Molecular Phylogeny of the Fundulidae (Teleostei, Cyprinodontiformes) Based on the Cytochrome b Gene

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I. Introduction

The family Fundulidae has a long and complex taxonomic history. After being included in the cyprinodontid subfamily Fundulinae by Myers (1931), the genera Adinia, Fundulus, Lucania, Leptolucania, and Plancterus were elevated to family status (Fundulidae) by Parenti (1981) in her major revision of order Cyprinodontiformes. To these extant genera, a few fossil forms, generally attributed to either Fundulus or Parafundulus, are also added to the family (Eastman, 1917; Miller, 1945; Parenti, 1981). A Central American family, Profundulidae, which includes one genus Profundulus with five species, is generally considered a sister clade to fundulids and other cyprinodontoids (Fig. 1). The fundulid genera themselves have been the subject of extensive taxonomic work, with a special emphasis put on the most speciose genus of the family, Fundulus. Fundulus systematics dates as far back as Linnaeus. The genus was revised several times by researchers including Garman (1895), Jordan and Evermann (1896), Jordan et al. (1930), Hubbs (1931), Miller (1955), Farris (1968), Parenti (1981), and Wiley (1986). Allozymic (Cashner et al., 1992, for a review) and DNA (Bernardi and Powers, 1995) data have also been added to the list of characters used to unravel the phylogenetic relationships among Fundulidae. This chapter gives a general overview of the major phylogenetic issues relevant to the family and presents molecular data that will address some of these issues.

Fundulidae is a relatively large group of cyprinodontiform fishes that live in fresh, brackish, and coastal marine waters. They are distributed over Central and North America, and their tolerance for high salinity probably explains their presence on Cuba and Bermuda (Fig. 1). An introduced population of F. heteroclitus is also found in southern Spain (Bernardi et al., 1995). Two species, F. parvipinnis and F. lima, are isolated on the western part of the North American continent, in California and Baja California (Mexico). Fundulids are oviparous, and their reproduction and egg development have been thoroughly studied (on earth as well as in space!) (Hubbs and Burnside, 1972; Koenig and Livingstone, 1976; Taylor et al. 1977, Hoffman et al., 1977). Other aspects of fundulid biology have also been studied such as hybridization (Hubbs and Drewry, 1959; Setzer, 1970), behavior (Foster, 1967), and karyology (Chen, 1971; Chen and Ruddle, 1970). F. heteroclitus is probably the best-studied fish model for enzyme kinetics and expression. Overall, this group has been
thoroughly studied in almost every possible aspect, however, its phylogenetic relationships, so essential for comparative studies, are still poorly understood.

II. Morphology

Parenti (1981) defines the family using two morphological synapomorphies: "(1) inner arms of the maxillaries directed anteriorly, and often pronounced hooks; and (2) snout pointed and drawn anteriorly with the autopalatine projecting and not articulating with the lateral ethmoid." Wiley (1986) agrees with this definition but questions the validity of the second character. He proposes, however, another morphological character to support the family: "in all fundulids, the epipleural ribs overlap the pleural ribs and are either directly connected to the parapophysis (Adinia, Leptolucania) or to the parapophysis via connective tissue (Fundulus, Lucania, "Planterus")" (Wiley, 1986). Once the boundaries of the family are defined, the major issues concerning this family are the interrelationships of the different genera and the monophyletic status of Fundulus. Few attempts have been made to establish phylogenetic relationships among fundulid genera, the most precise ones being presented by Parenti (1981) and Wiley (1986) (Fig. 2). Wiley (1986) questions several morphological characters used by Parenti (1981) to derive phylogenetic relationships within the family and concludes "that the placement of nominal genera within the family is problematical and a solution must await additional characters."

The phylogenetic relationships among Fundulus species, however, have been studied in great detail. By removing Planterus from Fundulus, Parenti was able to find a single character in support of Fundulus monophyly, a broad articular surface on the second pharyngobranchial. This character is questioned by Wiley (1986), but no alternate character is proposed. In any case, both Parenti and Wiley have doubts about Fundulus monophyly. Indeed, Parenti (1981) says that "a more parsimonious interpretation would place some species of Fundulus as more closely related to Lucania, Leptolucania, or Adinia," and Wiley cannot show Fundulus "to be monophyletic and cannot exclude the possibility that it might be para- or polyphyletic" (Wiley, 1986). Fundulus is the most speciose genus of the family. Although Adinia, Leptolucania, Lucania, and Planterus comprise 5 or 6 species altogether, Fundulus alone includes more than 35 species. Studies on Fundulus relationships were first attempted by Miller.
(1955) who ranked 27 species in a tentative phylogenetic sequence and by Brown (1957) who placed, without explanation, these taxa into five subgenera: Fontinus, Fundulus, Planerus, Xenisma, and Zygonectes. Griffith (1972, 1974) established evolutionary relationships among the different taxa based on 70 characters, and Farris (1968), using morphological characters, placed Fundulus taxa into four monophyletic subgenera: Fundulus, Planerus, Xenisma, and Zygonectes. Lastly, Wiley (1986) provided a phylogenetic analysis of the genus using morphological characters. Wiley recognized the five subgenera described by Brown (1957) but did not find a place for Planerus and the West Coast Fundulus (i.e., F. parvipinnis and F. lima), which were assigned to the “other species” category.

III. Allozymes and DNA

Allozyme data have been used to study Fundulus phylogenetic relationships at the population (Powers and Place, 1978) and species level (Fleming et al., 1962; Duggins et al., 1989). More extensive investigations at the subgeneric and generic level were presented by Cashner and co-workers (Rogers and Cashner, 1987; Cashner et al., 1988; Grady et al., 1990; Cashner et al., 1992). Allozyme work not only provided support for the monophyletic status of subgenera Xenisma and Zygonectes, as well as a clarification of the relationships of taxa within these subgenera, but also provided a framework to better understand the biogeographical implications of Fundulus distributions (Cashner et al., 1992). At the DNA level, nuclear and mitochondrial markers have been used. The nuclear lactate dehydrogenase-B gene has extensively been studied by Powers and co-workers (1993 for a review), mostly in F. heteroclitus populations. Mitochondrial DNA (mtDNA) restriction fragment length polymorphisms (RFLPs) and sequences were also studied for the same populations (Gonzales-Villasenor and Powers, 1990; Bernardi et al., 1993). At a higher taxonomic level, mtDNA gene sequences were determined for the genera Crenichthys and Empetrichthys, which were confirmed as nonfundulids (Grant and Riddle, 1995), for nine species of Fundulus and for Planerus zebrinus (Bernardi and Powers, 1995). West Coast Fundulus were found to be very divergent, but because sequences from only two genera, Fundulus and Planerus, were analyzed, and Planerus was used as an outgroup, the monophyletic status of Fundulus could not be addressed. This chapter presents sequence data from all fundulid genera and one outgroup, Profundulus, and discusses the phylogenetic implications derived from these results.

IV. Fish Samples

Samples were obtained from all the extant fundulid genera. Adinia xenica, Fundulus olivaceus, F. chrysotus, and F. dispar were collected in Louisiana by B. J. Granier, Leptolucania ommata was collected in Alabama by R. Harper, F. notatus and F. cattenatus were collected in Texas by A. Stock and D. W. Stock, F. lima was collected in San Ignacio, Baja California Sur, Mexico, by C. H. Stowell, and F. parvipinnis was collected in Santa Barbara, California, by S. Anderson. DNA sequences from Planerus zebrinus and DNA from Profundulus punctatus were made available by C. Grant. DNA was extracted from liver tissue following Bernardi and Bernardi (1990).

V. DNA Sequences

The polymerase chain reaction (PCR) (Saiki et al., 1988) was used to amplify a 270-bp region of the cytochrome b gene, beginning at the human amino acid 34. Primers and PCR protocols followed Kocher et al. (1989) and Palumbi et al. (1991). Sequencing and PCR primers used were CB2-H, CB1-L, and GLUDG-L (Palumbi et al., 1991). Approximately 100 ng of DNA was used as template for 100-μl PCR reactions containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% (w/v) gelatin, 200 mM each dNTP, 2.5 units of Taq DNA polymerase (Perkin-Elmer Cetus), and 1 μM each amplification primer. PCR products were used for Taq DyeDeoxy Terminator cycle-sequencing reactions (Applied Biosystems Inc.) and loaded on an automated sequencer (Applied Biosystems 373A). Cytochrome b sequences were aligned using the Navigator program (Applied Biosystems Inc.).

Phylogenetic analyses employed maximum parsimony (MP) using the Heuristic option of the PAUP program (phylogenetic analysis using parsimony, Swofford, 1993). The degree of confidence assigned to nodes in trees obtained by MP was determined by bootstrapping (Felsenstein, 1985) with 2000 replicates (Hedges, 1992). The topology-dependent cladistic permutation tail probability analysis (T-PTP) (Faith, 1991) was performed by randomly shuffling the data sets 99 times (after removing the outgroup sequence), using the RANDOMIZER package (Trueman, 1994) and these permuted data sets as input files in PAUP. Actual tree topologies were considered significantly better than random ones when less than 5% of the random sets produced shorter trees than the actual data. The maximum likelihood test of Kishino and Hasegawa (1989)
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* The number of substitutions between taxa are shown below the diagonal. Transition/transversion ratios are shown above the diagonal.
was also used to test significance between different trees. The test was performed using the corresponding option in the PHYLIP package (Felsenstein, 1989).

The 270-bp portion of the cytochrome b was analyzed (contact author for raw data). Of 263 aligned positions, 111 were variable and 92 were phylogenetically informative. The number of transitions was higher than the number of transversions (Table 1), with an average ratio of 3.5 (thus weighting ratios, when used, corresponded to 3 for transversions and 1 for transitions). This result indicates that data are likely to be close to the multiple-hit zone (Brown et al., 1982; Meyer and Wilson, 1990) and corroborates the idea that cytochrome b genes are often not ideal molecular markers for this level of phylogenetic analysis (Meyer, 1994). In the case reported here, cytochrome b data do not allow us to completely resolve the phylogenetic relationships among all taxa that were studied, but do allow us to statistically test some phylogenetic hypotheses.

VI. Phylogenetic Relationships

A single most parsimonious tree was obtained (unweighted tree length = 342 steps, consistency index = 0.49; weighted tree length = 538 steps). When transition/transversion weights were changed or removed, the topology of the tree remained mostly unchanged with the exception of the unstable position of *F. chrysotus*. Although the overall topology of the tree was stable, only a few clades showed high bootstrap support. A consensus tree (50% majority-rule consensus) of 2000 bootstrap replicates is shown in Fig. 3 (only bootstrap values higher than 50% are shown).

Three questions may be addressed from these results: (1) Are the West Coast *Fundulus* a sister clade to all other fundulids? (2) Is *Fundulus* monophyletic? (3) Are phylogenetic relationships among fundulids based on morphological and DNA characters concordant?

![Phylogenetic tree](image)

**FIGURE 3** Phylogenetic tree of the family Fundulidae obtained using 270 bp of the cytochrome b gene. Consensus tree (50% majority rule consensus) resulting from 2000 bootstrap replicates. The numbers at each branch indicate the result of a bootstrap analysis (2000 replicates) using maximum parsimony (heuristic search) when the bootstrap value was greater than 50%. Vertical bars correspond to the subgenera recognized by Farris (1968) whereas italic letters correspond to the subgenera described by Wiley (1986) (F, Fundulus; Z, Zygocetes; X, Xenisma; and O.S., Other species).
A. Are West Coast Fundulus a Sister Clade to All Other Fundulids?

As mentioned earlier, both Parenti (1981) and Wiley (1986) have questioned the monophyletic status of Fundulus. Indeed, only a single character was found by Parenti to supportFundulus monophyly. It is also worth noticing that within the family, the other genera only include one or two species whereas Fundulus comprises more than 35 species (Fig. 2). When using cytochrome b sequence data, 12 steps would have to be added to the most parsimonious tree to obtain a monophyletic Fundulus. In order to determine if these 12 steps are statistically significant, a topology-dependent cladistic permutation tail probability (T-PTP) test was performed (Faith, 1991; Halanych et al., 1995). Data reported here did not support a monophyletic Fundulus (data not shown).

If, as shown in Fig. 3, West Coast Fundulus are the sister clade of all other fundulids, then by definition the genus Fundulus is not monophyletic. Two species of West Coast Fundulus, F. lima and F. parvipinnis, live in an isolated area of the West Coast of the United States and Mexico. Although F. parvipinnis can live in fresh, brackish, or salt water, generally preferring brackish estuaries and sloughs along the coasts of California and Baja California (Mexico), F. lima live in freshwater lagoons in the Baja California desert close to San Ignacio. These species have been isolated from the rest of the group since the beginning of the Pliocene, 5.3 million years ago (Griffith, 1972). F. lima and F. parvipinnis may have migrated to the western part of the continent from the East Coast using a southern route before the closing of the Isthmus of Panama (Griffith, 1972). Although these species have tentatively been assigned to subspecies Xenisma by Farriss (1968), Wiley could not place them in any subspecies and prefers to include them in an undefined “other species” group (Wiley, 1986). Data show that the two West Coast species form a robust clade (supported in 100% of the bootstrap replicates) and that they are the sister group to all other species examined. Four supplementary steps would be necessary to disrupt this sistership. A T-PTP test showed that this result was highly significant (Fig. 4a). The maximum likelihood test of Kishino and Hasegawa (1989) also indicates that the phylogenetic trees have significantly different topologies. Two important implications can be derived from these results: (1) the West Coast Fundulus are shown to be the sister clade of all other fundulids and (2) Fundulus is not monophyletic.

B. Is the Genus Fundulus Monophyletic?

Because data show that West Coast Fundulus are not to be included in the genus, the next question is whether other Fundulus representatives form a monophyletic assemblage. The author analyzed data constraining the genus Fundulus (after removing the west coast species) to be monophyletic. However, these data were unable to provide statistically significant evidence for either hypothesis (Fig. 4b).

C. Are Phylogenetic Relationships among Fundulids Based on Morphological and DNA Characters Concordant?

1. Fundulidae

Fundulid relationships have been proposed by Parenti (1981) and Wiley (1986) (Fig. 2), however; neither
are supported by the author's data. Indeed, the phylogenetic relationships suggested by Parenti and Wiley require, respectively, 16 and 19 more steps than the relationships based on cytochrome b sequences (as shown in Fig. 3). Although the author's data do not support these relationships, no statistically supported alternative emerges from these data (most of the clades have low bootstrap support).

2. Fundulus

_Fundulus_ has been divided into three subgenera by most authors, _Fundulus, Xenisma_, and _Zygonectes_; two other subgenera have also been proposed, _Fontinus_ and _Plancterus_. Figure 3 compares the author's molecular results with previous subgeneric assignments based on morphological characters (Farris, 1968; Wiley, 1986). Representatives of subgenera _Fundulus, Xenisma_, and _Zygonectes_ were included in the analysis. The subgenus _Fundulus_, which is the least controversial of the groupings, is consistent for the three studies presented in Fig. 3. Molecular data support this group with high bootstrap values (94% of bootstrap replicates); however, data for more taxa are needed to confirm these results. Within _Zygonectes_, the striped species _F. notatus_ and _F. olivaceus_ are found to be sister taxa (95% bootstrap). This result is not surprising and is generally accepted (Farris, 1968; Wiley, 1986; Casheir et al., 1992). Another _Zygonectes_ representative, _F. chrysotus_, does not cluster with the remaining _Zygonectes_. However, as mentioned earlier, the branch leading to _F. chrysotus_ is unstable and data are not incompatible with a monophyletic _Zygonectes_. _F. catenatus_, a _Xenisma_ representative, is found to be the sister clade of subgenus _Fundulus_. This result is in disagreement with Farris (1968), who considers _Fundulus_ to be closely related to _Zygonectes_. The author's results are also in disagreement with the placement of the West Coast species in the subgenus _Xenisma_ (Farris, 1968). As mentioned earlier, _F. parvipes_ and _F. lima_ are found to be the sister clade of the rest of the _fundulids_.

_VII. Conclusion_

Fundulids have been the subject of several conflicting phylogenetic analyses making them a system of choice for molecular studies. Hypotheses based on morphology, behavior, and allozymic studies can be compared with molecular data, and the differences can be statistically tested. Our results are mostly in agreement with subgeneric assignments of different _Fundulus_ species. The subgenera _Fundulus_ and _Zygonectes_ are concordant between the different studies; only _Xenisma_ exhibits important differences among the analyses. At the other end of the hierarchical scale, the generic positions within the family are different between the two morphological studies and sequence data presented here. More taxa and more characters will be needed to clearly define relationships at the intrafamilial level. The West Coast _Fundulus_ species, previously assigned to the "other species" group by Wiley (1986), seem to form a monophyletic sister clade to all other _fundulids_ (or at least all other _fundulids_ studied here). This finding could be the result of long time and geographical isolation of _F. lima_ and _F. parvipes_ from the rest of the group, which would produce long branches that might artificially group the two clades. However, for both species the branch length is less than the average branch length of other taxa making this possibility unlikely. If West Coast _Fundulus_ are a sister clade to all other _fundulids_, as data suggest, some taxonomic revisions concerning these two species may have to be considered. Furthermore, it has been shown that _F. lima_ and _F. parvipes_ occur a basal position, making them good indicators for the time of divergence of the family. The family _Fundulidae_ would have diverged before the divergence of the West Coast _fundulids_ from the rest of the family, approximately 5 million years ago.

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