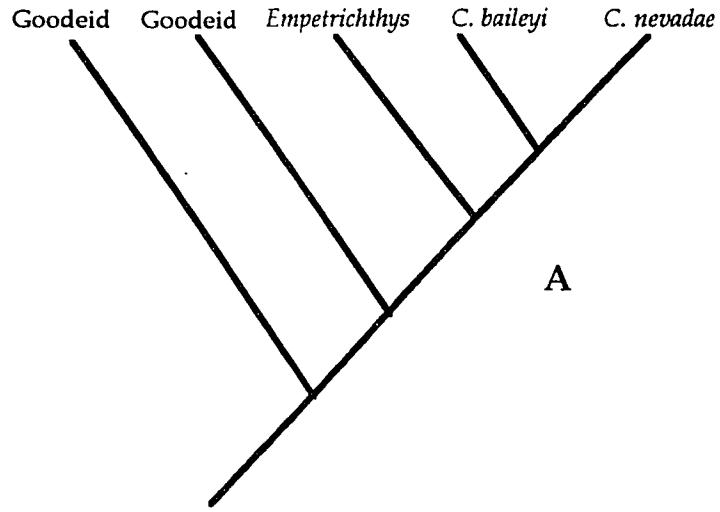


Figure 6. Alternative hypotheses of the phylogenetic affinities of *Crenichthys* and *Empetrichthys*.

A *Crenichthys* and *Empetrichthys* sister taxon affinity with the Mexican Plateau goodeids would suggest an interesting set of evolutionary hypotheses because *Crenichthys* and *Empetrichthys* would then be the only oviparous genera in an otherwise viviparous family. If *Crenichthys* and *Empetrichthys* share a common ancestor with all the Mexican Plateau goodeids (Fig. 7) then oviparity in *Crenichthys* and *Empetrichthys* is likely to be the primatively retained trait (Fig. 8). If *Crenichthys* and *Empetrichthys* share a common ancestor with only a subset of the goodeids (Fig. 7), then oviparity in *Crenichthys* and *Empetrichthys* is likely to be secondarily derived (Fig. 8).



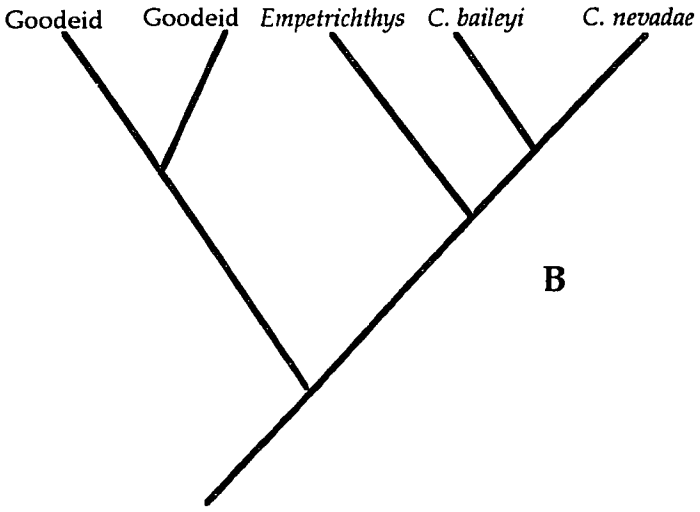


Figure 7. Two alternative hypotheses of plausible evolutionary histories of *Crenichthys* and *Empetrichthys* within the goodeid clade. (A) *Crenichthys* and *Empetrichthys* are derived from a lineage of extant goodeids. (B) The common ancestor of *Crenichthys* and *Empetrichthys* preceded the radiation of extant goodeid lineages.

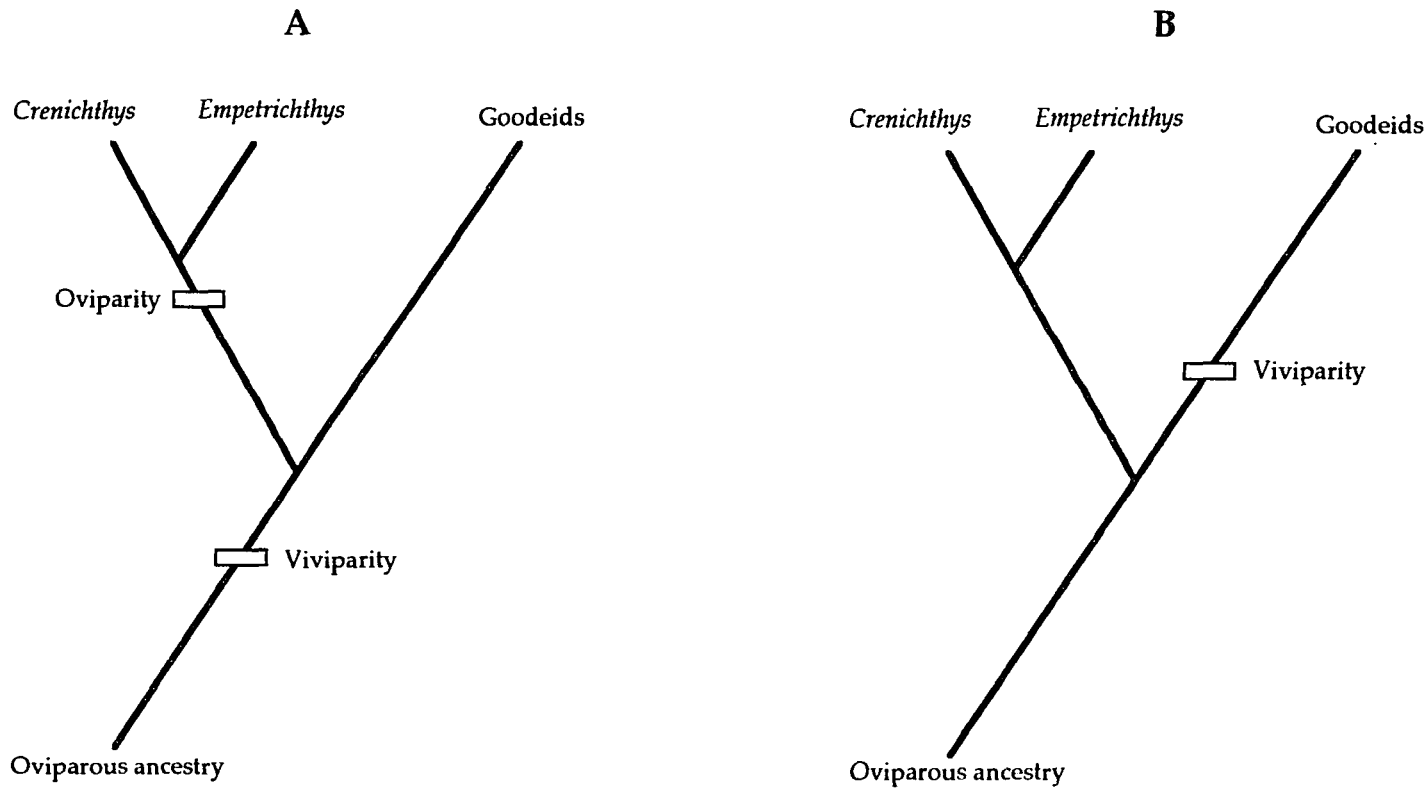


Figure 8. Two hypotheses of the origins of oviparity and viviparity in the goodeids, *Crenichthys* and *Empetrichthys*. (A) Viviparity arose prior to the vicariance of *Crenichthys* and *Empetrichthys* with oviparity appearing in *Crenichthys* and *Empetrichthys*. (B) Viviparity arose in the non-*Crenichthys*/*Empetrichthys* goodeids only.

CHAPTER 2

MATERIALS AND METHODS

All previous taxonomic studies were based on phenotypic characteristics such as skeletal morphology. With the advent of Polymerase Chain Reaction (PCR) and mitochondrial DNA (mtDNA) sequencing, we can use genomic characteristics for an independent phylogenetic test of the alternative hypotheses presented. Cytochrome-b has been successfully used for phylogenetic reconstruction of mammals which range in times of divergence from 5 - 60 million years (Irwin et al. 1991) and on cichlid fish (Meyer et al. 1990) with an estimated divergence time ranging from 200,000 - 4 million years. Furthermore, the cytochrome-b universal primers L14115 and H14542 have been shown to amplify a wide variety of fish mtDNA's (Kocher et al. 1989; Meyer et al. 1990).

Phylogenetic analysis based on mitochondrial DNA could yield a substantially different result then those previously suggested. For this reason the apolocheiloid *Cynolebias whitei* and the profundulid *Profundulus punctatus* were included in the analysis. All other cyprinodontiforms included belong to families that have been postulated as sister groups to *Crenichthys* and *Empetrichthys* (Table 1). The goodeids *Ilyodon furcidens* and *Skiffia multipunctata* have be shown to be highly divergent from one another (Grudzien et al. 1992), and therefore represent a wide range of the divergence present within the Goodeidae.

COLLECTION OF SPECIMENS

All work was done in accordance with a protocol approved by the University of Nevada, Las Vegas Animal Care and Use Committee and followed regulations set by the Animal Welfare Act. Collected animals were euthanized in the field; purchased and donated animals were euthanized upon arrival. The fish and amphibian anesthesia MS-222 (3-Aminobenzoic Acid Ethyl Ester Tricane Methanesulfate) was used in lethal doses (4 grams / liter H_2O) to euthanize all specimens utilized in this study. The specimens were placed in the solution until all opercular movement had ceased.

With the exception of *Cyprinodon nevadensis*, collected specimens (Table 2) were captured using an un-baited fry trap. *Cyprinodon nevadensis* was captured using a seine under the protocols and permits of Dr. David Soltz (Cal. State Long Beach).

The author was an additional collector under a State of Nevada Dept. of Wildlife permit (# S 6942) granted to Dr. James E. Deacon (UNLV) to collect *Crenichthys nevadae* and *Empetrichthys latos*. The stipulations required the presence of Jim Heinrich (Nevada Dept. of Wildlife) and that no more than 3 specimens for each species be collected. The only other Federally protected species, *Crenichthys baileyi*, was taken from a captive population maintained at the University of Nevada, Las Vegas animal research facility.

All other specimens, purchased or donated, were aquarium stock shipped live via express mail. All acquisition data on specimens used in this project was kept on an Excel (Macintosh Spreadsheet) file common to the UNLV molecular systematics laboratory. Each specimen was issued a catalogue or LVT (Las Vegas Tissue) number (Appendix). All specimens were documented by photograph. Voucher specimens for each species were initially fixed in formalin and preserved in 70% ethanol. Voucher specimens are kept in WHI 214 (UNLV).

Table 2. Specimens used in study with catalogue number.

Terminal taxon represented	Taxon Code	Species	Cat. #
Undetermined	none	*Crenichthys baileyi*	LVT 1749
		Crenichthys nevadae	LVT 1759
		Empetrichthys latos	LVT 1763
Cyprinodontines	CDN	*Cyprinodon nevadensis*	LVT 743
Fundulines	FUN	*Fundulus zebrinus*	LVT 430
		Fundulus parvipinnis	Sequence donated
		Fundulus heteroclitus	Sequence donated
Goodeids	GOO	*Ilyodon furcidens*	LVT 1745
		Skiffia mulitipunctata	LVT 1743
Poecilliids	PCL	*Gambusia affinis*	LVT 1734
		*Xiphophorus helleri**	LVT 1756
		*Poecilia reticulata**	LVT 1730
Profundulus	PRO	*Profundulus punctatus*	LVT 1748
Aploceiloids	APL	*Rivulus cylindraceus**	LVT 1737
		Cynolebias whitei	LVT 1739
Cyprinodontiformes	CPR	*Lythrurus roseipinnis*	Sequence donated
		Cyprinus carpio	Sequence in database

* Not used in the final analysis.

LABORATORY PROCEDURES

Tissues were collected immediately after euthanization. Brain, heart, and skeletal muscle were collected using flame sterilized instruments under a dissection scope. The tissues were stored in labeled nunc Cryogenic tubes. These and all catalogued tissues from the UNLV molecular systematics laboratory are stored in a cryogenic (-70° C) freezer in WHI 214 (UNLV).

DNA extraction was performed using a standard phenol / chloroform technique (Hillis et. al 1990). Approximately 100 mg of tissue were ground in STE buffer (Sodium chloride, Tris, and EDTA) using a tissue miser. The solution was incubated with SDS (Sodium lauryl sulfate) and Proteiniase K for 2 hours at 55° C, followed by extractions with equal volumes of PCI (Phenol, chloroform, and Isoamyl alcohol, with a ratio 25:24:1) for 5 minutes at room temperature; then centrifuged for 5 minutes to separate aqueous and non-aqueous layers. The aqueous (top) layer was pipetted off, discarding the non-aqueous layer using appropriate toxic waste disposal procedures. The PCI steps were then repeated, using the aqueous layer. The product was washed twice using the same steps but substituting CI (Chloroform and isoamyl alcohol) to remove all traces of phenol. The product was chilled (-20° C) overnight with 10% by volume 2M sodium chloride and diluted 2:1 with 100% ethanol in order to precipitate the DNA. The precipitate was then centrifuged and the liquid was discarded. The DNA was dried using a heated vacuum centrifuge (Savant Speed Vac) then rehydrated overnight with 250 μl purified water. The final product is the total genomic prep which serves as a template for PCR double strand amplification.

Polymerase Chain Reaction (PCR) is the process by which specified segments of DNA were amplified (replicated). The process (White et al. 1989) for double strand amplification requires two primers. Primers are oligonucleotides which hybridize to the

template DNA. PCR uses the thermally tolerant enzyme *Taq* DNA polymerase. The PCR reaction is run in a Perkin Elmer thermal cycler. This device heats and cools micro centrifuge tubes to specific temperatures at specific intervals for a specific number of cycles. Each cycle ideally doubles the amount of product; therefore the PCR can amplify a particular DNA fragment several million fold (Fig. 9).

Approximately 300 bases of the cytochrome-*b* gene of the mitochondrial genome (Fig. 10) were used as the data set for this study. The primers (Table 3) are termed "L" and "H" for the light and heavy strand of the mt DNA. Different primer combinations for double strand (ds) DNA amplifications were used at different times in the study. Primers L14115 and H14542 were used to amplify small pieces (315 base pairs) to get initial data. Primers L14724 and H15915 were used to amplify the entire gene from which other portions could be obtained at a later time (Fig. 10).

Double strand PCR amplifications were tested by running 4 μl of product diluted 1:1 with 1.5 M sucrose in an agarose electrophoresis gel. A 1 Kilobase size standard was run along with DNA products. The gel was stained in ethidium bromide and observed under UV light. A bright orange band of the appropriate size indicates the presence of the correct double stranded (ds) DNA product. The results from the gels were documented with Polaroid photographs.

The ds DNA was washed by centrifugation to remove unincorporated nucleotides and contaminants. The DNA was placed in a centricon tube with double distilled water and centrifuged at 4000 rpm's for 25 minutes. This was repeated twice then the centricon tube was placed upside-down and centrifuged at 750 rpm's for 3 minutes, resulting in approximately 40 μl of concentrated DNA.

Sequencing requires the use of single stranded (ss) DNA. This was accomplished by PCR with either one primer or using a primer and a limiting (1/10 concentration) primer (Table 3). The non-limiting primer may be the same as one used in the ds DNA reactions or internal to the ds DNA primers. The limiting primer (when used) runs in the same

Table 3.

PCR PRIMERS

Primer #	Sequence
Cyt b L Primers	
L 14115	CGA AGC TTG ATA TGA AAA ACC ATC GTT G
GL 14724	GTA ACT TGA AAA ACC ACC GTT G
Cyt b H Primers	
H 14542	GCA GCC CCT CAG AAT GAT ATT TGT CCT C
GH 15149	AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A
GH 15915	CAA CGA TCT CCG GTT TAC AAG AC

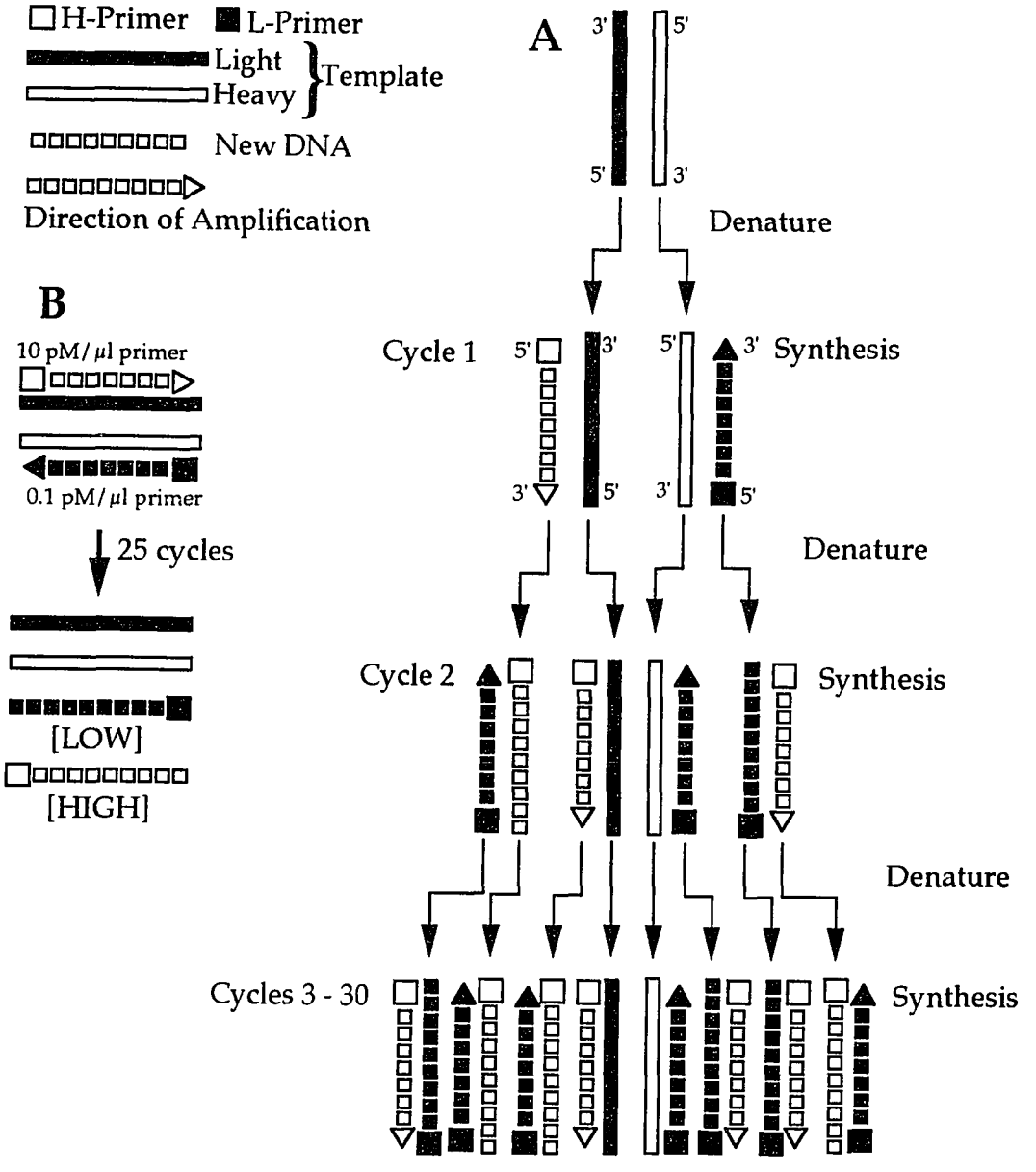


Figure 9. PCR amplification of A, double strand, and B, single strand DNA.

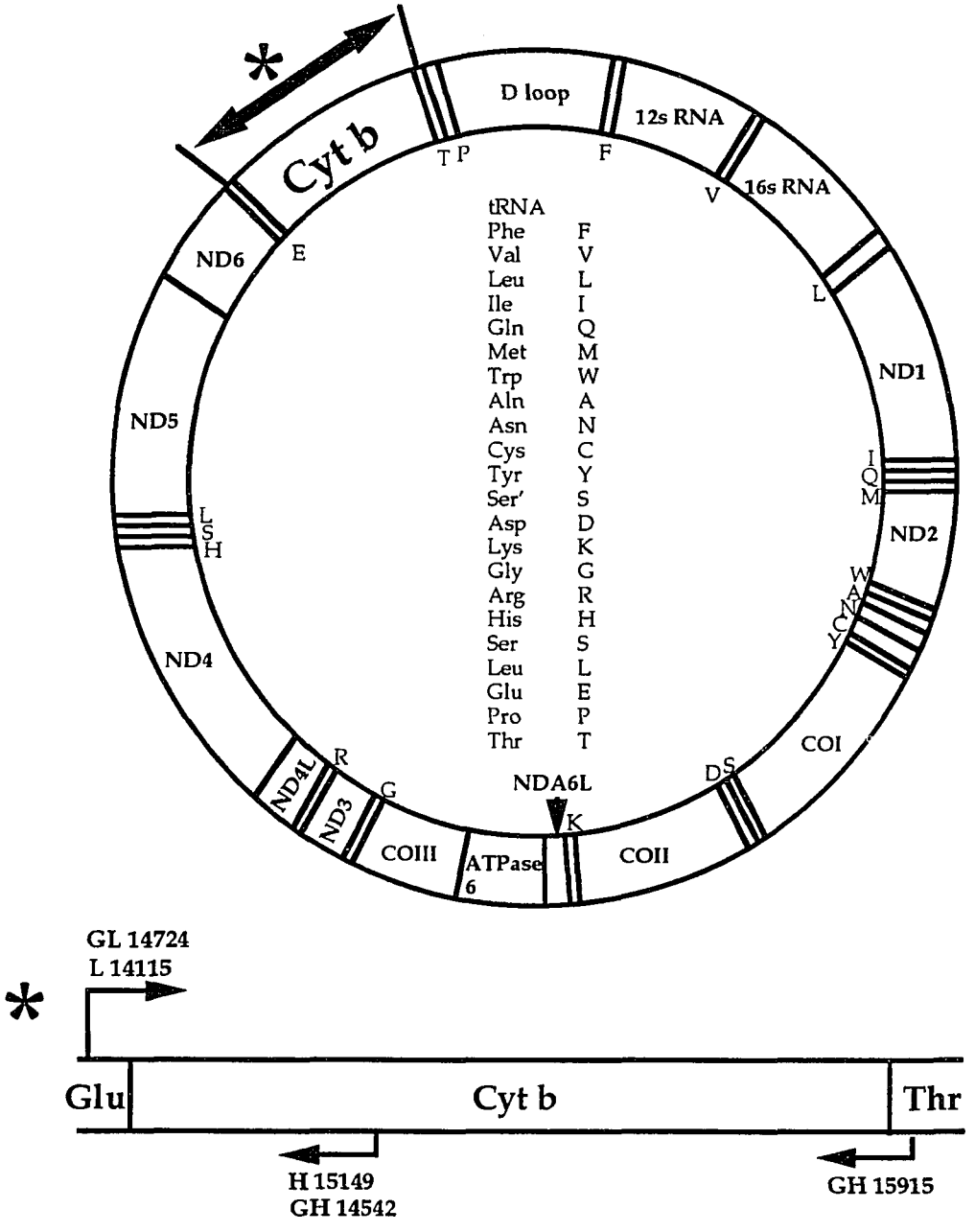


Figure 10. Mitochondrial DNA map with the Cyt b region enlarged using the primers shown.

direction as the Sequencing primer. It may be the same primer used in the ds DNA reactions or internal to the ds DNA primers. Limiting primers were only used when single strand amplifications were unattainable from the single primer method. The single strand product was tested and washed using the same procedures as with the double strand product. Single strand product bands in the agarose gel are not as bright as the double strand.

The ss DNA purified by the centricon process was used in the sequencing reaction. Sequencing was performed using the Sanger dideoxy method by protocols provided in USB (United States Biochemical) sequencing kits. 7 μl ss DNA were combined with 1 μl sequencing primer [10 pM/μl] and 2 μl of 5X sequencing reaction buffer in order to anneal the primer to the template. This mixture was incubated at 65° C for 2 minutes then allowed to cool for 30 minutes. The mixture was then briefly centrifuged (touch spin) to insure that it was completely at the bottom of the micro centrifuge tube. The Sequenase mixture is composed of 1.0 μl of Sequenase (vers. 2.0) and 6.5 μl enzyme dilution buffer. 3 μl of DuPont NEN's Sequetide (S^{35} labeled dATP plus non-labeled dCTP, dGTP, and dTTP nucleotides) were added to the end labeled templates followed by 2 μl of the Sequenase mixture. This reaction, which builds a new strand of DNA containing S^{35} labeled nucleotides, was allowed to incubate for 3 to 5 minutes. Tubes labeled with catalogue numbers and the corresponding terminal nucleic acids (A, C, G, or T), contained 2.5 μl of the appropriate termination mix. The termination mix includes dideoxynucleotides, which when incorporated, terminate the DNA extension. The termination mixes were pre-warmed to 37° C 2 minutes prior to addition of the pulse-labeling reaction. 3.5 μl pulse-labeling reaction were added to the termination mix using standard radiation safety procedures. The termination reactions were incubated at 37° C for 5 minutes before the addition of 4 μl of stop solution. The end products were four mixtures of radio-labeled DNA of random lengths which corresponded to an A, C, G, or T

termination. Upon completion, all samples were stored in a Plexiglas radiation container and frozen at -20° C for up to 14 days.

A denaturing urea-acrylimide electrophoresis gel was used to separate the DNA fragments by size. The small pore size in combination with high voltage allows one to distinguish fragments which differ by one nucleotide in size. Two clean glass plates (35 cm X 20 cm & 32.5 cm X 20 cm) with two plastic spacers (0.2 mm in thickness) were clamped lengthwise. The top, shorter plate was treated with 500 μl Sigmacote (Sigma Chemical Corp.) to prevent sticking. The acrylimide gel (Sequagel by National Diagnostics) was prepared by combining 16.8 ml gel concentrate (acrylimide), 46.2 ml diluent (urea), 7 ml 10X TBE (Tris, boric acid, disodium EDTA), 560 μl 10% AP (ammonium persulfate), and 25 μl TEMED. The TEMED was added last since it is the hardening agent. The acrylimide mixture was immediately injected between the glass plates by syringe from the overlap. Great care was taken to leave no air bubbles in the gel. The comb was placed approximately 0.5 cm into the gel, flat side in. This was done to leave a flat surface at the top of the gel. The gel was left for one hour to harden. The comb was then removed and replaced tooth side in to form the wells. The gel was clamped vertically into the gel apparatus, long plate facing out, with an aluminum heat sink against the back plate. 250 ml 1/2X TBE were placed in the top and 250 ml 1X TBE were placed in the bottom of the apparatus. The leads from the power supply were attached (red on the bottom). The power supply was set at 2200 volts, 80 milliamps, and 80 watts. The gel was warmed to 40° C by switching the power supply on prior to adding the labeled DNA. The labeled DNA was thawed, then heated to 80° C for two minutes. The power supply was then switched off. 3.5 μl DNA was placed in each well in alphabetic (A, C, G, T) order from left to right. The power supply was switched on after loading. At that time the loading sequence was logged.

Two dyes, bromophenol blue (dark blue) and xylene cyanol FF (light blue) are included in the stop solution which was added to the labeled DNA to terminate the

sequencing reaction. These dyes serve as electrophoretic markers. The bromophenol blue is of the lower molecular weight. It is lighter than one nucleotide; thus, it reaches the bottom of the gel prior to the first DNA fragment.

Two types of gels were run, these are termed short and long. A short gel was run until the dark blue dye reached the bottom. A long gel was run past the bottom in order to read nucleotides that couldn't be distinguished at the top of a short gel. A long run was terminated when the second dye (light blue) reached the bottom of the gel. A single sequencing reaction yeilds approximately 100 additional readable bases when a long run is performed.

125 ml sodium acetate was added to the bottom of the gel apparatus when the gel was 2/3 complete. This slowed the rate of electrophoresis at the bottom of the gel which caused the bands to "stack" allowing more bands to be read.

Upon completion of a run, the gel apparatus was dismantled and the plates were placed on two stands, raising them above the counter. The comb and spacers were removed and the plates were then carefully separated. A sheet of Whatmann 3MM chromatography paper was carefully placed directly onto the gel. The gel stuck to the paper and was peeled from the glass. A blotter was placed behind the chromatography paper and cellophane was placed over the gel. The gel was then dried using a Savant slab gel drier. Heat and vacuum were applied for 2 hours. The cellophane was removed and the gel was allowed to air dry for 20 minutes. It was then cut to size and, in complete darkness, placed in a film cassette against autoradiographic (X-ray) film. The film was exposed overnight. All radioactive waste, liquid and solid, was disposed of properly. The film was developed the next day using standard photographic development procedures. The autoradiograph was dried and then labeled with a felt marker.

SEQUENCE ENTRY AND ALIGNMENT

The gels were read from bottom to top as shown in figure 11. An IBI gel reader with MacVector software was used. The autoradiograph was placed on the reader which resembles a light table. A stylus was touched to the bands directly and digitized into a file. The sequence was saved and reentered for verification.

The sequence from *Gambusia affinis* (LVT 1734) was translated into protein format in MacVector and aligned with *Cyprinus carpio* (GenBank accession # x61010). This allowed testing for frame shift errors and provided a standard sequence to use in editing and aligning additional sequences. Sequence files were also converted into a DOS text file and opened in Sequaid, a program which allows alignment by eye.

PHYLOGENETIC ANALYSIS

All aligned sequences were loaded into the software MacClade (Maddison and Maddison 1992). MacClade provides a format for character weighting, character manipulation, and tree manipulation. MacClade files load directly into PAUP (Swofford 1991). In PAUP (Phylogenetic Analysis Using Parsimony), a variety of parsimony tree building and testing techniques were performed. With the complete data set, character state change charts were run in MacClade in order to address levels of signal and noise in the data set (Swofford and Olsen, 1990). An over abundance of character states, or saturation at a particular codon, indicates noise or "homoplasy" in the dataset. Alternatively, a lack of character state changes is uninformative. A data set that has the appropriate amount of character state changes is considered to have the optimal signal to noise ratio therefore yielding an unambiguous analysis.

Character weighting is the common way in which noisy characters can be filtered

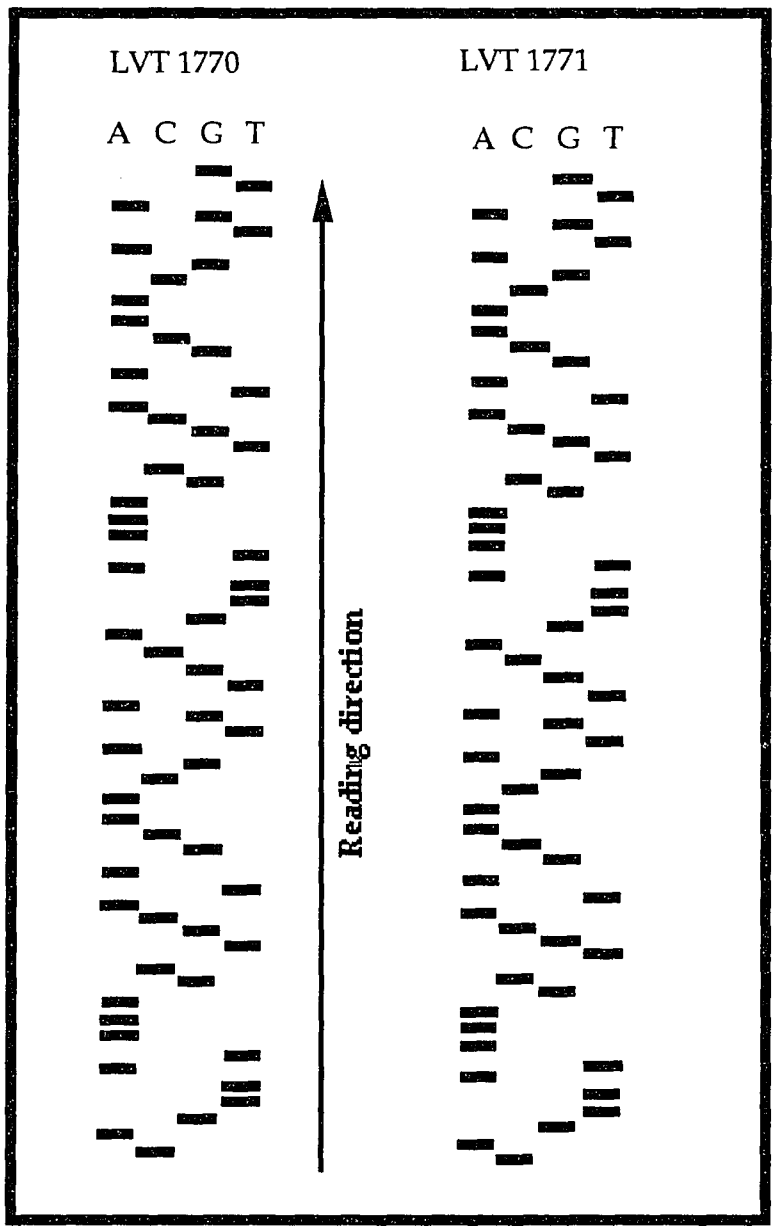


Figure 11. Sequence gel for LVT 1770 and LVT 1771. Sequence gels are read from the bottom up. Both LVT 1770 and LVT 1771 have the following sequence: CAGTTATA AGCTGCATAGCAACGATGATGCAGTTATAAAGCTGCATAGCAACGATGAG.

(Huelsenbeck and Hillis 1993). Noisy data are reduced in subsequent analyses providing greater phylogenetic resolution. The following nucleotide properties were considered in weighting: Transitions, which are purine to purine or pyrimidine to pyrimidine changes, are more common than transversions, purine to pyrimidine or pyrimidine to purine (Swofford and Olsen, 1990). Therefore, a common strategy to reduce noise in a data set is to ignore transition substitutions (i.e. Transversion Parsimony).

Parsimony analysis, which attempts to find a set of trees with the fewest character state changes, was run in PAUP, the heuristic TBR (tree bisection, reconstruction) option used. TBR is a branch swapping heuristic algorithm which finds the shortest tree by repeatedly bisecting the unrooted tree, then reconnecting the two subtrees by the joining pair of branches. All possible bisections and reconnections are evaluated (Swofford and Olsen 1990).

Bootstrap analysis provides an indicator of data set robustness (phylogenetic signal). While not a confidence indicator, bootstrap analysis tests how well all nodes in a tree are supported by the data. The support of tree nodes is evaluated by resampling columns of data from the dataset with replacement (Felsenstein, 1985). The Bootstrap TBR option was used with 500 replicates.

Heuristic trees were manipulated, using MacClade (Madison and Madison, 1992), as an additional test of data set robustness. The *Empetrichthys* and *Crenichthys* clades were joined to alternative taxa to determine the number of additional steps required for a given topology.

In addition to parsimony analysis, pairwise sequence divergence was calculated using the maximum likelihood approach (DNADIST in PHYLIP v3.5; Felsenstein, 1992). The 'T' option was set to 10 in order to provide a partial correction for bias in transition versus transversion rates in teleostean mtDNA (Thomas and Beckenbach, 1989). Neighbor-Joining (Saitou and Nei, 1987), a phylogenetic analysis that relaxes the

assumptions of equivalent rates of base substitution between taxa, was performed with the sequence divergence matrix obtained from PHYLIP.

CHAPTER 3

RESULTS

The data set of aligned sequences (Fig. 12) reveals numerous base substitutions. *Fundulus parvipinnis* and *Fundulus heteroclitus*, which are sequences graciously donated by Giacomo Bernardi, are missing the first 56 characters. *Crenichthys nevadae* is missing the first 38 bases and *Rivulus cylindracious* is missing the first 25 bases of the data set. *Profundulus punctata* is missing the last 80 characters and *Skiffia lermae* is missing the last 50. While these gaps in the data set are less than ideal, maximum parsimony algorithms can accommodate missing data.

PHENETIC ANALYSIS

The initial pair-wise distance matrix using maximum likelihood for all characters unordered is shown in Table 4. *Rivulus cylindracious.* is 31.0% divergent from the other Alpocheiloid *Cynolebious whitei.* This is a higher divergence than from the outgroup *Lythrurus roseipinnis* (28.4%). The high levels of divergence of *Rivulus cylindracious* with respect to all taxa including *Cynolebious whitei* suggest that the Aplocheiloids are a polyphyletic group, and that *Rivulus* is not an appropriate representative. *Rivulus cylindracious* was eliminated from subsequent analyses because it was not relevant to the basic question. Alternatively, these values may indicate a high level of saturation of base

Taxa	Sequence			
Gambusia affinis	?GAAACG???	??CTAGTGGA	TCTTCCCGCT	CCTGTCAACA
Ilyodon furcidens	GCT...?AAT	CT.....A..	C.....A...	..GT.?..T.
Crenichthys baileyi	GCT..TCAAT	CGA.C.....	C..C..AC.C	..CA.....
Crenichthys nevadae	.??????...	..????????	??????????	????????..
Empetrichthys latos	.??????...	..???..A..	.T...?A..C	..CA.....
Fundulus zebrinus	GC.....ACG	C...T..A.?	C.....A..C	..A..?....
Cyprinodon nevadensis	GC....AACG	CA..G.....	C..C..A..A	..A..A....
Cyprinus carpio	GCT....ACG	CA.....T..	C..A..AA.A	..ATC.....
Lythrurus roseipinnis	GCT..T.ACG	CAT....C..	C.....AA.A	..ATC...T.
Rivulus cylindracious	.??????...	..????????	?????.TA.A	...CCT..T.
Cynolebious whitei	.??????..C	ATT....A..	C..C..TA..	...A....T.
Skiffia multipunctata	.??????...	..????????	??????????	????.A.CAT
Profundulus punctatus	GC...T.AT.	CTT.G..A..	C..C..A..C	...A.T..T.
Fundulus heteroclitus	??????????	??????????	??????????	??????????
Fundulus parvipinnis	??????????	??????????	??????????	??????????

Gambusia affinis	TCTCAGCCTG	ATGAAACTTT	GGTTCCCTTC	TAGGACTTTG
Ilyodon furcidens	.......T..	....	........C.	....
Crenichthys baileyi	....	....	..A..T..C.	....G.....
Crenichthys nevadae	....	....	..G.....C.	....?.....
Empetrichthys latos	....	..?.......	..A.....C.	....G.....
Fundulus zebrinus	.TC.G.T...	....	.....TT.AT	....C..G..
Cyprinodon nevadensis	....C..T..	....	..C.......	....G..A..
Cyprinus carpio	.......A..	....	..A.....C.	.......A..
Lythrurus roseipinnis	.......AAT	C	..A.....G.	.G...T.G..
Rivulus cylindracious	.....A.???	.......AAC	..C..T..CT	....CA.A.?
Cynolebious whitei	.T.....T..	......T...	..C..T..A.	....G..A..
Skiffia multipunctata	.......T..	.........?	.?C.....C.	AT........
Profundulus punctatus	....	..?...T...	..C..T....	....TT....
Fundulus heteroclitus	??????????	??????....	..C.....CT	....CT.G..
Fundulus parvipinnis	??????????	??????....	..A..A..A.	.....T.A..

Gambusia affinis	CCTTATTACT	CAGATCCTGA	CCGGCCTTTT	CCTAGCAATG
Ilyodon furcidens	....GC.G.C	..A.....A.	.?.....A..	TT.......A
Crenichthys baileyi	.T.??C.G.C	..A.....A.	.......A..	T........A
Crenichthys nevadae	.T.G?C.G.C	..A.....A.	.......A..	T........A
Empetrichthys latos	....GC.G.C	..A.....A.	.G.....A..	.........A
Fundulus zebrinus	.T.A...G.C	.....TT...	.A..TT.A..	TT....T..A
Cyprinodon nevadensis	T..A...G.C	..AG..T.A.	.G..A..A..	TT.G......
Cyprinus carpio	.T.A.....C	..A..TT.A.	.......A..	......C..A
Lythrurus roseipinnis	T..A......	......T.A.	.T..AT.A..	......C..A
Rivulus cylindracious	.T.A...GTA	..A...T.A.	.T..A..C..	T..T.....A
Cynolebious whitei	TT.A....?.	..A..TT.A.	.T..AT.A..	T..T..T..A
Skiffia multipunctata	....GC.G.C	..A..T..A.	.T.....A..	..?T.....A
Profundulus punctatus	...A...GTC	..A..TT.A.	....T..A..	......T..A
Fundulus heteroclitus	TT.A...G.C	..A.....A.	.A...T.A..	T.....T..A
Fundulus parvipinnis	T...G.GT.C	.....TT...	....AT.A..	T.....T..A

Figure 12. Sequence alignments. Matching characters to the top sequence, *Gambusia affinis*, are represented by a dot; missing data is represented by a question mark.

Taxa	Sequences			
Gambusia affinis	CACTACACCT	CTGATATCTC	TACAGCATTC	TCATCTGTCG
Ilyodon furcidens	..T.....	.C..C..T..	C.T.....T	.....C..T.
Crenichthys baileyi	..T..T....	....C..T..	..T.....	.....C....
Crenichthys nevadae	..T..T....	....C..T..	..T..?....	.....C..T.
Empetrichthys latos	.....T....	....C..T..	..T.....	.....C..T?
Fundulus zebrinus	.....T..T.	.....T..	.....C...	..G..C..A.
Cyprinodon nevadensis	.....T..?.	.A..C..T..	...C.?....	.....C..T.
Cyprinus carpio	.....	.A..C..T..	A..C.....	.....TA
Lythrurus roseipinnis	.....	.C..C.....	G..C.....T	.....C...A
Rivulus cylindracious	..T...?..C	.C.....TA.	..T.....T	?.T..C..T.
Cynolebious whitei	.....A.	....C.....	C.....T..T	..T..C....
Skiffia multipunctata	..T..T..T.	....C..T.A	..T.....	.....
Profundulus punctatus	.....	.....	A.T.....T	..C..C....
Fundulus heteroclitus	..T..T..T.	.C..C..T..	.....	.....A..T.
Fundulus parvipinnis	.....	....C.....	C.....T	.....A..TA

Gambusia affinis	CCCATATTTG	CCGAGACGTT	AACTATGGCT	GACTCATCCG
Ilyodon furcidens	.A..C..C..	...T..T..A	.....C....	....T...?.
Crenichthys baileyi	.A..C..C..	...T..T..G	.....A.	.G.....
Crenichthys nevadae	.A..C..C..	...T..T..G	.....G.	.....
Empetrichthys latos	.G.....C..	...T..T..G	.....A.	.G.....
Fundulus zebrinus	....C.....	T.....A	.....	....T..T..
Cyprinodon nevadensis	.T..C.....	...C.....	..T.....	.....
Cyprinus carpio	....C..C..	.....A	..T..C....	....A.....
Lythrurus roseipinnis	.A.....	...T.....C	.....T.	....T..T..
Rivulus cylindracious	AA..C.....	...G.....C	...C....T.	.....T..
Cynolebious whitei	.A.....C..	.....A	..T.....	....G..T..
Skiffia multipunctata	.AT.....	...T.....A	..T..C....	.....
Profundulus punctatus	TA.....	.....A	..T.....T.	....G.....
Fundulus heteroclitus	....C..C..	.....T..A	..T.....	..T.A..T..
Fundulus parvipinnis	.....	...T.....G	..T.....T.	..T.A..T..

Gambusia affinis	CAACATACAC	GCCAACGGGG	CCTCTTTCTT	TTTTATTTGC
Ilyodon furcidens	A.....	.....A.	....C.....	C..CG.C..T
Crenichthys baileyi	A..T.....	.....A.	.T..GC.T..	C...G.C...
Crenichthys nevadae	A..T.....	.....A.	.T..AC.T..	C...G.C..?
Empetrichthys latos	A..T.....	.....A.	.T..AC.T..	C...G.C...
Fundulus zebrinus	T..T.....T	..T..T..A.	....C.....	.....
Cyprinodon nevadensis	A.....G..T	..T..T..A.	.A...??...	C.....C..T
Cyprinus carpio	T..TG.....	.....A.	.A..A.....	C..C.....
Lythrurus roseipinnis	A..T.....T	.....A.	.A..A..T..	C..C..C..T
Rivulus cylindracious	...T..T..T	..A..T....	.T..A..T..	C..C..C..T
Cynolebious whitei	T..T....G.	..A.....C.	....C..T..	C.....C..T
Skiffia multipunctata	A..T..G...	.....T..A.	.....C....	...C..C...
Profundulus punctatus	...T.....	.....A..A.	.T..A.....	.....
Fundulus heteroclitus	T..T..G...	.....	.....	C.....T
Fundulus parvipinnis	AT.T.....T	..T.....A.	.....A..	C.....

Figure 12. Sequence alignments (cont.)

Taxa	Sequences			
Gambusia affinis	ATCTACCTAC	ACATCGGCCG	AGGACTATAC	TACGGCTCCT
Ilyodon furcidens	..T..TT.T.	.......T..	...C....CA	??????????
Crenichthys baileyi	.G...T.AT.	....T...T.	...C.....T	C.T......A
Crenichthys nevadae	.....T.A??	?...T...T.	...C.....T	C.T......A
Empetrichthys latos	......TAC.	....T...T.	...C......	?.T......A
Fundulus zebrinus	.....TA.T.	.T.....A..	...G..G...	..T.....A?
Cyprinodon nevadensis	..T.....G.	.T.....T..	...C..TG.T	..T.....
Cyprinus carpio	......A...	......C...	...C......	.....A..A.
Lythrurus roseipinnis	..T..TA...	.T..T.CT..	C..T..T...	.....A..T.
Rivulus cylindracious	.....TA.T.	.T..T.CT..	.?.CT....T	C.T..T....
Cynolebious whitei	..T..TA...	.T.....	C..TT....T	..T.....
Skiffia multipunctata	......T.C.	.T..T.?...	...C.?G...	..T...????
Profundulus punctatus	T.A...T.T.	.......A..	...C......	..T.....A.
Fundulus heteroclitus	..T..T..T.	....T..A..	...CT....T	.....T..A.
Fundulus parvipinnis	..T..T..T.	.T.....A..	...CT....T	..T..A..T.

Taxa				
Gambusia affinis	ACCTATTTAA	AGAGACATGA	AACACTGGTG	TAATC
Ilyodon furcidens	??????????	????.....	....T...G.	.T...
Crenichthys baileyi	T.TC..A..T	....	..T.T...A.	.T???
Crenichthys nevadae	T.TC?.AC.?	.?...?????	??????????	?????
Empetrichthys latos	T?TC..AC??	?...G.?.?.	...GT...A.	.T..T
Fundulus zebrinus	?.....A...	...A....?.	..?GT...?.	.T??T
Cyprinodon nevadensis	..???.A..?	????..C...	..TGTG..A.	..TG?
Cyprinus carpio	....T.AC..	...A..C...	....T.....	..G..
Lythrurus roseipinnis	......AC..	...A..C...	....T...A.	.TG.T
Rivulus cylindracious	....?.AC..	..C?..G...	.....G..A.	.????
Cynolebious whitei	.T..C.A...	...A.....	..TGTC..C.	.TG.T
Skiffia multipunctata	??????????	??????????	??????????	?????
Profundulus punctatus	.T..T.A...	...A.....	..T.T...A.	.C...
Fundulus heteroclitus	....T.A...	...A.....	...GTA....	.T...
Fundulus parvipinnis	....T.AC..	GACA.....	..TGTG..A.	.T...

Figure 12. Sequence alignments (cont.)

Table 4.

Taxon	Sequence Divergence (%)*														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 *Gambusia affinis*		6.2	12.5	9.4	11.0	7.6	6.5	6.8	8.6		6.9	7.7	8.0	6.2	9.0
2 *Ilyodon furcidens*	23.2		6.3	5.0	5.3	9.4	8.1	9.8	11.8		12.2	6.5	8.0	10.0	10.9
3 *Crenichthys baileyi*	27.9	18.0		0.6	3.8	14.4	11.5	14.3	17.2		14.1	8.5	8.8	12.9	18.1
4 *Crenichthys nevadae*	23.6	13.7	3.3		1.9	11.8	9.0	11.8	15.1		11.8	7.3	7.8	11.9	16.5
5 *Empetrichthys latos*	24.3	17.9	8.5	6.0		12.6	10.9	14.4	16.0		13.1	5.6	7.6	13.0	14.8
6 *Fundulus zebrinus*	25.8	29.6	31.9	31.7	31.8		5.4	7.4	9.2		7.1	13.3	11.0	4.4	9.4
7 *Cyprinodon nevadensis*	27.3	27.6	29.1	24.3	28.9	26.0		8.7	10.7		9.2	9.4	7.9	8.0	8.9
8 *Cyprinus carpio*	22.3	23.4	31.0	24.1	28.0	27.7	26.5		3.5		6.4	11.6	10.2	8.3	9.9
9 *Lythrurus roseipinnis*	31.5	28.4	35.9	33.0	32.5	32.9	30.7	20.1			7.0	13.4	9.5	7.0	7.1
10 *Rivulus cylindracious*	37.7	32.8	34.3	29.3	39.1	37.8	33.8	34.5	28.4						
11 *Cynolebious whitei*	30.4	29.8	33.8	32.1	35.8	27.2	31.3	30.5	27.4	31.0		11.8	9.2	5.0	7.8
12 *Skiffia multipunctatus*	23.8	20.5	22.2	20.3	18.5	33.0	25.9	26.4	35.6	38.4	34.0		11.8	11.0	12.1
13 *Profundulus punctata*	24.1	28.1	27.4	25.9	25.9	27.7	31.8	27.0	30.3	31.8	24.7	29.2		9.6	10.8
14 *Fundulus heteroclitus*	26.3	21.5	26.8	23.9	30.4	21.0	26.1	21.7	31.3	30.8	25.2	28.8	30.5		5.7
15 *Fundulus parvipinnis*	30.4	28.2	34.6	33.7	33.7	27.4	28.2	28.3	25.5	38.0	26.2	34.9	29.6	23.4	

*Pair-wise distance matrix using maximum likelihood. The lower diagonal represents all codon position unordered. The upper diagonal represents first and second codon position unordered with the third codon position excluded. *Rivulus cylindracious* was eliminated from subsequent analyses; therefore, the third codon position excluded matrix lacks this taxa.

substitutions at certain nucleotide positions. Transition substitutions at the third codon position are most likely to demonstrate a saturation effect among distantly related taxa. Therefore, another distance matrix was generated after eliminating the third codon base position characters (Table 4). This matrix was used to construct a neighbor-joining tree (Fig. 13) The branch lengths are proportional to estimated levels of sequence divergence.

PARSIMONY ANALYSIS

Charting character state changes from trees derived by parsimony analysis provides a good indicator of the signal to noise ratio. These charts are histograms of the frequency of how often characters change states. Charts that are skewed strongly to the left indicate strong signal. Charts that are of an approximately "bell curve" distribution indicate saturation, or a noisy data set. Figure 14 is the most parsimonious tree with all characters unordered (i.e. generalized parsimony). The numbers at various nodes are the results of bootstrap analysis. Only bootstrap values over 50% are shown. The chart from that tree (Fig. 15) is not highly skewed to the left when ignoring the invariant characters. This indicates a poor signal to noise ratio. Further analysis in determining where saturation is occurring is shown on figure 16. The total character state changes for each codon position is shown at the top. The third codon position is responsible for the vast majority of character state changes. Figure 16 also shows the character state changes at the third codon position only. This is approaching a "bell curve" distribution; thus the third codon position unordered is not an informative portion of this data set over the entire tree. Tree manipulations (Fig. 17), show the number of additional steps from the most parsimonious tree that are required when forcing the *Empetrichthys-Crenichthys* clade to form a sister taxon relationship with *Fundulus heteroclitus*, *F. parvipinnis*, and *F. zebrinus*.

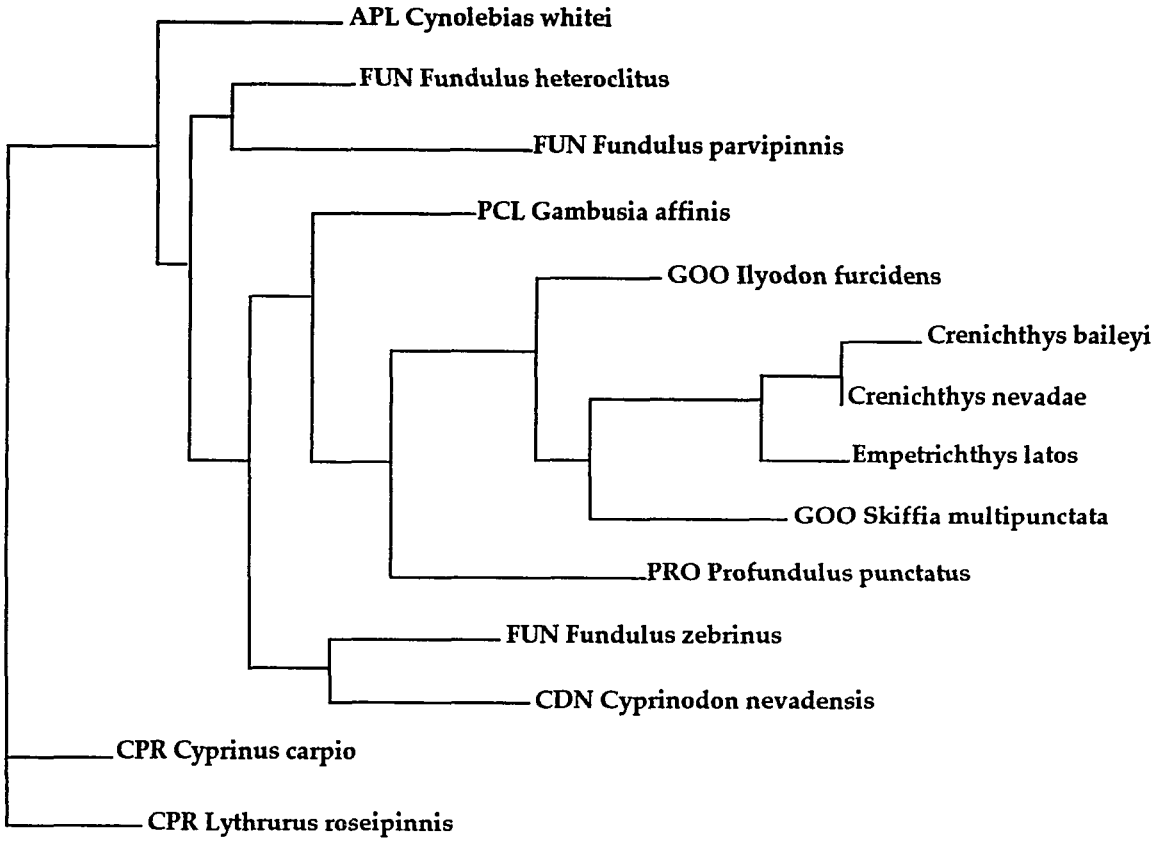


Figure 13. The neighbor-joining tree based on the distance matrix generated after excluding the third codon position characters (Table 4).

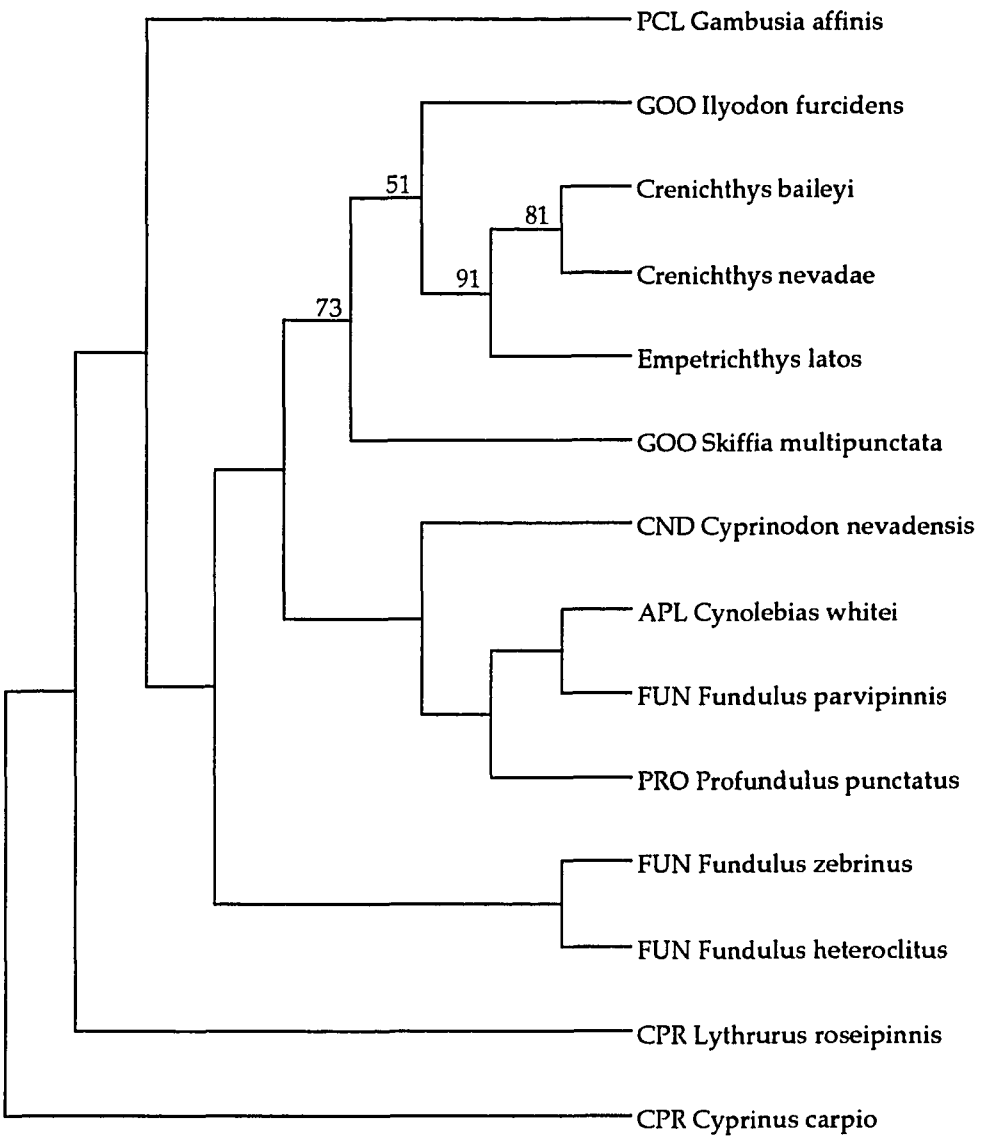


Figure 14. The most parsimonious tree (TBR branch swapping) for all characters unordered. TBR bootstrap (200 reps) with values over 50% shown.

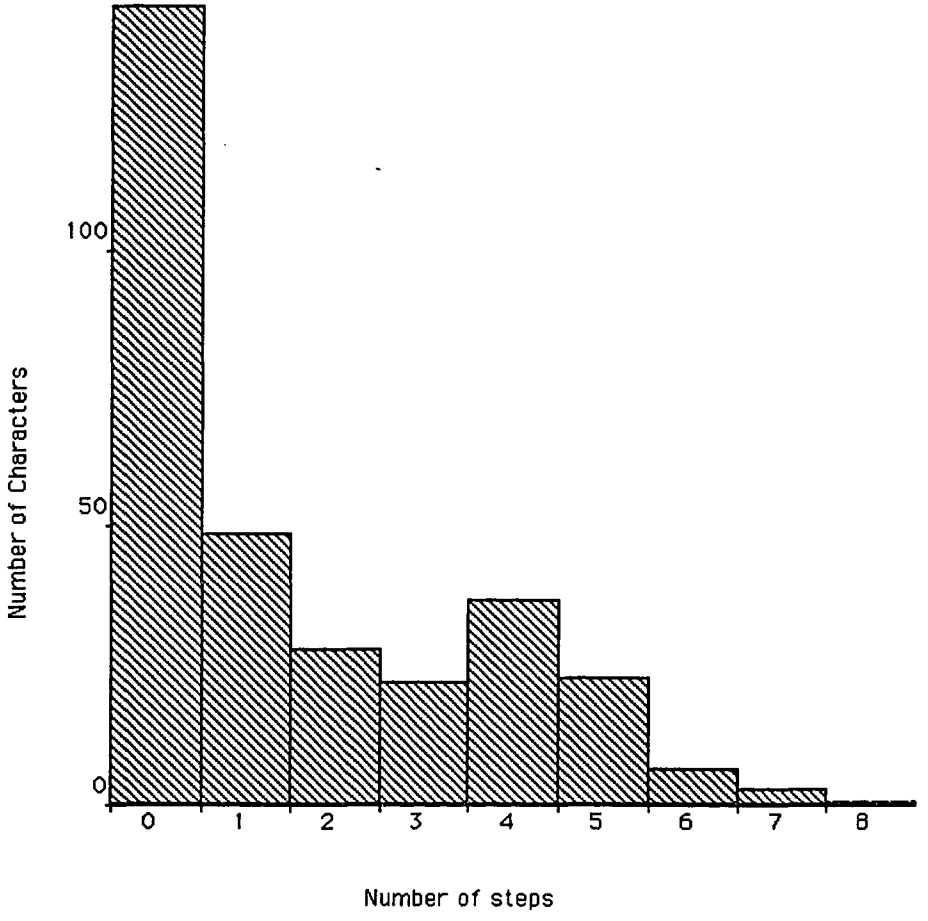


Figure 15. A histogram of the number of times each character changed states calculated from the dataset (Fig. 12) over the most parsimonious tree (Fig, 14).

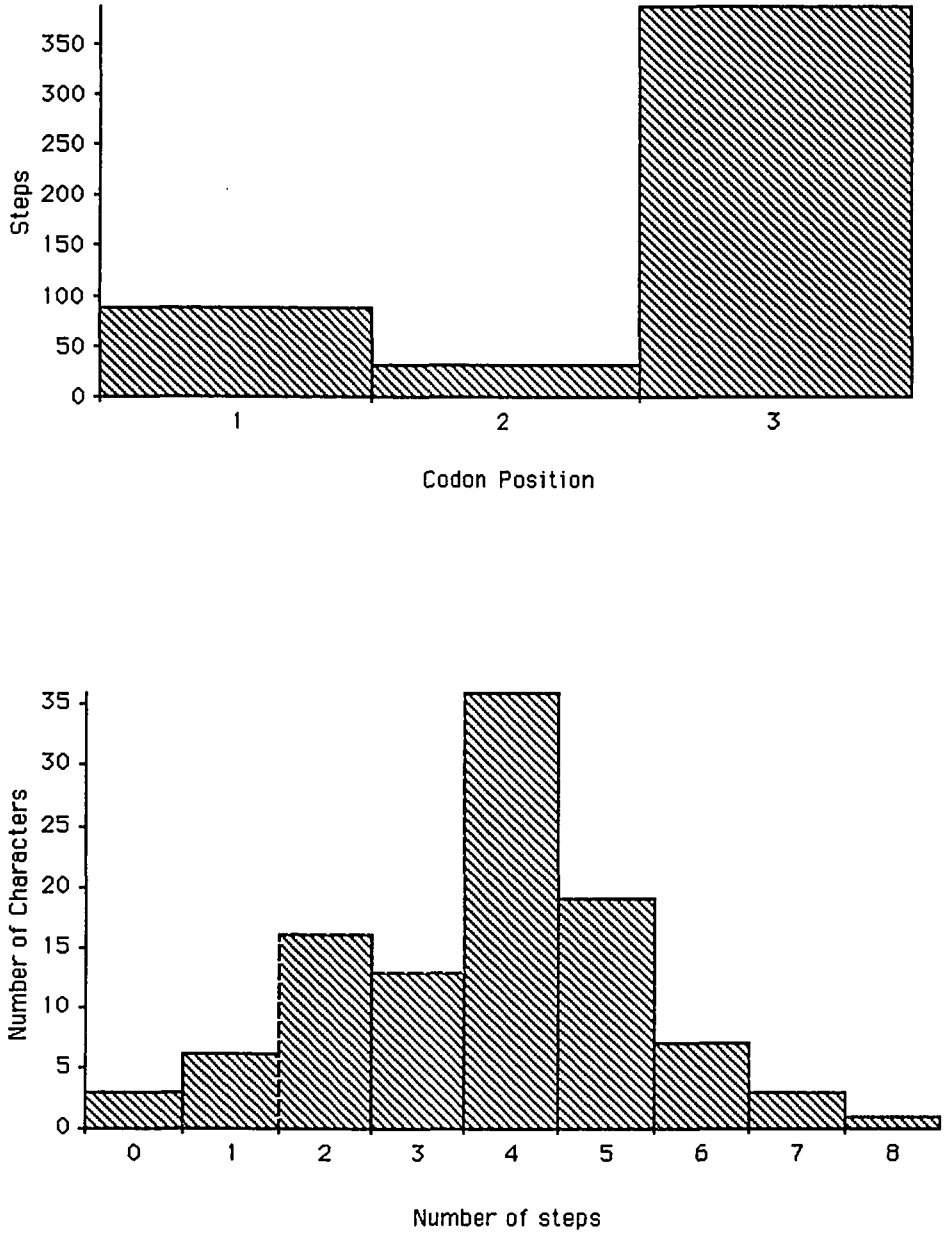


Figure 16. Histograms of the number of character state changes (steps) charted over the most parsimonious tree of the complete data set (Fig. 14). The total number of character state changes by codon position are charted (top), and the third codon position only charted (bottom).

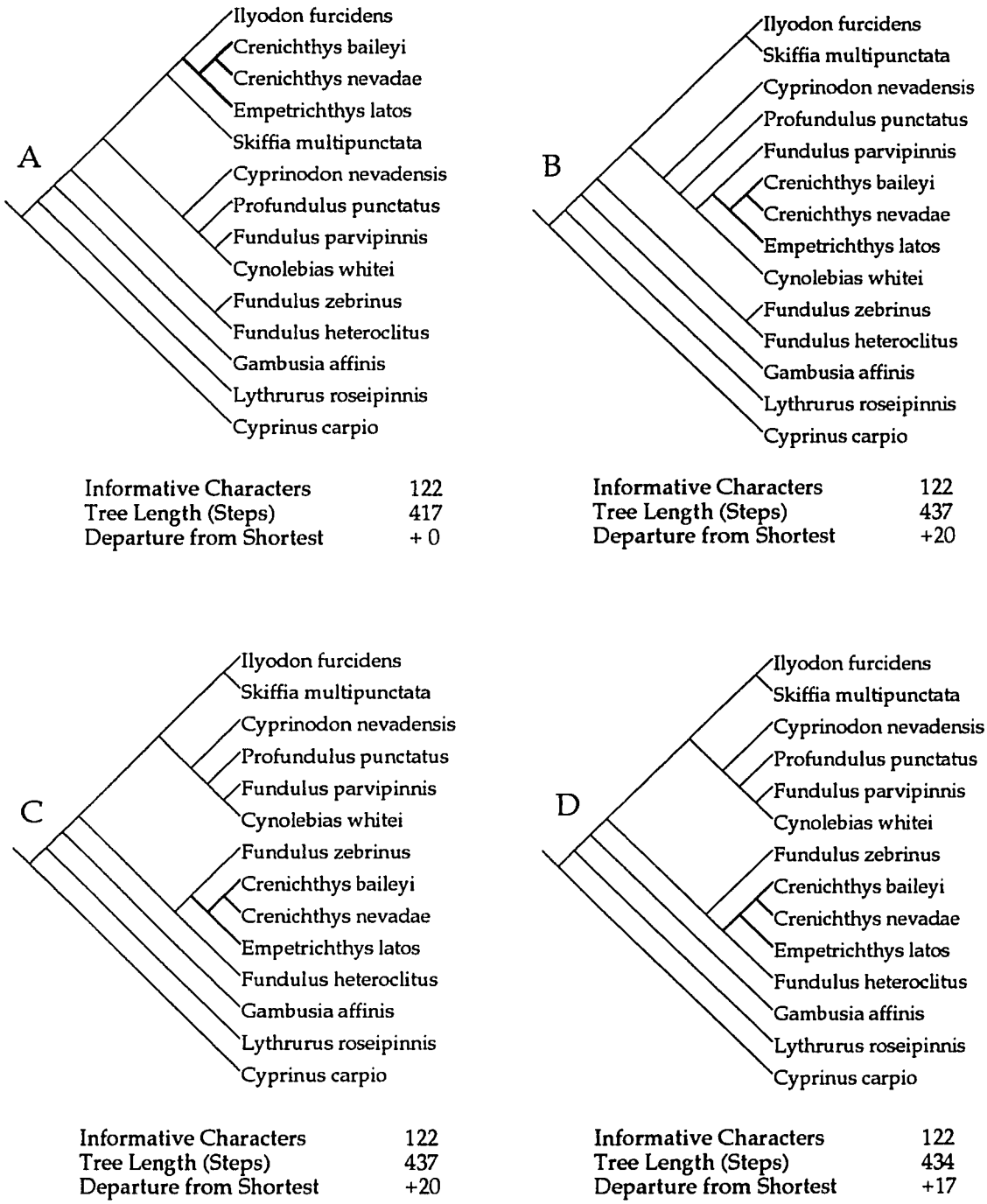


Figure 17 Tree manipulation for all characters unordered for A, most parsimonious, B, (*Crenichthys* and *Empetrichthys*) clade sister with (*Fundulus parvipinnis*), C, (*Fundulus zebrinus*), and D, (*Fundulus heteroclitus*).

A single most parsimonious tree (Fig. 18) is generated under a mixed weighting approach (generalized parsimony for first and second codon positions, and transversion parsimony for the third codon position characters). The charts for this tree (Fig. 19) show that this data set has a higher signal to noise ratio than was demonstrated for the previous analysis. The top chart (Fig. 19) represents all three codon positions, with the transversion substitutions only at the third position; the bottom chart shows only third codon position changes. Both are skewed to the left, giving an indication of a good signal to noise ratio. The tree manipulations (Fig. 20), show 13 more informative characters over the all previous generalize parsimony analysis. This is due to the inability of MacClade to exclude uninformative transversion characters.

In the final analysis, the first and second codon position characters are unordered (generalized parsimony), and third codon position characters are excluded. The chart for the most parsimonious tree (Fig. 21), is highly skewed to the left. This data set is the most conservative. The tree manipulations for the third codon position excluded with first and second codon positions unordered (Fig. 22) reveal that there are only 31 informative characters. Nevertheless, a relationship between *Crenichthys* and *Empetrichthys* with the goodeids is strongly supported by bootstrap analysis (Fig. 23) and tree manipulations (Fig. 22).

The full character generalized parsimony data set has 122 phylogenetically informative characters. Characters are phylogenetically informative when they have at least two non-autapomorphic (above a terminal node) states. The tree length increased by 17 - 20 steps when forcing the *Crenichthys* and *Empetrichthys* clade to form sister relationships with various *Fundulus* species (Fig. 17). The mixed parsimony data set has 135 informative characters. Tree lengths increase by 14 - 17 steps when forcing the *Crenichthys* and *Empetrichthys* clade to form sister relationships with the *Fundulus* species (Fig. 20). The reduced character generalized parsimony data set has only 31 informative characters. Tree lengths increase by 9 - 10 steps when forcing the

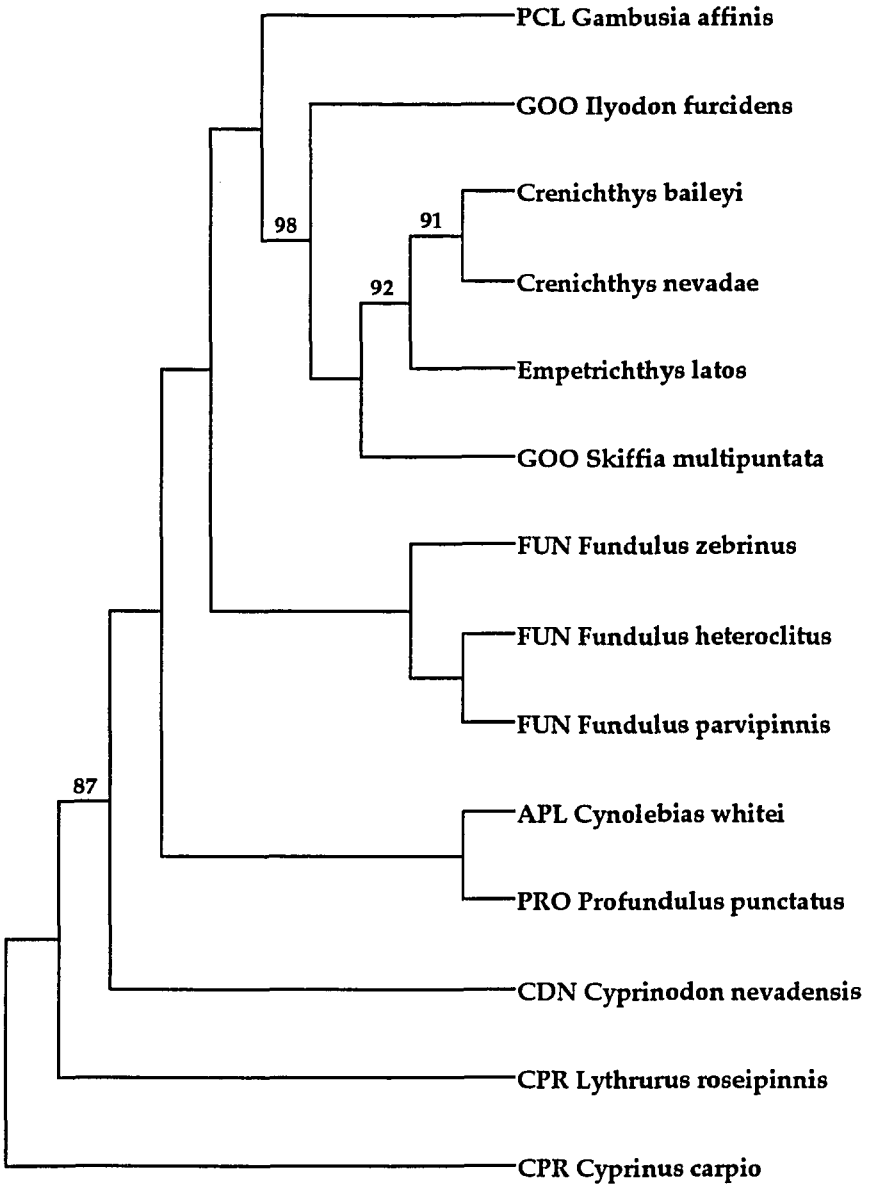


Figure 18. Most parsimonious tree (Branch swapping TBR) for third codon position transversions only, first and second codon positions unordered. TBR Bootstrap (200 reps) values over 50% shown.

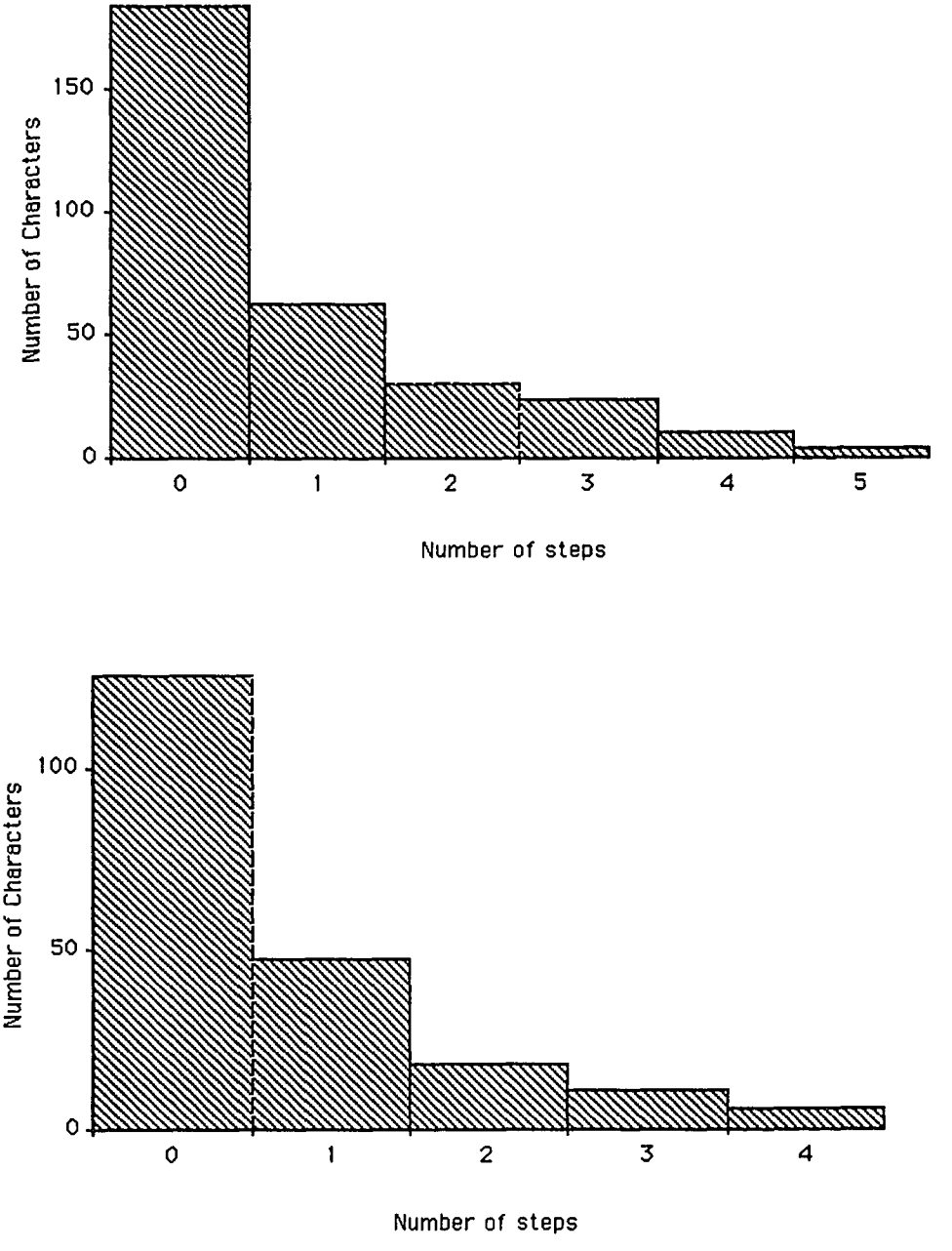


Figure 19. Histograms of the number of character state changes (steps) for first and second codon positions unordered, third codon positon tranversions only, charted over the most parsimonious tree (Fig. 18). The top represents all three codon positions while the bottom represents the third codon position only.

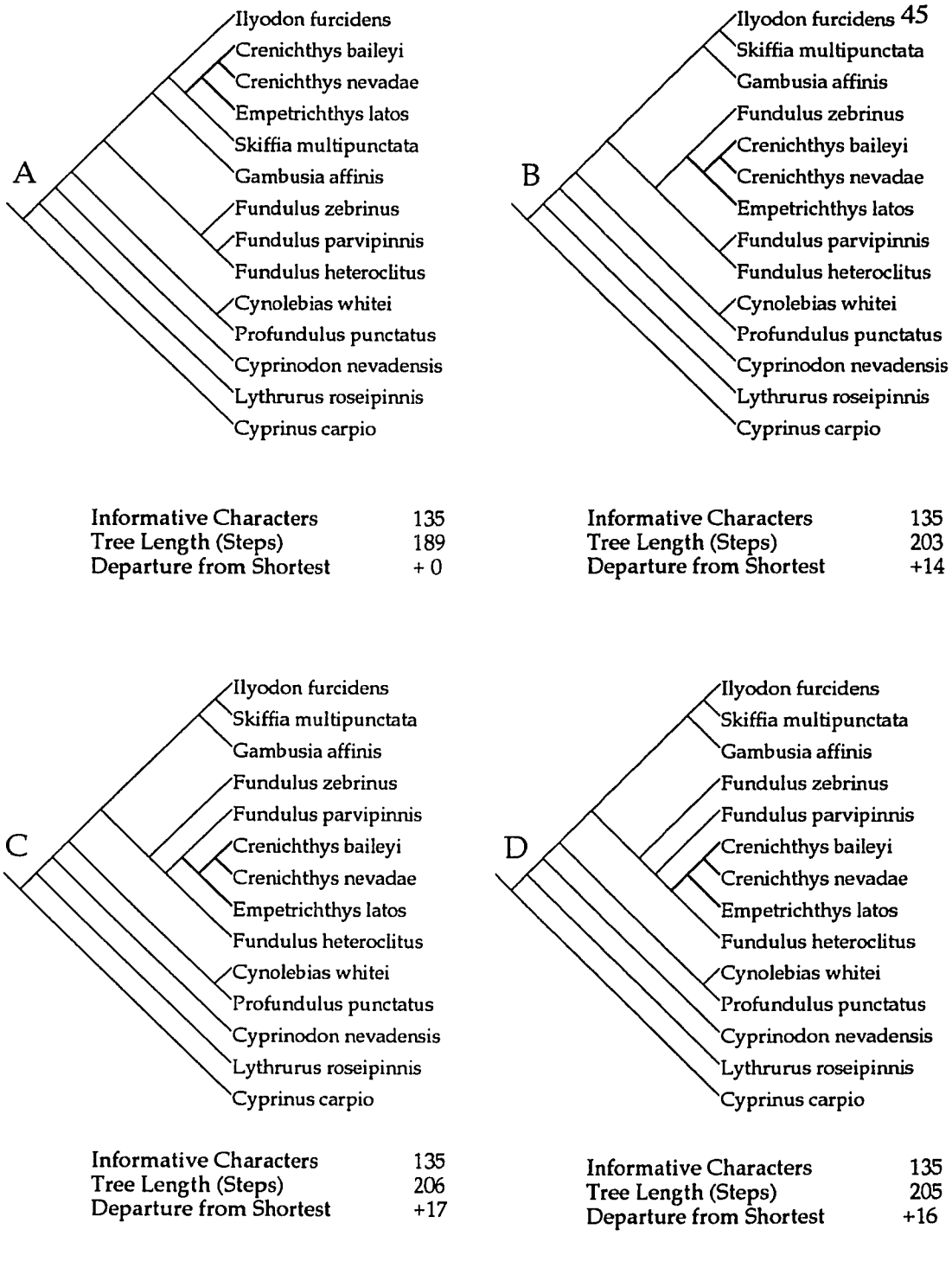


Figure 20. Tree manipulation for third codon transversions only, first and second codon positions unordered for A, most parsimonious, B, (*Crenichthys* and *Empetrichthys*) clade sister with (*Fundulus zebrinus*), C, (*Fundulus parvipinnis*) and D, (*Fundulus heteroclitus*).

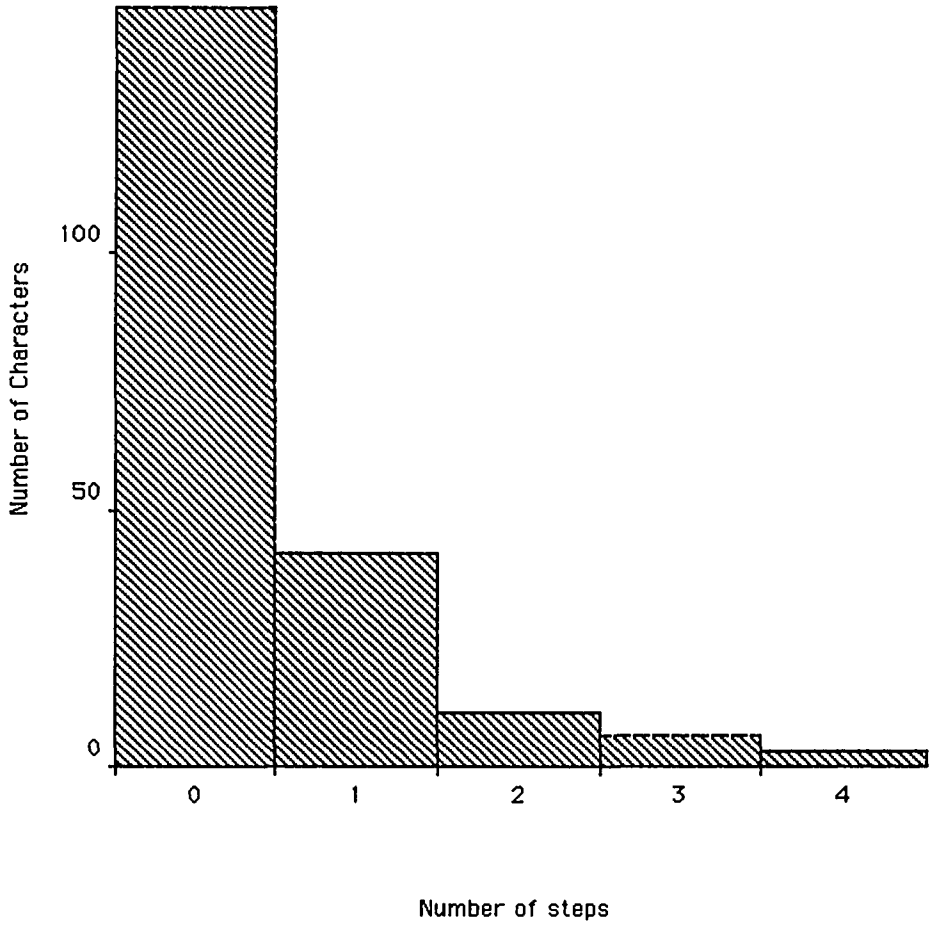
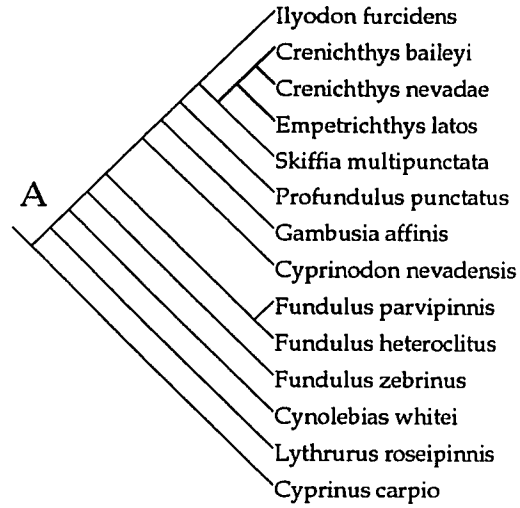
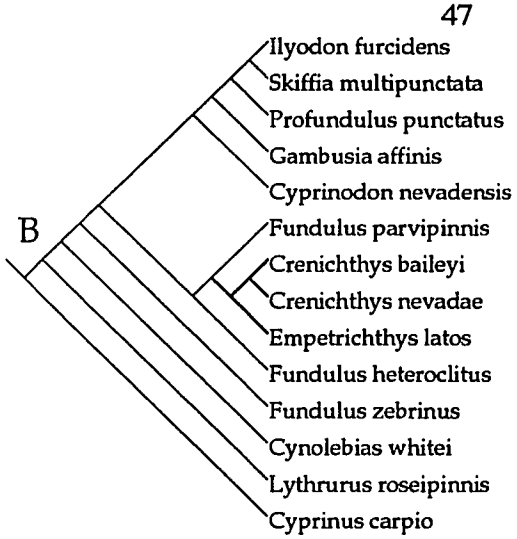


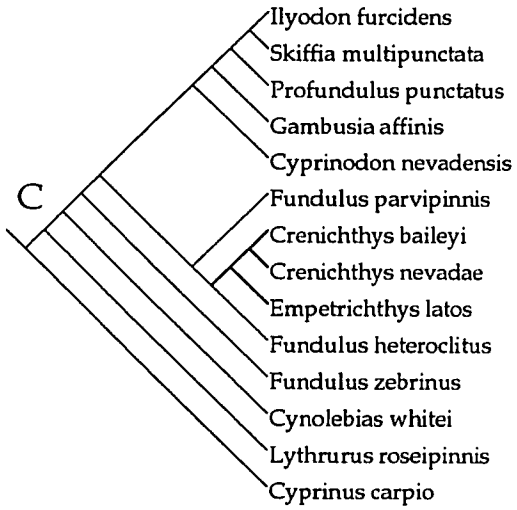
Figure 21. Histogram of character state changes (steps) for first and second codon positions unordered, third codon position excluded, charted over the most parsimonious tree (Fig. 23).



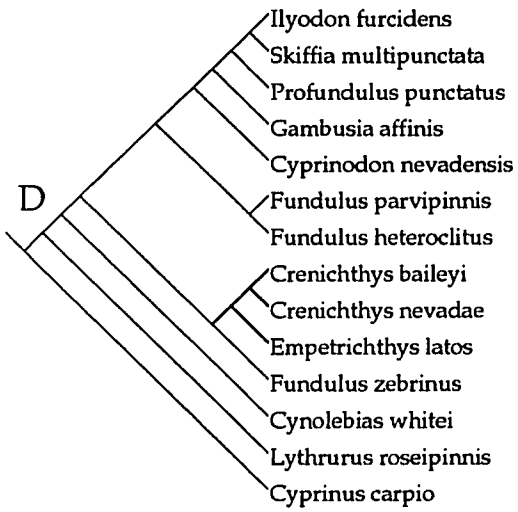
Informative Characters	31
Tree Length (Steps)	61
Departure from Shortest	+ 0



Informative Characters	31
Tree Length (Steps)	71
Departure from Shortest	+10



Informative Characters	31
Tree Length (Steps)	71
Departure from Shortest	+10



Informative Characters	31
Tree Length (Steps)	70
Departure from Shortest	+ 9

Figure 22. Tree manipulation for third codon position excluded, first and second codon position unordered, for A, most parsimonious, B, (*Crenichthys* and *Empetrichthys*) clade sister with (*Fundulus parvipinnis*), C, (*Fundulus heteroclitus*), and D, (*Fundulus zebrinus*).

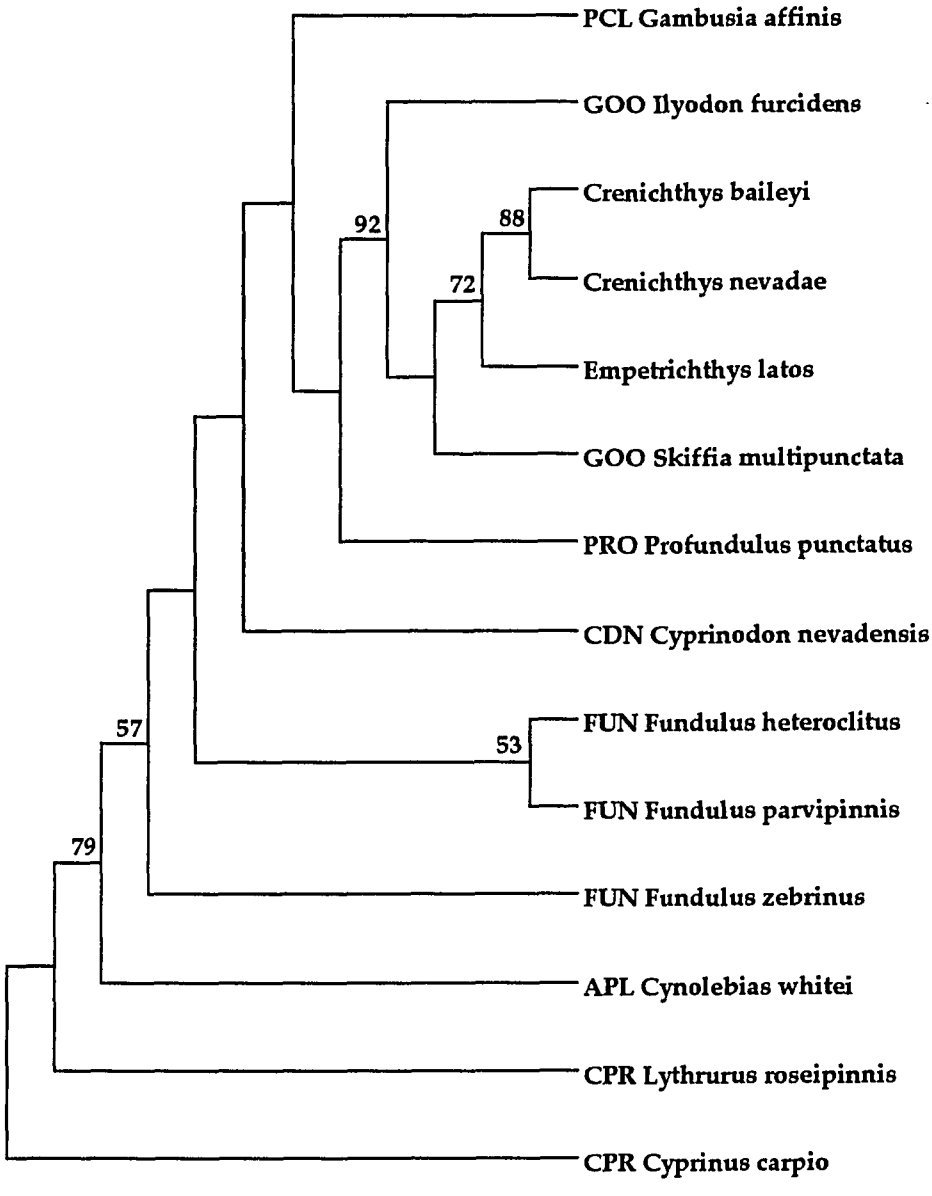


Figure 23. Most parsimonious tree (branch swapping TBR) for third codon position excluded, first and second codon positions unordered. TBR bootstrap (200 reps) values over 50% shown.

Crenichthys and *Empetrichthys* clade to form sister relationships with the *Fundulus* species (Fig. 22). Increased tree lengths in all cases support the hypothesis that *Crenichthys* and *Empetrichthys* are most closely related to the goodeids.

CHAPTER 4

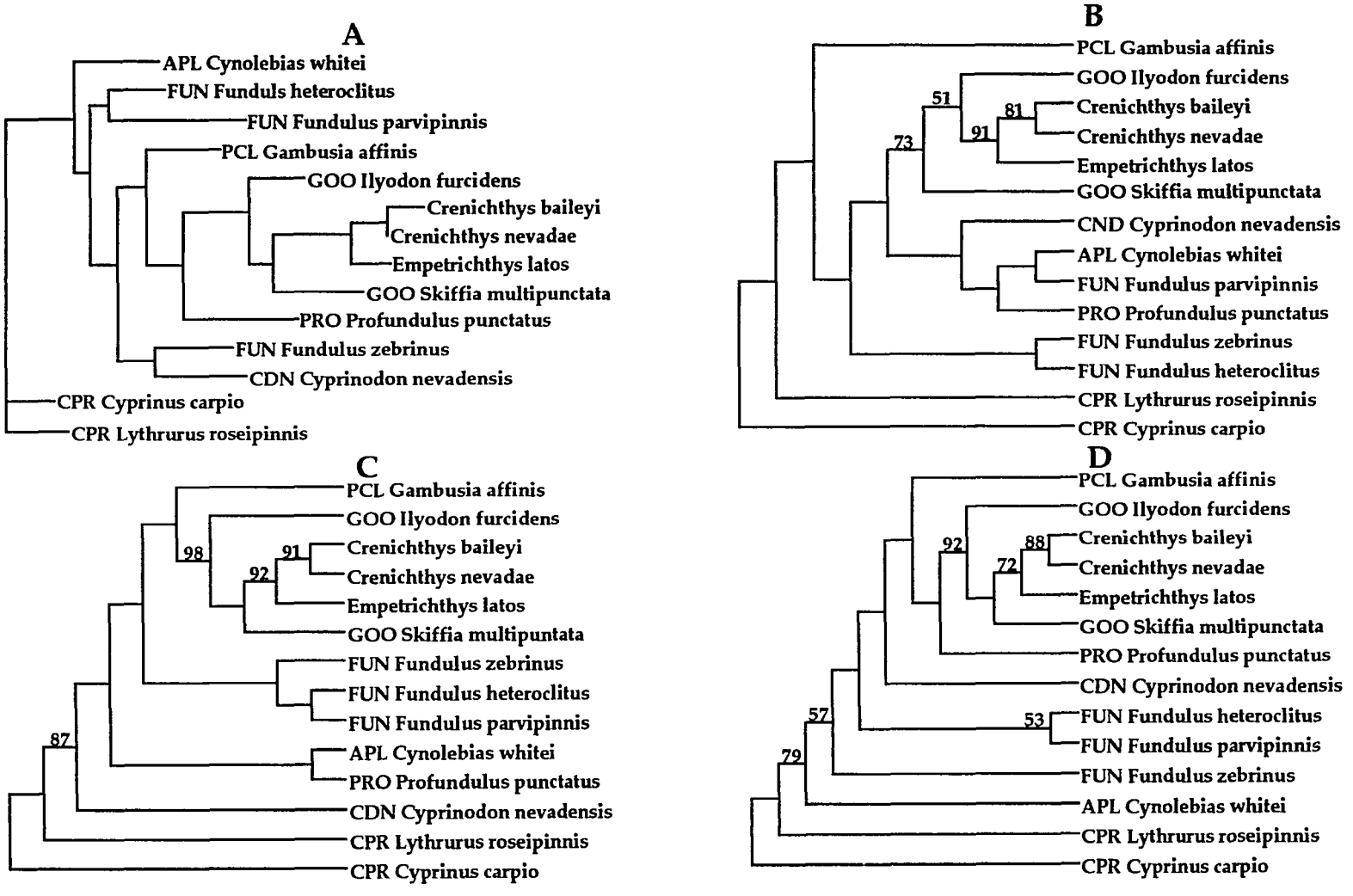
DISCUSSION

TREE TOPOLOGIES

The distance matrix (Table 4) shows relatively low divergence levels among *Crenichthys baileyi, C. nevadae*, and *Empetrichthys latos* (3.3% - 8.5% for all codon positions unordered). The next lowest values are between *Crenichthys baileyi, C. nevadae, Empetrichthys latos* and the goodeids *Ilyodon furcidens* and *Skiffia multipunctata* (13.7% - 22.2%). Neighbor-joining tree (Fig. 23 A) supports a relationship between *Crenichthys* and *Empetrichthys* and the goodeid clade.

All parsimony analyses (Fig 24 B-D) concur that *Crenichthys baileyi, C. nevadae* and *Empetrichthys latos*, are most closely related to the goodeids. The alternative hypothesis that would align *Crenichthys* and *Empetrichthys* with the fundulines is therefore rejected based on this analysis. These data do not unambiguously align *Crenichthys* and *Empetrichthys* with a particular goodeid. *Ilyodon furcidens* is immediately ancestral to *Crenichthys* and *Empetrichthys* in the generalized parsimony analysis using all characters (Fig. 24 B) The other two parsimony analyses and the neighbor-joining analysis suggest that *Skiffia multipunctata* is immediately ancestral to *Crenichthys* and *Empetrichthys*. None of these analyses provide a high level of bootstrap support however.

Figure 24. A compiled figure containing Figures 13, 14, 18, and 23. (A) The neighbor-joining tree from distance matrix (Table 4) using maximum likelihood. The upper diagonal was used, which was calculated with the third codon position excluded. Branch lengths are proportional to genetic divergence. (B - D) The most parsimonious trees with TBR branch swapping. TBR bootstrap (500 reps) values over 50% shown. (B) All codon positions unordered. (C) First and second codon positions unordered, third codon position transversions only. (D) First and second codon positions unordered, third codon position excluded.


A
APL Cynolebias whitei
FUN Funduls heteroclitus
FUN Fundulus parvipinnis
PCL Gambusia affinis
GOO Ilyodon furcidens
Crenichthys baileyi
Crenichthys nevadae
Empetrichthys latos
GOO Skiffia multipunctata
PRO Profundulus punctatus
FUN Fundulus zebrinus
CDN Cyprinodon nevadensis
CPR Cyprinus carpio
CPR Lythrurus roseipinnis
B
PCL Gambusia affinis
GOO Ilyodon furcidens
51
81
Crenichthys baileyi
91
Crenichthys nevadae
73
Empetrichthys latos
GOO Skiffia multipunctata
CND Cyprinodon nevadensis
APL Cynolebias whitei
FUN Fundulus parvipinnis
PRO Profundulus punctatus
FUN Fundulus zebrinus
FUN Fundulus heteroclitus
CPR Lythrurus roseipinnis
CPR Cyprinus carpio
C
PCL Gambusia affinis
GOO Ilyodon furcidens
98
91
Crenichthys baileyi
92
Crenichthys nevadae
Empetrichthys latos
GOO Skiffia multipuntata
FUN Fundulus zebrinus
FUN Fundulus heteroclitus
FUN Fundulus parvipinnis
87
APL Cynolebias whitei
PRO Profundulus punctatus
CDN Cyprinodon nevadensis
CPR Lythrurus roseipinnis
CPR Cyprinus carpio
D
PCL Gambusia affinis
GOO Ilyodon furcidens
92
88
Crenichthys baileyi
72
Crenichthys nevadae
Empetrichthys latos
GOO Skiffia multipunctata
PRO Profundulus punctatus
CDN Cyprinodon nevadensis
57
53
FUN Fundulus heteroclitus
FUN Fundulus parvipinnis
79
FUN Fundulus zebrinus
APL Cynolebias whitei
CPR Lythrurus roseipinnis
CPR Cyprinus carpio

Bootstrap values vary with the level of conservation in the data sets. For generalized parsimony analysis, relatively high bootstrap values are found for the goodeid-*Crenichthys* and *Empetrichthys* clade. This is due to the low level of genetic divergence between these species. For the mixed parsimony analysis, high bootstrap values are found for the *Crenichthys -Empetrichthys* -goodeid clade as well as the outgroups *Lythrurus roseipinnis.* and *Cyprinus carpio.* For the final analysis, bootstrap values drop a little for the least divergent taxa *C. baileyi, C. nevadae* and *E. latos.* This is probably due to a loss of information with the exclusion of the third codon position. The only other significant bootstrap value is that of the outgroup node. The presence of other bootstrap values suggests a gain in information quality at deeper levels of divergence, yet the gains are not significant.

EVOLUTIONARY SIGNIFICANCE

Unfortunately, this data set does not provide the resolution needed to determine whether *Crenichthys* and *Empetrichthys* are derived from a single lineage of goodeids or if the *Crenichthys* and *Empetrichthys* lineage had an ancestral split prior to the evolution of extant goodeids in Mexico (Fig. 7). While the neighbor-joining tree and all heuristic trees show *Crenichthys* and *Empetrichthys* derived from a goodeid, the bootstrap consensus is an un-resolved polytomy between *Ilyodon furcidens, Skiffia multipunctata,* and the *Crenichthys* and *Empetrichthys* clade.

Knowing whether *Crenichthys* and *Empetrichthys* are ancestral to or derived from the extant goodeids would provide a method of evaluating whether oviparity in *Crenichthys* and *Empetrichthys* is a primitively retained or derived trait (Fig. 8). *Crenichthys* and *Empetrichthys* being ancestral to extant goodeids would support the hypothesis that oviparity is the primitivly retained trait in this clade. However,

Crenichthys and *Empetrichthys* being derived from an extant goodeid would support the hypothesis that oviparity is secondarily derived in this lineage.

Mexican Plateau goodeid fishes have a split anal fin which facilitates internal fertilization. Males possess an internal muscular organ for reproductive function. Females possess a single median ovary which is the product of the union of rudimentary lateral organs (Fitzsimons 1972). Goodeid larvae have trophotaeniae (Turner 1937), modified external gut villi which function as a placenta (Lombardi and Wourms 1985). These traits are unique to the goodeids and are not found in *Crenichthys* and *Empetrichthys* .

Environmental models for oviparity and viviparity have been proposed (Wourms et al. 1988). The advantages of viviparity are many (Wourms et al. 1988). Young are protected from predators through gestation. The young are released in the wild at a later stage in larval development and are better suited for changing environments and evading predators. Normally maternal nutrient transfer increases the rate of development, but if resources are limited then development may be retarded. Broods are portable, they can be moved when conditions are deteriorating. This portability makes viviparous fishes excellent colonizers. For these reasons viviparous fishes are selected for changing or "fringe" environments. The main disadvantage of viviparity is that the clutch size is considerably smaller than in oviparous fishes (Wourms et al. 1988). It follows that in stable environments such as large lakes and rivers, oviparous fishes would be selected.

HISTORICAL BIOGEOGRAPHY

The oldest *Empetrichthys* fossil, *E. erdisi*, was found in Miocene strata of southern California (Link 1982). The oldest goodeid fossil, *Tapatia occidentalis*, was found in the Miocene of the Mexican Plateau (Alverez and Arriola 1972). Two historical biogeographical hypotheses have been proposed. Parenti (1981) hypothesized a

continuous ancestral distribution of goodeid fishes from the Mexican plateau northward to the Death Valley system. This continuous distribution was present no later than the Miocene and became disjunct in the late Tertiary. Her hypotheses are based on corresponding floral distributions of dicot forests proposed by Axelrod (1979).

Alternatively, Miller and Smith (1986) and Minkley et al. (1986) attributed the disjunction of *Crenichthys* and *Empetrichthys* and the Mexican Plateau goodeids to tectonics. According to this hypothesis, ancestral *Empetrichthys* occurred on the west side of a fault while the ancestral Mexican Plateau goodeids occurred on the east side. The western plate shifted northward while the eastern plate shifted southward during the Miocene, carrying the organisms and their all fossil evidence with them. Based on paleomagnetic reconstructions, southern California and Baja formations may have moved 2000 km from their original site (Beck and Plumley 1979). Rocks forming San Miguel Island are from Eocene deposits 3800 km south of the island's present location, off the coast of southern California (Champion et al. 1981). Minkley et al. (1986) attribute other species disjunction to these plate movements, including the East - West disjunction of *Fundulus.*

PROPOSED FUTURE RESEARCH

Due to the high levels of divergence for all taxa other than the goodeids, *Crenichthys,* and *Empetrichthys* (Table 4), the deeper phylogenetic nodes are unstable. The lack of bootstrap support (Fig. 24), for taxa other than the outgroups, further shows that this portion of the mitochondrial genome is evolving too quickly to test these relationships.

The node which determines whether *Crenichthys* and *Empetrichthys* are ancestral or derived from the Mexican Plateau goodeids lacks sufficient bootstrap support. In future research all 16 genera of goodeids should be tested with *Crenichthys* and

Empetrichthys. A suitable outgroup, such as the Poeciliid *Gambusia affinis*, should be used to polarize characters. The entire Cytochrome-b gene could yield the needed information; alternatively a slower evolving gene such as ribosomal RNA would be useful. Knowing whether *Crenichthys* and *Empetrichthys* are ancestral to or derived from the Mexican Plateau goodeids would answer some interesting questions and could spawn new research topics.

If *Crenichthys* and *Empetrichthys* are ancestral to the Mexican Plateau goodeids, then their oviparity is probably a primatively retained trait. Future physiological and morphological research of these two species in comparison with the Mexican Plateau goodeids could yield valuable information on the origins of viviparity. Inoperative egg-laying genes could be found using *Crenichthys* and *Empetrichthys* for alignment.

If *Crenichthys* and *Empetrichthys* are derived from the Mexican Plateau goodeids, then their oviparity is probably secondarily derived. The egg envelope in the Mexican Plateau goodeids is very thin and has unique features (Riehl and Greven 1992). *Crenichthys* and *Empetrichthys* egg morphology could be tested for remnants of these features. The egg size is unusually large (1.4 - 2 mm in diameter) in *Crenichthys* and *Empetrichthys* (Baugh et al. 1985). This could be due to a viviparous ancestry. In a case where oviparity is secondarily derived, the egg-laying genetic machinery may have been intact and switched back on. In this scenario the mutation would have arisen in a female. A male could still internally fertilize the female as with the internally fertilizing oviparous *Tomerus gracilus* (Constnatz 1989). This mutation could have been selected in response to environmental stress. Possibly the inoperable live-bearing genes could be found and aligned with the Mexican Plateau goodeids. Future physiological, morphological, and genetic research on *Crenichthys* and *Empetrichthys* could provide insights on the origins of viviparity and oviparity.

Taxonomically, it could be argued that *Crenichthys* and *Empetrichthys* would be placed in the family Goodeidae. While this would have a strong phylogenetic argument,

the Mexican Plateau goodeids have very distinctive reproductive morphology and behavior, which is not shared by *Crenichthys* and *Empetrichthys*. Families may arise when a new species takes a trajectory which radiates into a sufficiently distinctive group. It could be argued that *Crenichthys* and *Empetrichthys* are sufficiently distinct to be placed in their own family Empetrichthyidae (Miller and Smith 1986).

CONCERNS FOR CONSERVATION

Recently one species and two subspecies of *Empetrichthys* became extinct. The only surviving subspecies, *E. latos latos*, which is listed as endangered, survives in artificial refugia (Soltz and Naiman 1978). *Crenichthys baileyi* is listed as endangered while the only other species, *C. nevadae* is listed as threatened. Excessive water usage by ranchers and introduction of exotic fish and frog species are the major contributing factors in the endangerment of *Crenichthys* and *Empetrichthys* (Minkley and Deacon 1968; Williams and Wilde 1981; Williams and Williams 1981). The genus *Empetrichthys* is over five million years old. It may be the oviparous ancestor of a highly derived group of viviparous fishes, or derived from that group reclaiming its distant oviparous strategy. Either way they have persisted in a harsh environment through at least five million years of climatic change. Due to their uniqueness, *Crenichthys* and *Empetrichthys* should be placed near the top of the conservation priority list.

APPENDIX I. Specimen and Tissue Data

Common Name	*Scientific Name*	Taxa	Master #	Cat. #	Country	State	County
plains killifish	*Fundulus zebrinus*	CDT	LVT-00429	MEA-135	USA	NM	Eddy
plains killifish	*Fundulus zebrinus*	CDT	LVT-00430	MEA-136	USA	NM	Eddy
plains killifish	*Fundulus zebrinus*	CDT	LVT-00431	MEA-137	USA	NM	Eddy
Amargosa Pupfish	*Cyprinodon nevadae amargosus*	CDN	LVT-00742	ECG-001	USA	CA	Inyo
Amargosa Pupfish	*Cyprinodon nevadae amargosus*	CDN	LVT-00743	ECG-002	USA	CA	Inyo
Amargosa Pupfish	*Cyprinodon nevadae amargosus*	CDN	LVT-00744	ECG-003	USA	CA	Inyo
Amargosa Pupfish	*Cyprinodon nevadae amargosus*	CDN	LVT-00745	ECG-004	USA	CA	Inyo
Guppy	*Poecilia reticulata*	PCL	LVT-01730	ECG005	MEXICO		
Guppy	*Poecilia reticulata*	PCL	LVT-01731	ECG006	MEXICO		
Guppy	*Poecilia reticulata*	PCL	LVT-01732	ECG007	MEXICO		
Mosquito fish	*Gambusia affinis*	PCL	LVT-01733	ECG008	USA	NV	CLARK
Mosquito fish	*Gambusia affinis*	PCL	LVT-01734	ECG009	USA	NV	CLARK
Mosquito fish	*Gambusia affinis*	PCL	LVT-01735	ECG010	USA	NV	CLARK
Cuban Killifish	*Rivulus cylindraceus*	APL	LVT-01736	ECG011	CUBA		
Cuban Killifish	*Rivulus cylindraceus*	APL	LVT-01737	ECG012	CUBA		
Cuban Killifish	*Rivulus cylindraceus*	APL	LVT-01738	ECG013	CUBA		
White's Pearlfish	*Cynolebias whitei*	APL	LVT-01739	ECG014	BRAZIL		
White's Pearlfish	*Cynolebias whitei*	APL	LVT-01740	ECG015	BRAZIL		
White's Pearlfish	*Cynolebias whitei*	APL	LVT-01741	ECG016	BRAZIL		
Sploched Skiffia	*Skiffia multipunctata*	GOO	LVT-01742	ECG017	MEXICO		
Sploched Skiffia	*Skiffia multipunctata*	GOO	LVT-01743	ECG018	MEXICO		

Sploched Skiffia	*Skiffia multipunctata*	GOO	LVT-01744	ECG019	MEXICO		
Goldbreast Splitfin	*Ilyodon furcidens*	GOO	LVT-01745	ECG020	MEXICO		
Goldbreast Splitfin	*Ilyodon furcidens*	GOO	LVT-01746	ECG021	MEXICO		
Goldbreast Splitfin	*Ilyodon furcidens*	GOO	LVT-01747	ECG022	MEXICO		
Brownspotted Killifish	*Profundulus punctatus*	PRO	LVT-01748	ECG023	MEXICO		
White River Spring Fish	*Crenichthys baileyi grandis*	CDT	LVT-01749	ECG024	USA	NV	NYE
White River Spring Fish	*Crenichthys baileyi grandis*	CDT	LVT-01750	ECG025	USA	NV	NYE
California Killifish	*Fundulus parvipinnis*	CDT	LVT-01751	ECG026	USA	CA	ORANGE
California Killifish	*Fundulus parvipinnis*	CDT	LVT-01752	ECG027	USA	CA	ORANGE
California Killifish	*Fundulus parvipinnis*	CDT	LVT-01753	ECG208	USA	CA	ORANGE
California Killifish	*Fundulus parvipinnis*	CDT	LVT-01754	ECG029	USA	CA	ORANGE
California Killifish	*Fundulus parvipinnis*	CDT	LVT-01755	ECG030	USA	CA	ORANGE
Green Swordtail	*Xiphophorus helleri*	PCL	LVT-01756	ECG031			
Green Swordtail	*Xiphophorus helleri*	PCL	LVT-01757	ECG032			
Green Swordtail	*Xiphophorus helleri*	PCL	LVT-01758	ECG033			
Railroad Valley Springfish	*Crenichthys nevadae*	CDT	LVT-01759	ECG034	USA	NV	NYE
Railroad Valley Springfish	*Crenichthys nevadae*	CDT	LVT-01760	ECG035	USA	NV	NYE
Railroad Valley Springfish	*Crenichthys nevadae*	CDT	LVT-01761	ECG036	USA	NV	NYE
Devel's Hole Pupfish	*Cyprinodon diablos*	CDT	LVT-01762	ECG037	USA	NV	NYE
Pahrump Poolfish	*Empetrichthys latos*	CDT	LVT-01763	ECG038	USA	NV	CLARK
Pahrump Poolfish	*Empetrichthys latos*	CDT	LVT-01764	ECG039	USA	NV	CLARK
Pahrump Poolfish	*Empetrichthys latos*	CDT	LVT-01765	ECG040	USA	NV	CLARK

Master #	Locality	Date	Time
LVT-00429	wash through cattle pasture into Black R. - into Pecos R.	9-Apr-92	700
LVT-00430	wash through cattle pasture into Black R. - into Pecos R.	9-Apr-92	700
LVT-00431	wash through cattle pasture into Black R. - into Pecos R.	9-Apr-92	700
LVT-00742	Amargosa Riv. south of Tecopa at 127 bridge	23-Apr-92	1200
LVT-00743	Amargosa Riv. south of Tecopa at 127 bridge	23-Apr-92	1200
LVT-00744	Amargosa Riv. south of Tecopa at 127 bridge	23-Apr-92	1200
LVT-00745	Amargosa Riv. south of Tecopa at 127 bridge	23-Apr-92	1200
LVT-01730	Jewels of the sea	21-Aug-92	14:00
LVT-01731	Jewels of the sea	21-Aug-92	14:00
LVT-01732	Jewels of the sea	21-Aug-92	14:00
LVT-01733	Flamingo wash at Maryland Pky behind Toys R Us	21-Aug-92	16:00
LVT-01734	Flamingo wash at Maryland Pky behind Toys R Us	21-Aug-92	16:00
LVT-01735	Flamingo wash at Maryland Pky behind Toys R Us	21-Aug-92	16:00
LVT-01736	Purchased from Denis Lomax of Aurora Colorado	1-Sep-92	
LVT-01737	Purchased from Denis Lomax of Aurora Colorado	1-Sep-92	
LVT-01738	Purchased from Denis Lomax of Aurora Colorado	1-Sep-92	
LVT-01739	Purchased from Denis Lomax of Aurora Colorado	1-Sep-92	
LVT-01740	Purchased from Denis Lomax of Aurora Colorado	1-Sep-92	
LVT-01741	Purchased from Denis Lomax of Aurora Colorado	1-Sep-92	
LVT-01742	Donated by Bob Mckean of Greenwich CT	24-Sep-92	
LVT-01743	Donated by Bob Mckean of Greenwich CT	24-Sep-92	

LVT-01744	Donated by Bob Mckean of Greenwich CT	24-Sep-92	
LVT-01745	Donated by Bob Mckean of Greenwich CT	24-Sep-92	
LVT-01746	Donated by Bob Mckean of Greenwich CT	24-Sep-92	
LVT-01747	Donated by Bob Mckean of Greenwich CT	24-Sep-92	
LVT-01748	Donated by Dale Weber of Novato CA (Bay Area Killifish Assn.)	14-Oct-92	
LVT-01749	Kept a the UNLV Animal Facility (Fran Taylor)	14-Oct-92	
LVT-01750	Kept a the UNLV Animal Facility (Fran Taylor)	14-Oct-92	
LVT-01751	Donated by David Soltz CA State Long Beach, Cerritos channel near Alamitos Bay (Joe Sisneros)	22-Oct-92	
LVT-01752	Donated by David Soltz CA State Long Beach, Cerritos channel near Alamitos Bay (Joe Sisneros)	22-Oct-92	
LVT-01753	Donated by David Soltz CA State Long Beach, Cerritos channel near Alamitos Bay (Joe Sisneros)	22-Oct-92	
LVT-01754	Donated by David Soltz CA State Long Beach, Cerritos channel near Alamitos Bay (Joe Sisneros)	22-Oct-92	
LVT-01755	Donated by David Soltz CA State Long Beach, Cerritos channel near Alamitos Bay (Joe Sisneros)	22-Oct-92	
LVT-01756	Jewels of the sea	11-Nov-92	
LVT-01757	Jewels of the sea	11-Nov-92	
LVT-01758	Jewels of the sea	11-Nov-92	
LVT-01759	Big Spring outlet at Lock's Ranch on highway 6	15-Nov-92	1400
LVT-01760	Big Spring outlet at Lock's Ranch on highway 6	15-Nov-92	1400
LVT-01761	Big Spring outlet at Lock's Ranch on highway 6	15-Nov-92	1400
LVT-01762	Devel's Hole, in Ash Meadows South of Pahrump; Found dead, witness: Doug Threeloff	14-Mar-93	1030
LVT-01763	Corn Creek off US-95 Near Mt Charlston, Middle pool	18-Mar-93	1200
LVT-01764	Corn Creek off US-95 Near Mt Charlston, Middle pool	18-Mar-93	1200
LVT-01765	Corn Creek off US-95 Near Mt Charlston, Middle pool	18-Mar-93	1200

Master #	Habitat	Preparation	Tissues	Storage
LVT-00429	mesquite, yucca scrub	Tissues in LN2		
LVT-00430	mesquite, yucca scrub	Tissues in LN2		
LVT-00431	mesquite, yucca scrub	10% formalin wh		
LVT-00742	Turbid, gravel & Silt, Tamarisk	frozen	H, B, M	ULTRA COLD
LVT-00743	Turbid, gravel & Silt, Tamarisk	frozen	H, B, M	ULTRA COLD
LVT-00744	Turbid, gravel & Silt, Tamarisk	frozen	H, B, M	ULTRA COLD
LVT-00745	Turbid, gravel & Silt, Tamarisk	frozen	Whole	ULTRA COLD
LVT-01730	Captive	FROZEN	H,B.M	ULTRA COLD
LVT-01731	Captive	FROZEN	H,B.M	ULTRA COLD
LVT-01732	Captive	PRESERVED	WHOLE	ETOH
LVT-01733	stream	FROZEN	H,B,M	ULTRA COLD
LVT-01734	stream	FROZEN	H,B,M	ULTRA COLD
LVT-01735	stream	PRESERVED	WHOLE	ETOH
LVT-01736	Captive	FROZEN	H,B.M	ULTRA COLD
LVT-01737	Captive	FROZEN	H,B.M	ULTRA COLD
LVT-01738	Captive	PRESERVED	WHOLE	ETOH
LVT-01739	Captive	FROZEN	H,B.M	ULTRA COLD
LVT-01740	Captive	FROZEN	H,B.M	ULTRA COLD
LVT-01741	Captive	PRESERVED	WHOLE	ETOH
LVT-01742	Captive	FROZEN	H,B.M	ULTRA COLD
LVT-01743	Captive	FROZEN	H,B.M	ULTRA COLD

LVT-01744	Captive	PRESERVED	WHOLE	ETOH
LVT-01745	Captive	FROZEN	H,B.M	ULTRA COLD
LVT-01746	Captive	FROZEN	H,B.M	ULTRA COLD
LVT-01747	Captive	PRESERVED	WHOLE	ETOH
LVT-01748	Captive	FROZEN	H,B.M	ULTRA COLD
LVT-01749	Captive	FROZEN	H,B.M	ULTRA COLD
LVT-01750	Captive	FROZEN	H,B.M	ULTRA COLD
LVT-01751	Esturary, Brackish water	FROZEN	H,B.M	ULTRA COLD
LVT-01752	Esturary, Brackish water	FROZEN	H,B.M	ULTRA COLD
LVT-01753	Esturary, Brackish water	FROZEN	H,B.M	ULTRA COLD
LVT-01754	Esturary, Brackish water	PRESERVED	WHOLE	ETOH
LVT-01755	Esturary, Brackish water	PRESERVED	WHOLE	ETOH
LVT-01756	Captive	FROZEN	H,B.M	ULTRA COLD
LVT-01757	Captive	FROZEN	H,B.M	ULTRA COLD
LVT-01758	Captive	PRESERVED	WHOLE	ETOH
LVT-01759	warm clear spring water	FROZEN	H,B.M	ULTRA COLD
LVT-01760	warm clear spring water	FROZEN	H,B.M	ULTRA COLD
LVT-01761	warm clear spring water	PRESERVED	WHOLE	ETOH
LVT-01762	Devel's Hole Hot spring	FROZEN	WHOLE	ULTRA COLD
LVT-01763	Bull frog infested sping pond @19-C	FROZEN	H,B.M	ULTRA COLD
LVT-01764	Bull frog infested sping pond @19-C	FROZEN	H,B.M	ULTRA COLD
LVT-01765	Bull frog infested sping pond @19-C	PRESERVED	WHOLE	ETOH

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