

The phylogenetic position of the zebrafish (*Danio rerio*), a model system in developmental biology: an invitation to the comparative method

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SUMMARY

The zebrafish, *Danio* (*Brachydanio*) *rerio*, has become one of the most widely studied model systems in developmental biology. We present a DNA-based phylogeny of zebrafish and other species of the genus *Danio*, and the genera *Rasbora*, *Puntius* and *Cyprinus*. Homologous regions of the large (16S) mitochondrial ribosomal RNA gene were amplified by the polymerase chain reaction and directly sequenced. The phylogeny revealed: (i) the zebrafish, *Danio* (*Brachydanio*) *rerio*, is identical in its 16S sequence to its aquarium breeding morph, the leopard danio; (ii) the pearl danio (*Danio albolineatus*) is more closely related to the zebrafish than the giant danio (*Danio aequipinnatus*); and (iii) species of the genus *Rasbora* (*heteromorpha*, *trilineata*, *elegans*, *pauciperforata*, *dorsiocellata*) are more closely related to the danios than members of the genus *Puntius* (*tetrazona*, *conchonius*) and *Cyprinus*, the carp. All of these species are readily available in the aquarium trade, easily kept and bred in captivity, and amenable to developmental work. It is hoped that this molecular phylogeny will invite developmental biologists to use the comparative method to ask questions about function (e.g. cellular and genetic aspects) and evolution of zebrafish developmental biology in a phylogenetic context.

1. INTRODUCTION

The comparative method has enjoyed a long successful history in classical biology and also in developmental biology at the cellular and molecular level (Baldwin 1902; Waddington 1957; Gould 1977; Goodwin *et al.* 1983; Raff & Kaufman 1983; Arthur 1984; for a recent review see Harvey & Pagel 1991). It was Darwin's favourite method of research and remains the most widely used technique for answering questions about change and evolution in biology and the relation of development and evolution (Ghiselin 1984; and see references in Harvey & Pagel 1991). A recent resurgence of interest in the comparative method involved finding techniques that are based on explicit evolutionary and statistical models (Brooks & McLennan 1991; Harvey & Pagel 1991). The aim of this approach is to study evolution of the phenotypic and genetic traits of organisms based on knowledge of the phylogenetic relations among the species compared. In developmental biology, comparisons tend to be made across large evolutionary distances, often between phyla, and focusing on leech, sea urchin, fruit fly, frog, chicken, mouse and rat. The new interest in the development of zebrafish adds this species to the list of vertebrate model systems.

Fine-grained (on a phylogenetic level) comparisons of developmental patterns among closely related species (e.g. mouse and rat) can provide insights that are not easily gained from comparisons among distantly related species. For example, the unexpectedly large differences in larval morphology of sea urchins

suggests that a few molecular changes can produce strikingly different phenotypes (Strathmann *et al.* 1992; Raff 1992). Closely related species have genes in common because they share recent common ancestors; some of these genes might be absent or much modified among distant relatives. Homology at the phenotypic

Table 1. *Systematic position and current scientific and common names of species in this study*

Order Cypriniformes
Family Cyprinidae
Subfamily Rasborinae
<i>Danio rerio</i> (= <i>Brachydanio rerio</i> : zebrafish)
<i>Danio rerio</i> 'frankei' (= <i>Brachydanio rerio</i> : leopard danio)
<i>Danio albolineatus</i> (= <i>Brachydanio albolineatus</i> : pearl danio)
<i>Danio aequipinnatus</i> (= <i>malabaricus</i> : giant danio)
<i>Rasbora heteromorpha</i> (the rasbora)
<i>Rasbora dorsiocellata</i> (eyespot rasbora)
<i>Rasbora pauciperforata</i> (red-striped rasbora)
<i>Rasbora elegans</i> (elegant rasbora)
<i>Rasbora trilineata</i> (scissor-tailed rasbora)
Subfamily Cyprininae
<i>Puntius tetrazona</i> (tiger barb)
<i>Puntius conchonius</i> (rosy barb)
<i>Cyprinus carpio</i> (carp)
Order Characiformes
Family Prochilodidae
<i>Prochilodus</i> sp.
Family Ctenoluciidae
<i>Boulengerella</i> sp.



D. rerio	GCCTGCCAGTACAATA-GTTTAAACGGCCGGGATTTTGACCGTGCAAAAGTAGGCGCAATCACTCGTCTTTAAATAGGACCTGTATGAATGGCCAAACGAGGGCTTAACTGTCTCC	[120]
D. rerio "f"	GCCTGCCAGTACAATA-GTTTAAACGGCCGGGATTTTGACCGTGCAAAAGTAGGCGCAATCACTCGTCTTTAAATAGGACCTGTATGAATGGCCAAACGAGGGCTTAACTGTCTCC	
D. albolineatus	GCCTGCCAGTACAATA-GTTTAAACGGCCGGGATTTTGACCGTGCAAAAGTAGGCGCAATCACTCGTCTTTAAATAGGACCTGTATGAATGGCCAAACGAGGGCTTAACTGTCTCC	
D. aequipinnatus	GCCTGCCAGTACAATA-GTTTAAACGGCCGGGATTTTGACCGTGCAAAAGTAGGCGCAATCACTCGTCTTTAAATAGGACCTGTATGAATGGCCAAACGAGGGCTTAACTGTCTCC	
R. heteromorpha	GCCTGCCAGTACAATA-GTTTAAACGGCCGGGATTTTGACCGTGCAAAAGTAGGCGCAATCACTCGTCTTTAAATAGGACCTGTATGAATGGCCAAACGAGGGCTTAACTGTCTCC	
R. dorsiozellata	GCCTGCCAGTACAATA-GTTTAAACGGCCGGGATTTTGACCGTGCAAAAGTAGGCGCAATCACTCGTCTTTAAATAGGACCTGTATGAATGGCCAAACGAGGGCTTAACTGTCTCC	
R. pauciperforata	GCCTGCCAGTACAATA-GTTTAAACGGCCGGGATTTTGACCGTGCAAAAGTAGGCGCAATCACTCGTCTTTAAATAGGACCTGTATGAATGGCCAAACGAGGGCTTAACTGTCTCC	
R. elegans	GCCTGCCAGTACAATA-GTTTAAACGGCCGGGATTTTGACCGTGCAAAAGTAGGCGCAATCACTCGTCTTTAAATAGGACCTGTATGAATGGCCAAACGAGGGCTTAACTGTCTCC	
R. trilineata	GCCTGCCAGTACAATA-GTTTAAACGGCCGGGATTTTGACCGTGCAAAAGTAGGCGCAATCACTCGTCTTTAAATAGGACCTGTATGAATGGCCAAACGAGGGCTTAACTGTCTCC	
P. tetrazona	GCCTGCCAGTACAATA-GTTTAAACGGCCGGGATTTTGACCGTGCAAAAGTAGGCGCAATCACTCGTCTTTAAATAGGACCTGTATGAATGGCCAAACGAGGGCTTAACTGTCTCC	
P. conchonus	GCCTGCCAGTACAATA-GTTTAAACGGCCGGGATTTTGACCGTGCAAAAGTAGGCGCAATCACTCGTCTTTAAATAGGACCTGTATGAATGGCCAAACGAGGGCTTAACTGTCTCC	
P. carpio	GCCTGCCAGTACAATA-GTTTAAACGGCCGGGATTTTGACCGTGCAAAAGTAGGCGCAATCACTCGTCTTTAAATAGGACCTGTATGAATGGCCAAACGAGGGCTTAACTGTCTCC	
Boulengerella	GCCTGCCAGTACAATA-GTTTAAACGGCCGGGATTTTGACCGTGCAAAAGTAGGCGCAATCACTCGTCTTTAAATAGGACCTGTATGAATGGCCAAACGAGGGCTTAACTGTCTCC	
Prochilodus	GCCTGCCAGTACAATA-GTTTAAACGGCCGGGATTTTGACCGTGCAAAAGTAGGCGCAATCACTCGTCTTTAAATAGGACCTGTATGAATGGCCAAACGAGGGCTTAACTGTCTCC	
D. rerio	CCCATCAAGTCAGTGAATTTGATCTATCCGTGCAGAAAGCGGATATAATAACAAGCAGAGAAGCCCTTTGGAGCTTAAAGTACAAGTTTAAATTTACATCAAGCAACC----	[240]
D. rerio "f"	CCCATCAAGTCAGTGAATTTGATCTATCCGTGCAGAAAGCGGATATAATAACAAGCAGAGAAGCCCTTTGGAGCTTAAAGTACAAGTTTAAATTTACATCAAGCAACC----	
D. albolineatus	CCCATCAAGTCAGTGAATTTGATCTATCCGTGCAGAAAGCGGATATAATAACAAGCAGAGAAGCCCTTTGGAGCTTAAAGTACAAGTTTAAATTTACATCAAGCAACC----	
D. aequipinnatus	CCCATCAAGTCAGTGAATTTGATCTATCCGTGCAGAAAGCGGATATAATAACAAGCAGAGAAGCCCTTTGGAGCTTAAAGTACAAGTTTAAATTTACATCAAGCAACC----	
R. heteromorpha	CCCATCAAGTCAGTGAATTTGATCTATCCGTGCAGAAAGCGGATATAATAACAAGCAGAGAAGCCCTTTGGAGCTTAAAGTACAAGTTTAAATTTACATCAAGCAACC----	
R. dorsiozellata	CCCATCAAGTCAGTGAATTTGATCTATCCGTGCAGAAAGCGGATATAATAACAAGCAGAGAAGCCCTTTGGAGCTTAAAGTACAAGTTTAAATTTACATCAAGCAACC----	
R. pauciperforata	CCCATCAAGTCAGTGAATTTGATCTATCCGTGCAGAAAGCGGATATAATAACAAGCAGAGAAGCCCTTTGGAGCTTAAAGTACAAGTTTAAATTTACATCAAGCAACC----	
R. elegans	CCCATCAAGTCAGTGAATTTGATCTATCCGTGCAGAAAGCGGATATAATAACAAGCAGAGAAGCCCTTTGGAGCTTAAAGTACAAGTTTAAATTTACATCAAGCAACC----	
R. trilineata	CCCATCAAGTCAGTGAATTTGATCTATCCGTGCAGAAAGCGGATATAATAACAAGCAGAGAAGCCCTTTGGAGCTTAAAGTACAAGTTTAAATTTACATCAAGCAACC----	
P. tetrazona	CCCATCAAGTCAGTGAATTTGATCTATCCGTGCAGAAAGCGGATATAATAACAAGCAGAGAAGCCCTTTGGAGCTTAAAGTACAAGTTTAAATTTACATCAAGCAACC----	
P. conchonus	CCCATCAAGTCAGTGAATTTGATCTATCCGTGCAGAAAGCGGATATAATAACAAGCAGAGAAGCCCTTTGGAGCTTAAAGTACAAGTTTAAATTTACATCAAGCAACC----	
P. carpio	CCCATCAAGTCAGTGAATTTGATCTATCCGTGCAGAAAGCGGATATAATAACAAGCAGAGAAGCCCTTTGGAGCTTAAAGTACAAGTTTAAATTTACATCAAGCAACC----	
Boulengerella	CCCATCAAGTCAGTGAATTTGATCTATCCGTGCAGAAAGCGGATATAATAACAAGCAGAGAAGCCCTTTGGAGCTTAAAGTACAAGTTTAAATTTACATCAAGCAACC----	
Prochilodus	CCCATCAAGTCAGTGAATTTGATCTATCCGTGCAGAAAGCGGATATAATAACAAGCAGAGAAGCCCTTTGGAGCTTAAAGTACAAGTTTAAATTTACATCAAGCAACC----	
D. rerio	AAG-----TGATTAAGCTAGTAAAATGATAACTACCTTCCGTTGGGGCCACCGAGTAAAAAAACAACCTCCAAGCGGAAACGGG--CACAGCC--CTAAACCAAGA-GAA	[360]
D. rerio "f"	AAG-----TGATTAAGCTAGTAAAATGATAACTACCTTCCGTTGGGGCCACCGAGTAAAAAAACAACCTCCAAGCGGAAACGGG--CACAGCC--CTAAACCAAGA-GAA	
D. albolineatus	AAG-----TGATTAAGCTAGTAAAATGATAACTACCTTCCGTTGGGGCCACCGAGTAAAAAAACAACCTCCAAGCGGAAACGGG--CACAGCC--CTAAACCAAGA-GAA	
D. aequipinnatus	AGG-----CACAACCTAGTAAAATGATAACTACCTTCCGTTGGGGCCACCGAGTAAAAAAACAACCTCCAAGCGGAAACGGG--CACAGCC--CTAAACCAAGA-GAA	
R. heteromorpha	AGT-----TCTTAAACCTAGTAAAATGATAACTACCTTCCGTTGGGGCCACCGAGTAAAAAAACAACCTCCAAGCGGAAACGGG--CACAGCC--CTAAACCAAGA-GAA	
R. dorsiozellata	AGT-----TCTTAAACCTAGTAAAATGATAACTACCTTCCGTTGGGGCCACCGAGTAAAAAAACAACCTCCAAGCGGAAACGGG--CACAGCC--CTAAACCAAGA-GAA	
R. pauciperforata	AAT-----TCTTAAACCTAGTAAAATGATAACTACCTTCCGTTGGGGCCACCGAGTAAAAAAACAACCTCCAAGCGGAAACGGG--CACAGCC--CTAAACCAAGA-GAA	
R. elegans	AGT-----CCTTAAACCTAGTAAAATGATAACTACCTTCCGTTGGGGCCACCGAGTAAAAAAACAACCTCCAAGCGGAAACGGG--CACAGCC--CTAAACCAAGA-GAA	
R. trilineata	AGT-----CCTTAAACCTAGTAAAATGATAACTACCTTCCGTTGGGGCCACCGAGTAAAAAAACAACCTCCAAGCGGAAACGGG--CACAGCC--CTAAACCAAGA-GAA	
P. tetrazona	AGCAACTCCTTTTAACTAGTAAAATGATAACTACCTTCCGTTGGGGCCACCGAGTAAAAAAACAACCTCCAAGCGGAAACGGG--CACAGCC--CTAAACCAAGA-GAA	
P. conchonus	AGCAACTCCTTTTAACTAGTAAAATGATAACTACCTTCCGTTGGGGCCACCGAGTAAAAAAACAACCTCCAAGCGGAAACGGG--CACAGCC--CTAAACCAAGA-GAA	
P. carpio	AGCAACTCCTTTTAACTAGTAAAATGATAACTACCTTCCGTTGGGGCCACCGAGTAAAAAAACAACCTCCAAGCGGAAACGGG--CACAGCC--CTAAACCAAGA-GAA	
Boulengerella	AGCAACTCCTTTTAACTAGTAAAATGATAACTACCTTCCGTTGGGGCCACCGAGTAAAAAAACAACCTCCAAGCGGAAACGGG--CACAGCC--CTAAACCAAGA-GAA	
Prochilodus	AGCAACTCCTTTTAACTAGTAAAATGATAACTACCTTCCGTTGGGGCCACCGAGTAAAAAAACAACCTCCAAGCGGAAACGGG--CACAGCC--CTAAACCAAGA-GAA	
D. rerio	ACATCTCTAAGCCTCAGAACACCTGACCAAGACGATCCGGC---CTATAAGCCGATCAACGAAACCAAGTTACCTTAGGATTAACAGCGCAATCCTCTTCAAGTCCATATCGACGAG	[480]
D. rerio "f"	ACATCTCTAAGCCTCAGAACACCTGACCAAGACGATCCGGC---CTATAAGCCGATCAACGAAACCAAGTTACCTTAGGATTAACAGCGCAATCCTCTTCAAGTCCATATCGACGAG	
D. albolineatus	ACATCTCTAAGCCTCAGAACACCTGACCAAGACGATCCGGC---CTATAAGCCGATCAACGAAACCAAGTTACCTTAGGATTAACAGCGCAATCCTCTTCAAGTCCATATCGACGAG	
D. aequipinnatus	ACATCTCTAAGCCTCAGAACACCTGACCAAGACGATCCGGC---CTATAAGCCGATCAACGAAACCAAGTTACCTTAGGATTAACAGCGCAATCCTCTTCAAGTCCATATCGACGAG	
R. heteromorpha	ACATCTCTAAGCCTCAGAACACCTGACCAAGACGATCCGGC---CTATAAGCCGATCAACGAAACCAAGTTACCTTAGGATTAACAGCGCAATCCTCTTCAAGTCCATATCGACGAG	
R. dorsiozellata	ACATCTCTAAGCCTCAGAACACCTGACCAAGACGATCCGGC---CTATAAGCCGATCAACGAAACCAAGTTACCTTAGGATTAACAGCGCAATCCTCTTCAAGTCCATATCGACGAG	
R. pauciperforata	ACATCTCTAAGCCTCAGAACACCTGACCAAGACGATCCGGC---CTATAAGCCGATCAACGAAACCAAGTTACCTTAGGATTAACAGCGCAATCCTCTTCAAGTCCATATCGACGAG	
R. elegans	ACATCTCTAAGCCTCAGAACACCTGACCAAGACGATCCGGC---CTATAAGCCGATCAACGAAACCAAGTTACCTTAGGATTAACAGCGCAATCCTCTTCAAGTCCATATCGACGAG	
R. trilineata	ACATCTCTAAGCCTCAGAACACCTGACCAAGACGATCCGGC---CTATAAGCCGATCAACGAAACCAAGTTACCTTAGGATTAACAGCGCAATCCTCTTCAAGTCCATATCGACGAG	
P. tetrazona	ACATCTCTAAGCCTCAGAACACCTGACCAAGACGATCCGGC---CTATAAGCCGATCAACGAAACCAAGTTACCTTAGGATTAACAGCGCAATCCTCTTCAAGTCCATATCGACGAG	
P. conchonus	ACATCTCTAAGCCTCAGAACACCTGACCAAGACGATCCGGC---CTATAAGCCGATCAACGAAACCAAGTTACCTTAGGATTAACAGCGCAATCCTCTTCAAGTCCATATCGACGAG	
P. carpio	ACATCTCTAAGCCTCAGAACACCTGACCAAGACGATCCGGC---CTATAAGCCGATCAACGAAACCAAGTTACCTTAGGATTAACAGCGCAATCCTCTTCAAGTCCATATCGACGAG	
Boulengerella	ACATCTCTAAGCCTCAGAACACCTGACCAAGACGATCCGGC---CTATAAGCCGATCAACGAAACCAAGTTACCTTAGGATTAACAGCGCAATCCTCTTCAAGTCCATATCGACGAG	
Prochilodus	ACATCTCTAAGCCTCAGAACACCTGACCAAGACGATCCGGC---CTATAAGCCGATCAACGAAACCAAGTTACCTTAGGATTAACAGCGCAATCCTCTTCAAGTCCATATCGACGAG	
D. rerio	GGGTTTACGACCTCGATGTTGGATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGG	[538]
D. rerio "f"	GGGTTTACGACCTCGATGTTGGATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGG	
D. albolineatus	GGGTTTACGACCTCGATGTTGGATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGG	
D. aequipinnatus	GGGTTTACGACCTCGATGTTGGATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGG	
R. heteromorpha	GGGTTTACGACCTCGATGTTGGATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGG	
R. dorsiozellata	GGGTTTACGACCTCGATGTTGGATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGG	
R. pauciperforata	GGGTTTACGACCTCGATGTTGGATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGG	
R. elegans	GGGTTTACGACCTCGATGTTGGATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGG	
R. trilineata	GGGTTTACGACCTCGATGTTGGATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGG	
P. tetrazona	GGGTTTACGACCTCGATGTTGGATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGG	
P. conchonus	GGGTTTACGACCTCGATGTTGGATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGG	
P. carpio	GGGTTTACGACCTCGATGTTGGATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGG	
Boulengerella	GGGTTTACGACCTCGATGTTGGATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGG	
Prochilodus	GGGTTTACGACCTCGATGTTGGATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGG	

Figure 1. Aligned partial large mitochondrial ribosomal sequences of zebrafish and other species considered in this study. Species names can be inferred from table 1.

and genetic level (Patterson 1988) is often more problematic to assess among distantly related species, particularly for families of genes that underwent repeated duplication events, such as homeobox genes (see, for example, Scott & Carroll 1987; Kappen *et al.* 1989; Runstadler & Kocher 1991; Murtha *et al.* 1991; Krauss *et al.* 1992; Marx 1992; Tabin 1992; Schubert *et al.* 1993). Homology of genes among distant species, such as between *Drosophila* and zebrafish, is often ambiguous. It would appear that much insight into the functional aspects (both cellular and molecular) of development could be gained if several closely related species were compared, making the search for and identification of homologous genes simpler. Potential inferences about genotype-phenotype relations are difficult to test, but may be more tractable in a set of comparisons among closely related species whose evolutionary relations are known (Raff 1992).

The zebrafish, *Danio (Brachydanio) rerio* Hamilton-Buchanan 1822, is one of the most widely used model systems in the developmental biology of lower vertebrates. Since the pioneering work of George Streisinger, literally hundreds of publications on zebrafish have appeared (Westerfield 1989), and knowledge about cellular and molecular aspects of zebrafish development continues to increase very rapidly. Part of the reason for the success of the zebrafish as a model system is that they are readily available in pet stores throughout the world and are easy to breed in captivity, which ensures a steady supply of embryos. Many of the advantages of zebrafish are also present in closely related species of fish, providing ideal conditions for a comparative approach.

The zebrafish *Danio ('Brachydanio') rerio* belongs to the family Cyprinidae in the order Cypriniformes. This family of fish contains about 1600 species (Nelson

1984) and is one of the most successful families in terms of species number and geographic distribution. Several genera in addition to *Danio* are assigned to the subfamily Rasborinae (table 1), many of which are common in India, Pakistan, Nepal, Bangladesh, Sri Lanka, Thailand, the Malay Peninsula, Sumatra and the Yunnan province of China (Barman 1991). The genus *Danio* was first described by Hamilton-Buchanan in 1822. Weber and de Beaufort (1916) first proposed *Brachydanio* as subgenus of *Danio*. Since then, the classification of these genera has been reviewed repeatedly (see, for example, Hora & Mukerji 1934), most recently by Barman (1991) who eliminated the genus *Brachydanio* by synonymizing with it *Danio*. *Danio* is therefore the valid genus name for the zebrafish (*D. rerio*) as well as for the pearl danio (*D. albolineatus*). This recent revision also found *Danio malabaricus* Jerdon 1849 to be synonymous with *D. aequipinnatus* McClelland 1839, the latter name having precedence. Despite this taxonomic work, the phylogeny of danios and their phylogenetic relations to other cyprinid fishes remained untested.

Traditionally, systematists have used phenotypic characters to establish classifications and phylogenetic relations among species. Recent molecular approaches, namely the phylogenetic analysis of homologous DNA sequences to establish phylogenetic relations, have provided a successful alternative approach. Mitochondrial DNA in particular has been widely used to establish relationships among closely related species (see, for example, Meyer 1993*b*). We analysed partial sequences of the large mitochondrial ribosomal RNA gene to determine the phylogenetic position of zebrafish in relation to other species in its genus and several other genera of cyprinid fishes.

The sequences reported in this paper have been deposited in the GenBank database under accession numbers L14487–L14499.

2. MATERIALS AND METHODS

Table 1 lists the species used in this study and their current classification. Specimens were commercially obtained from several localities (Berkeley, Albany and San Francisco in California; Long Island in New York; and Lübeck in Germany) to survey potential intraspecific variation. Two specimens of zebrafish (WT, AB lines) were also obtained from the Kimmel laboratory at the University of Oregon. Four specimens of *D. rerio*, three specimens of *D. rerio* 'frankei', the aquarium breeding morph of the zebrafish, three specimens of *D. albolineatus*, and two specimens of *D. aequipinnatus* (= *malabaricus*) were sequenced. For the other species, one specimen per species was sequenced. The 16S sequence of the carp (*Cyprinus carpio*) was obtained from GenBank (accession no. X61010). Two distantly related tetras (order Characiformes) of the genera *Prochilodus* and *Boulengerella* were used as outgroups in our phylogenetic analysis.

Specimens were preserved in 70% (by volume) ethanol or frozen, and total genomic DNA was extracted from white muscle or liver tissue by Proteinase K/SDS dissolution and purified by phenol–chloroform extraction and ethanol precipitation (Kocher *et al.* 1989; Sambrook *et al.* 1989). The polymerase chain reaction (PCR) (Saiki *et al.* 1988) was used

to amplify a segment of the large (16S) mitochondrial ribosomal gene. Double-stranded amplifications were done in 25 µl volumes containing (in millimoles per litre): 67 Tris (pH 8.8), 6.7 MgCl₂, 16.6 (NH₄)₂SO₄, 10 2-mercaptoethanol, 1 of each dNTP, 1 µM of each primer, 10–1000 ng genomic DNA, and 0.5 units of Taq Polymerase (Cetus Corp.). Primers used were 16Sar-L (5'-CGCCTGTTTATCAAAAACAT-3') (Palumbi *et al.* 1991) and 16Sbr-H (5'-CCGGTCTGAACTCAGATCAGGT-3') (Palumbi *et al.* 1991) to obtain an amplification product about 600 base pairs (b.p.) long. Gel purification (2.5% NuSieve-Agarose, in TAE buffer) of the double-stranded product was followed by generation of single-stranded DNA of both strands for direct sequencing, by using asymmetric PCR (Gyllenstein & Erlich 1988). Single-stranded DNA was concentrated and desalted in spin columns (Millipore: Ultrafree-MC 30000), and both strands were sequenced by the dideoxy method using a commercial kit (Sequenase, United States Biochemical).

The orthologous DNA sequences obtained were aligned, using default settings, by CLUSTAL (Higgins & Sharp 1988) and with a multiple sequence editor (ESEE: Cabot & Beckenbach 1989). Parsimony analyses were done by using maximum parsimony (MP) (PAUP: Swofford 1991). In PAUP, minimal trees were found with branch-and-bound, and bootstrap analyses (100 replications) were done with heuristic searches by using simple stepwise addition, the TBR branch-swapping algorithm and the MULPARS option. Indels were coded as single characters irrespective of their length. When indels of different length overlapped, each size class of indels was assigned a particular character state. Parsimony analyses were done treating characters as unordered and assigning different weights to transitions, transversion and indels; e.g. treating all kinds of substitutions equally, transversions and indels three times the weight of transitions, or transversions only.

Maximum likelihood analysis (ML) (Felsenstein 1981, 1991) was done using the PHYLIP package (DNAML Version 3.4) using default parameters: empirical base frequencies and a transition/transversion ratio of 2.

Neighbour-joining analysis (NJ) (Saitou & Nei 1987) was done with the PHYLIP package (NEIGHBOR, Version 3.4) (Felsenstein 1991). Distances were computed by using DNADIST with a maximum-likelihood multiple-substitution correction. Bootstrapping with 100 replications (Felsenstein 1985) was done with SEQBOOT in PHYLIP to estimate confidence in the phylogenetic trees.

3. RESULTS

Figure 1 shows the aligned sequences of the 14 species used in this study. Up to 538 b.p. (including indels) were determined for each species. This portion of the 16S gene corresponds about to positions 2510 to 3080 in the human complete mitochondrial DNA sequence (Anderson *et al.* 1981). We found no intraspecific differences in *D. albolineatus* and *D. aequipinnatus*, and only one transition among the four specimens of *D. rerio* and three specimens of *D. rerio* 'frankei' (the spotted aquarium breeding morph called the leopard danio) (position 261; figure 1). This position varied within *D. rerio* proper and within the leopard danio, supporting the notion that the leopard danio (figure 2) is not distinct from *D. rerio*.

The observed substitution pattern conforms to expectations based on prior knowledge about the evolutionary behaviour of this molecule (reviewed in

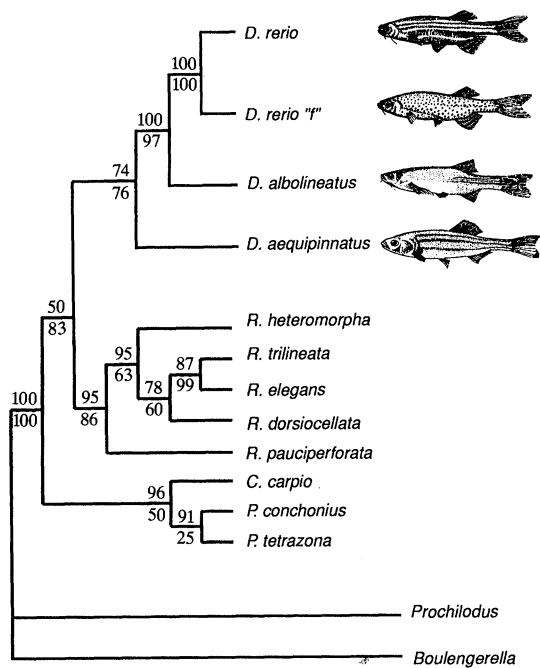


Figure 2. Molecular phylogenetic position of the zebrafish. The same cladogram was inferred by the neighbour-joining method (Saitou & Nei 1987), maximum-likelihood (Felsenstein 1981) and maximum-parsimony methods (PAUP: Swofford 1991). Maximum likelihood confirmed that all branches are significantly positive (at $p < 0.01$). The maximum-parsimony method (using no differential weights for transitions and transversions, calculated with PAUP (Swofford 1991)) found four equally parsimonious trees (length = 438 steps, consistency index (ci) = 0.662, ci excluding uninformative characters = 0.583). One of these trees is the topology shown, the other three varied in the relationships among the *Puntius* and *Cyprinus* and the parsimony bootstrap tree did not resolve (at the 50% level) the relationships among *Puntius* and *Cyprinus*. The numbers above the branches are bootstrap values (Felsenstein 1985) using the NJ, and those below are from the maximum parsimony bootstrap analyses.

Meyer 1993a). Single base pair substitutions are the most frequently observed type of change, whereas indels are much less frequent, as expected from other

studies of mitochondrial DNA in fish (reviewed in Meyer 1993a). Indels are more frequent in inferred loops than in stem regions (reviewed in Mindell & Honeycutt 1990, for example; Meyer 1993a), potentially retaining the secondary structure of the molecule. As is typical for mitochondrial DNA (Brown *et al.* 1982; reviewed in Meyer 1993a, b), transition substitutions outnumber transversions among closely related species. For example, *D. rerio* and *D. albolineatus* differ by a total of 33 substitutions, of which 25 (76%) are transitions and eight (24%) are transversions (table 2). As expected, transversions become more abundant between more distantly related species (e.g. carp and zebrafish: 36% of 65 changes were transversions).

The molecular phylogeny obtained agrees with the current classification (table 1; figure 2). The molecular tree (figure 2) is corroborated by all three phylogenetic methods used, and most branches are supported with high confidence (Felsenstein 1985), as indicated by the high bootstrap values. All variations of the parsimony analysis yielded congruent topologies with the one shown, except for the relationship between *Puntius* and *Cyprinus* which remains tentative. But the neighbour-joining and maximum-likelihood methods strongly favour the grouping shown (figure 2).

The phylogeny (figure 2) derived from the 16S sequences supports the sister group relationship of *D. rerio* and *D. albolineatus*, in agreement with their previous (before Barman 1991) placement into the now obsolete genus *Brachydanio*. Of the species included in this study, the giant danio is the most closely related species to these two. All species of the *Danio* and all species of *Rasbora* are placed into two distinct clades. The 16S phylogeny supports the classification of these two genera into the subfamily Rasborinae, and the position of the two genera *Cyprinus* and *Puntius* into the subfamily Cyprininae.

4. DISCUSSION

Biologists striving to understand what has shaped biological phenomena need to know the past to make

Table 2. Matrix of sequence differences of partial 16S mitochondrial DNA

(Values above the diagonal are uncorrected percent sequence difference including substitutions and indels, below are the total numbers of substitutions and transversions only.)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1 <i>Boulengerella</i>	—	13.4	15.3	17.6	17.4	16.7	19.5	17.8	19.4	17.4	18.6	18.9	18.5	16.8
2 <i>Prochilodus</i>	72/19	—	16.6	19.2	19.0	17.9	20.7	16.7	19.2	17.2	18.3	18.8	17.8	15.6
3 <i>C. carpio</i>	82/38	88/34	—	12.1	12.1	12.4	12.7	10.1	12.4	10.8	11.8	11.6	9.5	5.7
4 <i>D. rerio</i>	94/36	101/32	65/24	—	0.2	6.1	11.0	11.3	13.1	13.8	13.0	13.1	13.8	13.4
5 <i>D. rerio 'f'</i>	93/36	100/32	65/24	1/0	—	6.1	11.0	11.3	13.1	13.8	13.0	13.1	13.8	13.4
6 <i>D. albolineatus</i>	89/34	94/29	66/21	33/8	33/8	—	13.1	11.5	12.3	13.4	12.5	11.9	15.6	13.5
7 <i>D. aequipinnatus</i>	104/39	109/37	68/20	60/24	60/24	71/24	—	11.8	13.3	13.7	13.1	13.1	16.1	14.0
8 <i>R. heteromorpha</i>	95/37	88/29	54/17	61/24	61/24	62/22	64/25	—	5.5	7.4	3.5	6.5	14.6	11.9
9 <i>R. trilineata</i>	103/40	101/33	66/17	71/27	71/27	66/24	72/26	30/7	—	9.4	4.8	4.8	15.9	14.0
10 <i>R. pauciperforata</i>	93/37	91/33	58/16	75/23	75/23	72/23	74/22	40/10	51/11	—	7.0	9.2	14.5	10.8
11 <i>R. dorsiocellata</i>	99/41	96/31	63/18	70/26	70/26	67/24	71/29	19/8	26/6	38/12	—	5.5	16.7	13.3
12 <i>R. elegans</i>	101/41	99/33	62/16	71/26	71/26	64/23	71/27	35/6	26/5	50/10	30/5	—	14.8	13.2
13 <i>P. tetrazona</i>	101/42	96/34	52/18	75/25	75/25	84/28	87/29	79/27	86/29	79/27	90/31	80/29	—	9.7
14 <i>P. conchonus</i>	90/33	83/29	31/12	72/26	72/26	72/23	75/25	64/20	75/20	58/18	71/20	71/19	53/22	—

sense of the present. Phylogenies are the necessary first step in the comparative method, and can be used to distinguish between alternative hypotheses and competing models of evolution. Usually, species that share a recent common ancestor tend to be similar in their genes, their phenotypes, and probably in their development (but see del Pino & Elinson 1983; Wray & Raff 1991; Raff 1992). However, distantly related species that live in the same environment and experience the same selection pressures might evolve similar adapted phenotypes convergently. This is why the phenotype can be a misleading indicator of evolutionary relationships.

Many examples show that the evolution of developmental mechanisms and adult phenotypes do not proceed at a regular pace and that large morphological differences may be caused by small genetic differences. Hence, to infer descent and phylogeny from the phenotype can be misleading because the rate of morphological evolution can be so variable. Molecules tend to be more impartial tracers of evolutionary relationships. Murray (1989) demonstrated that a change in a single parameter of the reaction diffusion mechanism (e.g. for coat coloration in mammals) may be all that is required for switches between stripes and spots. The coloration of the striped zebrafish and one of its breeding morphs, the spotted leopard danio, might be brought about by similarly small genetic differences. Comparisons between closely related species allow us to test the generality of biological phenomena. An example where development shows much variation among closely related species involves the plasticity of the mode of development in many marine invertebrates and ascidians. Even in sister species of echinoderms the pronounced differences found between some larval forms, which start at the fourth cleavage, indicate that morphogenetic mechanisms of early embryonic stages can undergo drastic changes within short geological time spans (Wray & Raff 1991). Based on current knowledge of echinoderm phylogeny, direct development has evolved independently from a specialized feeding pluteus in at least six clades in sea urchins accompanied by analogous changes in gene expression (Wray & Raff 1991; Wray 1992). Some aquatic vertebrates, too, exhibit modified early development, e.g. hylid frogs (del Pino & Elinson 1983). Ballard (1981) pointed out the need for studies on the early development of more vertebrate species whose evolutionary relationships have been established by independent criteria.

In development (as in most fields of biology) strong inferences about a large number of species tend to be made from a relatively small number of model systems. There are very good practical reasons for this. The ground work necessary to establish a new model system and to start studies of the cellular and molecular genetic aspects of development are so prohibitive that most laboratories will not venture to do so. The practical advantages of zebrafish as a model system that have made it so popular in developmental biology (e.g. they are easily obtained and bred, and have clear eggs), apply to many other closely related species of fishes. Our phylogeny of the zebrafish is meant as an

invitation to study the biology of cyprinid fishes in a fine-grained evolutionary framework. When comparing zebrafish development with several other closely related species meaningful insights will surely be gained into the generality of zebrafish developmental biology and the evolution of development.

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