

Comparative osteology of the *Danio* (Cyprinidae: Ostariophysi) axial skeleton with comments on *Danio* relationships based on molecules and morphology

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Danio is a diverse group of small, colourful and easily bred freshwater fishes native to Southern Asia. Biological interest in danios has increased in recent years because the zebrafish, *Danio rerio*, has become an important model organism, particularly for studies of vertebrate developmental biology and genetics. Though several phylogenetic studies of *Danio* have been done on a subset of *Danio* species, the resulting phylogenies conflict in detail. To examine the utility of osteology for systematics of this group at the species level, we studied the axial skeleton for 11 species of *Danio*. We analyse our morphological data alone and combined with DNA sequence data for five gene sequences generated in earlier phylogenetic studies. The axial skeleton of *Danio* exhibits 14 characters that prove useful in phylogenetic analysis. Both molecular and morphological data support the monophyly of the danios included in our analysis and both data sets support the monophyly of two subclades: a deep-bodied group and a slender-bodied group. Separate analysis of molecular and morphological data sets show that the molecular data resolves relationships within the slender subclade whereas the topology of the deep-bodied subclade is determined by morphological data. © 2002 The Linnean Society of London. *Zoological Journal of the Linnean Society*, 2002, 135, 529–546.

ADDITIONAL KEYWORDS: Systematics – Weberian apparatus – caudal skeleton – *Danio rerio* – zebrafish – total evidence.

INTRODUCTION

Danio Hamilton 1822 is a diverse genus of small, colourful, freshwater fishes commonly found in streams and rice paddies of their native Southern Asia (McClure, 1998; Paxton & Eschmeyer, 1998). The ease with which danios can be maintained, bred and reared in captivity has made them popular with aquarists (Axelrod *et al.*, 1998) and biologists alike. In recent years, the zebrafish, *Danio rerio* Hamilton, has become an important model organism, especially for studies of vertebrate developmental biology and genetics (e.g. Detrich *et al.* 1999). As a result, our understanding of the developmental genetics of *D. rerio* has increased dramatically, although relatively little is known about the basic ecology or evolution of the zebrafish and its congeners (but see McClure, 1998). Our rapidly increasing understanding of zebrafish developmental genetics, however, provides

an unprecedented opportunity to investigate patterns and mechanisms of phenotypic transformation among closely related vertebrate species. Such comparative and evolutionary studies of danios require a species level phylogeny. While progress has been made in this regard (e.g. Meyer *et al.* 1993, 1995; Zardoya *et al.* 1996), the several available phylogenies, which rely almost entirely on DNA sequence data, conflict in detail and include only a fraction of *Danio* species. In this study we describe osteological variation found in the axial skeleton of danios and assess the phylogenetic utility at the species level of these morphological data. In combination with molecular data, our morphological data provide a well-supported phylogeny for the 11 species of *Danio* that we were able to obtain. Our study provides a foundation for a more comprehensive phylogenetic analysis of osteological data for *Danio*, pending completion of ongoing alpha taxonomic studies by others (e.g. F. Fang, M. Kottelat).

SYSTEMATIC BACKGROUND

Danios are ostariophysan fishes, which are the dominant group of freshwater fishes comprising approxi-

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mately 64% of all freshwater fishes (Nelson, 1994). The Ostariophysi include Gonorynchiformes (milkfish), Cypriniformes (minnow, carps), Characiformes (tetras, piranhas, etc.), Siluroidei (catfishes) and Gymnotoidea (knifefishes, electric eel). The Otophysi (Ostariophysi excluding Gonorynchiformes) is characterized by the presence of the Weberian apparatus, a physical connection between the gas bladder and the otolith of the inner ear through a series of small tendons and bones derived from the four anterior-most vertebrae (Rosen & Greenwood, 1970). The Weberian apparatus gives otophysans the ability to detect a broader range of sound frequencies, especially higher frequencies, than other fishes (Evans, 1925; Popper & Coombs, 1980) and has been thought by some to be responsible for the success of the group. The Weberian apparatus and the caudal skeleton, modified from elements of the terminal vertebrae, have often been informative in systematic analysis of teleost fishes (Weitzman, 1962; Cavender, 1966; Nybelin, 1971, 1973; Patterson & Rosen, 1977) and of the Ostariophysi (Cavender, 1991; Howes, 1978, 1980, 1981, 1991; Fink & Fink, 1981, 1996; Fink *et al.* 1984; Coburn & Cavender, 1992). However, relatively few studies (e.g. Buhan, 1970, 1972; Eastman, 1980; Arratia, 1983; Mayden, 1989) have explored the systematic utility of these regions at the species level.

Within the Otophysi, *Danio* is a member of the Cyprinidae (minnows), which includes more than 2000 species in 210 genera (Nelson, 1994). Though the monophyly of Cyprinidae has been substantiated (Cavender & Coburn, 1992; characters 1–4), relationships of taxa within the Cyprinidae remains problematic (Howes, 1991; Nelson, 1994). Nevertheless, the genus *Danio* has been placed either in the subfamily Danioninae (Howes, 1991; Rainboth, 1991), Rasborinae (Gosline, 1978), or the subfamily Leuciscinae, which includes the Rasborinae (= Danioninae) *s. l.* (Cavender & Coburn, 1992). The Rasborinae, though probably polyphyletic (Cavender & Coburn, 1992; Nelson, 1994), are considered to be basal within the Cyprinidae (Chen *et al.* 1984; Cavender & Coburn, 1992). Detailed discussions of cyprinid relationships can be found in Cavender & Coburn (1992) and Howes (1991).

Danio contains 51 nominal species of which approximately 26 are considered valid (Fang, 1997; Eschmeyer, 1998). The first phylogenetic study of *Danio* was carried out by Meyer *et al.* (1993) using sequence data from the 16S ribosomal gene. With additional sequence data from 16S and 12S ribosomal genes for nine species of *Danio*, Meyer *et al.* (1995) were able to generate a fully resolved phylogeny showing two major subclades, a deep-bodied and a slender-bodied group (McClure, 1998). In a reanalysis of data from Meyer *et al.* (1995), McClure (1998) was

unable to duplicate their exact phylogeny, though the general topology of two subclades was upheld. Zardoya *et al.* (1996) sequenced the genes *Sonic hedgehog* and *Hoxd 10* and generated phylogenies for each gene along with a combined analysis of the two. Each of these three analyses generated slightly different phylogenies; in particular resolution of the deep-bodied subclade is at issue, although the authors do not comment on these differences or provide a combined analysis of all gene sequence data. Despite differences in details, all of these previous studies demonstrate monophyly for the *Danio* species sampled.

MATERIAL AND METHODS

Over 60 specimens of three outgroup taxa and 11 *Danio* species, *D. rerio*, *D. albolineatus* Blyth, *D. pathirana* Kottelat & Pethiyagoda, *D. regina* Fowler, *D. devario* Hamilton, *D. pulcher* Smith, *D. kerri* Smith, *D. malabaricus* Jerdon, *D. quangbinhensis* (Tu, Thang & Khoa) (see Kullander, 2001), *D. aequipinnatus* McClelland, and *D. browni* Regan (*D. sp. cf. aequipinnatus* of McClure, 1998; see Fang, 2000a) were examined in this study (see material examined). Specimens were cleared and stained following the method described in Taylor & Vandyke (1985). Specimens are stored in either 100% or 75% glycerol with a small amount of thymol. Several disarticulated specimens were also examined. Specimens were viewed with a Wild M5 dissecting microscope. The Weberian apparatus, caudal and trunk vertebrae of articulated specimens were then drawn with the aid of a camera lucida. Two to seven specimens of each species were studied and, where possible, specimens from different age and size classes were also examined.

TERMINOLOGY

The Weberian ossicles were originally considered homologs of the mammalian inner ear bones and were thus named the malleus, incus, stapes and claustrum to reflect this relationship (Weber, 1820). Bridge & Haddon (1889) re-examined these bones and determined them modified processes, arches and ribs of the anterior four vertebrae and renamed them the tripus, intercalarium, scaphium and claustrum, respectively. These names continue to be used today, though other nomenclatures are sometimes employed (Harder, 1975). Terminology for the Weberian apparatus and anterior vertebrae herein follow that of Fink & Fink (1981). Nomenclature for the caudal skeleton follows that of Patterson (1968).

MATERIAL EXAMINED

Specimens for this study were from Cornell University Museum of Vertebrates (CU), American Museum of

Natural History (AMNH) and the Museum of Comparative Zoology (MCZ). All specimens were wild caught or are the F1 generation of wild-caught individuals. A list of specimens used for this study can be found in Table 1. Although some individuals in our sample were maintained in captivity, we do not expect that variation described below is due to growth in captivity. Spontaneous skeletal deformities are rare in F1 generation zebrafish but increase in frequency with further inbreeding (Piron, 1978). In a comparison of wild-caught and lab-reared zebrafish it has been shown that anomalies in the Weberian apparatus are rare (Ferreri *et al.*, 2000). Anomalies of the axial skeleton are apparently more common in the caudal skeleton, but are found in approximately the same frequencies in lab-reared and wild-caught zebrafish (Ferreri *et al.*, 2000). The most common anomalies pertain to the neural and haemal elements associated with the last four centra (Ferreri *et al.*, 2000).

PHYLOGENETIC ANALYSIS

Opsariichthys uncirostris Temminick & Schlegel and *Zacco temminickii* Temminick & Schlegel, long thought to be basal cypriniform fishes (Regan, 1911a,b; Greenwood *et al.*, 1966; Fink & Fink, 1981), were chosen as outgroups for the morphological portion of this study. These taxa, along with *Danio*, have generally been included in the Rasborinae (Chen *et al.*, 1984; Nelson, 1994) or a more inclusive Leuciscinae (Cavender & Coburn, 1992). *Rasbora paviei* Tirant, *Tanichthys albonubes* Lin, and *Pseudorasbora parva* Nichols were used as outgroups in previous molecular phylogenetic analyses (Meyer *et al.*, 1993, 1995;

Zardoya *et al.*, 1996; McClure, 1998). These taxa were not used to polarize the morphological data due to the presence of many apomorphic features relative to cypriniform fishes such as fusion of vertebrae numbers two and three (Fink & Fink, 1981; Cavender & Coburn, 1992); lack of forked supraneural (Cavender & Coburn, 1992), lack of reduced parapophysis associated with the fifth vertebral centra (Cavender & Coburn, 1992), and highly reduced first, second/third vertebrae (Fink & Fink, 1981).

Sequence data for two mitochondrial genes (12S ribosomal gene, 16S ribosomal gene) and three nuclear genes (*Hedgehog*, *Hoxd 10* and *Sonic Hedgehog*) were downloaded from GenBank. Sequences are primarily from Meyer *et al.* (1995) and Zardoya *et al.* (1996) with several modifications as follows. In Meyer *et al.* (1993) *D. malabaricus* and *D. aequipinnatus* are treated as synonyms though Jayaram, 1991) clearly showed these as two distinct species based on morphology and morphometric analysis. In the absence of voucher specimens to verify the identity of Meyer *et al.*'s (1993) *D. malabaricus*/*D. aequipinnatus* we have used the 16S sequence for *D. aequipinnatus* submitted by D. Parichy (unpub., GenBank #AF322664) while those for *D. malabaricus* are from Meyer *et al.* (1995; GenBank #U21384). Sequences of 12S and 16S ribosomal genes for *D. browni* (*D. sp. cf. aequipinnatus* of McClure, 1998) and 16S for *D. rerio* were obtained directly from M. McClure (see McClure, 1998). Sequence information of *D. cf. aequipinnatus* from Meyer *et al.* (1995) was not available. Sequences were aligned with Megalign from the DNASTAR package (Lasergene) using the Clustal method as well as visual inspection. Aligned sequences for all five genes, comprising 2189 bp, were combined into a single matrix using Microsoft Excel and MacClade (Maddison & Maddison, 1992).

Morphological characters from this study were augmented by several characters from McClure (1998, 1999). McClure's ontogenetic characters were not included however, because the ontogenetic series necessary to score these taxa were unavailable for more than half the taxa included in our study.

A combined matrix of molecular and morphological characters was created as recommended by Baker *et al.* (1998) and phylogenetic analysis carried out using PAUP* v4.0b5 (Swofford, 1999). Tests for homogeneity, such as those described by Bull *et al.* (1993), were not performed because we are convinced that data sets do not need to be congruent to be combined (Kluge, 1989; Kluge & Wolfe, 1993; Nixon & Carpenter, 1996; Cunningham, 1997) and that such tests are not necessary when dealing with mixed morphological and molecular data sets (Lovejoy, 2001). Heuristic searches (100 replicates of random taxon additions) were used to find the most parsimonious tree(s). All characters

Table 1. Specimens examined. Numbers in parenthesis indicate number of specimens examined in each lot

Ingroups:

- D. aequipinnatus*, AMNH15761 (2)
- D. albolineatus*, CU82547 (1), CU77841(4)
- D. devario*, CU77862(4), CU82548 (1)
- D. kerri*, CU82551 (4), CU82554 (3),
- D. malabaricus*, CU82549(3), MCZ52399 (2)
- D. pathirana*, CU85509 (5)
- D. pulcher*, CU77840 (3)
- D. quangbinhensis*, AMNH227907(3), AMNH227913(1)
- D. regina*, CU82550 (1), CU77870(2), MCZ47244 (2)
- D. rerio*, CU82546 (6), CU77867(1), CU77872(1)
- D. browni*, CU82553 (1), CU82552 (4), CU76960(2), CU77893(2)

Outgroups:

- Opsariichthys uncirostris*, MCZ32375 (2)
- Pseudorasbora parva*, MCZ32378 (2)
- Zacco temminickii*, CU37570 (1)

were unweighted and unordered. Bootstrap (100 replicates), tree lengths and consistency index values were calculated using PAUP*. Morphological and molecular data sets were also analysed independently to identify support for different nodes and the resolution that different data sets provide. Using the rooting options of PAUP*, individual molecular and morphological trees were rooted with a paraphyletic outgroup. For the combined analysis, rooting of the ingroup was carried out relative to a basal polytomy of all five outgroup taxa. Phylogenetic analysis of individual genes can be found in Meyer *et al.* (1995), Zardoya *et al.* (1996), and McClure (1998) and are thus not presented here. Rather than excluding taxa for which we do not have complete data, we use the phylogenetic trunk method outlined by Anderson (2001) to test for the effects of incomplete data on tree topology.

RESULTS

GENERAL ANATOMY OF THE WEBERIAN APPARATUS

The Weberian apparatus of *Danio* (Fig. 1) is typical of cyprinids and otophysans. As in other otophysans, the Weberian apparatus of *Danio* comprises the anterior four vertebrae and four ossicles, the tripus, intercalarium, scaphium and claustrum (Rosen & Greenwood, 1970; Fink & Fink, 1981, 1996). As in all other Cypriniformes, the Weberian apparatus of *Danio*

has its anterior two supraneurals expanded anteriorly to form a synchondral joint with neural arches three and four and its second lateral processes extended laterally into the somatic musculature (Fink & Fink, 1981, 1996). Neither *Danio*, nor *Opsariichthys* and *Zacco*, exhibit a free-floating supraneural recognized as a synapomorphy of the Leuciscinae (Cavender & Coburn, 1992). Some species of *Danio* do, however, have the forked supraneural with sides separated by a deep groove thought to be characteristic of all leuciscines (Chen *et al.*, 1984; Cavender & Coburn, 1992; but see below). *Danio* also exhibits the leuciscine synapomorphy of reduced parapophyses associated with the fifth vertebra (Fig. 2), which is thought to allow the fifth rib greater three-dimensional mobility (Cavender & Coburn, 1992).

VARIATION IN THE WEBERIAN APPARATUS OF *DANIO*

Os suspensorium

The os suspensorium is a modified fourth rib and parapophysis that supports the anterior gas bladder and dorsal mesentery. Little of the os suspensorium is visible from a lateral view without disarticulating the skeleton. In *D. albolineatus*, *D. kerri*, *D. pulcher* and *D. rerio* (Fig. 3b,d,g,j) there is a small flange on the most distal process of the os suspensorium (compare Fig. 3j and c, arrow 1; character 1, Table 2) in contrast

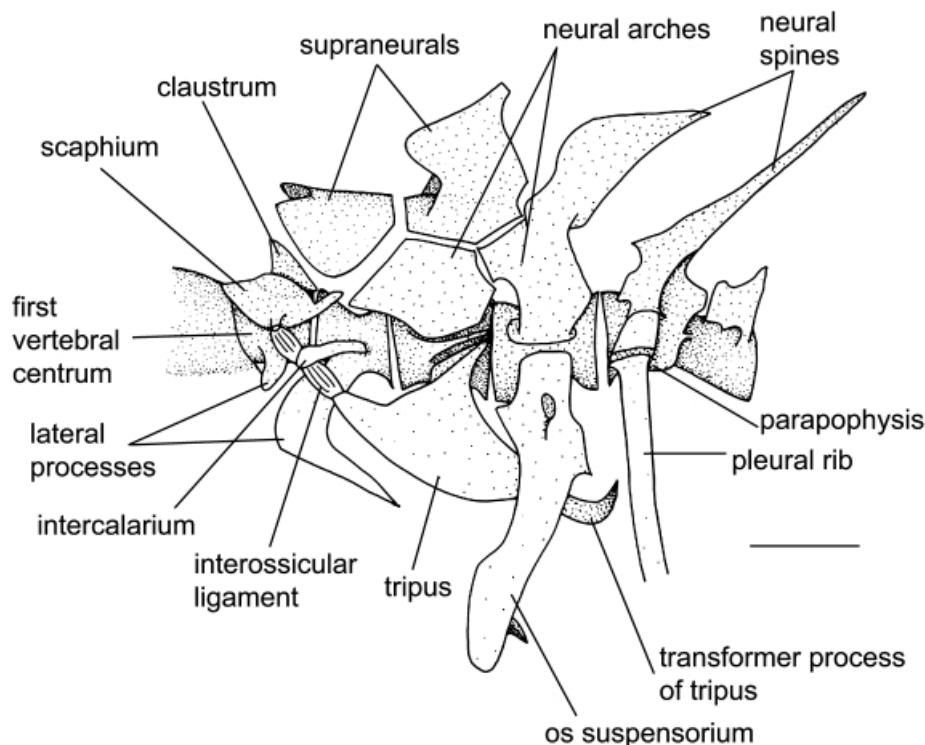


Figure 1. The Weberian apparatus of *D. rerio* (CU 82546), left lateral view. Scale bar = 0.5 mm.

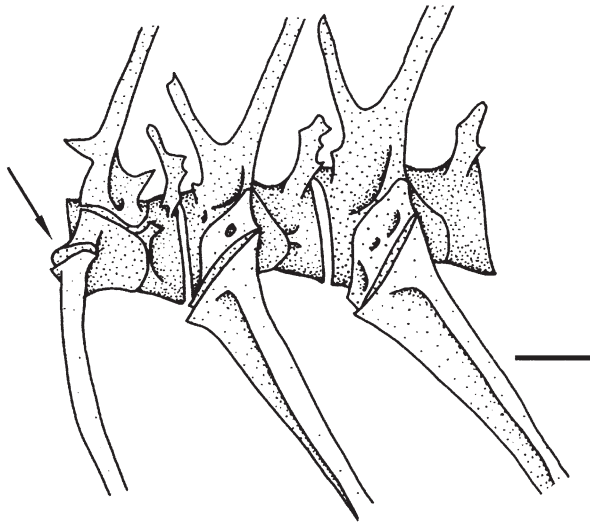


Figure 2. Reduced parapophysis of the fifth vertebral centrum (arrow). Compare to unreduced vertebral parapophyses of vertebrae six and seven (*D. malabaricus*; MCZ 52399). Scale bar = 0.5 mm.

to other *Danio* species, which do not have a visible flange (Fig. 3a,c,e,f,h,I,k). All *Danio* have pitting or a groove in the upper body of the os suspensorium that probably serves as an attachment for tendon or muscle serving the gas bladder (Evans, 1925). The shape and size of this groove varies within species.

Neural Arches and Spines

In otophysans the first neural arch has been modified into the scaphium and claustrum and the second has been modified into the intercalarium (Fink & Fink, 1996). Neural arches of the third and fourth centra are greatly enlarged. The shape of the third neural arch is conserved within and between species while that of the fourth is highly variable intraspecifically. The anterior margin of this fourth neural arch is often in contact with or slightly nested within the posterior margin of the third neural arch of large *D. kerri* specimens. The posterior margin of the fourth neural arch has processes extending from it that often become bigger and more elaborate in larger individuals. In *D. albolineatus*, *D. kerri*, *D. pulcher* and *D. rerio* (Fig. 3b,d,g,j) the fourth neural spine is broad and forms a fan or blade-like appearance (compare Fig. 3c and j, arrow 2; character 2, Table 2) in contrast to the fourth neural spine in other species where it is thin and narrows to a fine point distally (Fig. 3a,c,e,f,h,I,k).

Supraneurals

Primitively the larger supraneural of Leuciscinae (includes rasborins *sensu* Nelson, 1994) divides dor-

Table 2. Summary of morphological characters used in phylogenetic analysis

Character 1: medial flange on os suspensorium absent (0); present (1)
Character 2: blade-like fourth neural spine absent (0); present (1)
Character 3: anterior margin of large supraneural unfused (0); fused (1)
Character 4: posterior margin of large supraneural unfused (0); fused (1)
Character 5: large supraneural not reduced (0); reduced to a thin slip (1)
Character 6: large supraneural tall (0); short (1)
Character 7: ventral margin of small supraneural vertical (0); folds medially (1)
Character 8: medial ridge of small supraneural horizontal (0); saddle-like (1)
Character 9: ridge on tripus perpendicular to vertebral column absent (0); present (1)
Character 10: ascending process of intercalarium developed (0); reduced (1)
Character 11: lateral process of first centrum developed (0); reduced (1)
Character 12: total vertebrae count between 31 and 33 (0); 34–38 (1); greater than 38 (3)
Character 13: four hypurals in upper lobe of caudal fin (0); three hypurals (1)
Character 14: epural and neural arch of compound centrum abut (0); are separated (1)
Character 15: reticulations in anterior pigment pattern absent (0); present (1); vertical bars (3)
Character 16: postopercular spot absent (0); present (1)

sally into two thin slips of bone separated by a small dorsal groove (Fig. 4a; Cavender & Coburn, 1992). In all *Danio* specimens examined, the anterior margins of these slips of bone have become fused (Fig. 4b, arrow 1; character 3, Table 2). In *D. malabaricus*, *D. pathirana* and *D. regina* the posterior margins of these slips have also become fused leaving a bowl-shaped depression in the dorsal surface of the supraneural (Fig. 4c, arrow 2; character 4, Table 2). In *D. kerri*, *D. pulcher* and *D. rerio* the supraneural is fused into a thin slip along the dorsal margins (Fig. 4d, arrow 3; character 5, Table 2). In *D. albolineatus*, *D. kerri*, *D. pulcher* and *D. rerio* this supraneural is also foreshortened relative to other danios examined (character 6, Table 2).

In *D. quangbinhensis* and *D. aequipinnatus* the ventral-most margins of this supraneural are curved medially while on other species these edges are nearly vertical (Fig. 4b, arrow 5; character 7, Table 2). In most species the smaller, more anterior supraneural is nearly horizontal and has no medial ridge. However, in two species, *D. kerri* and *D. pulcher*, there is a

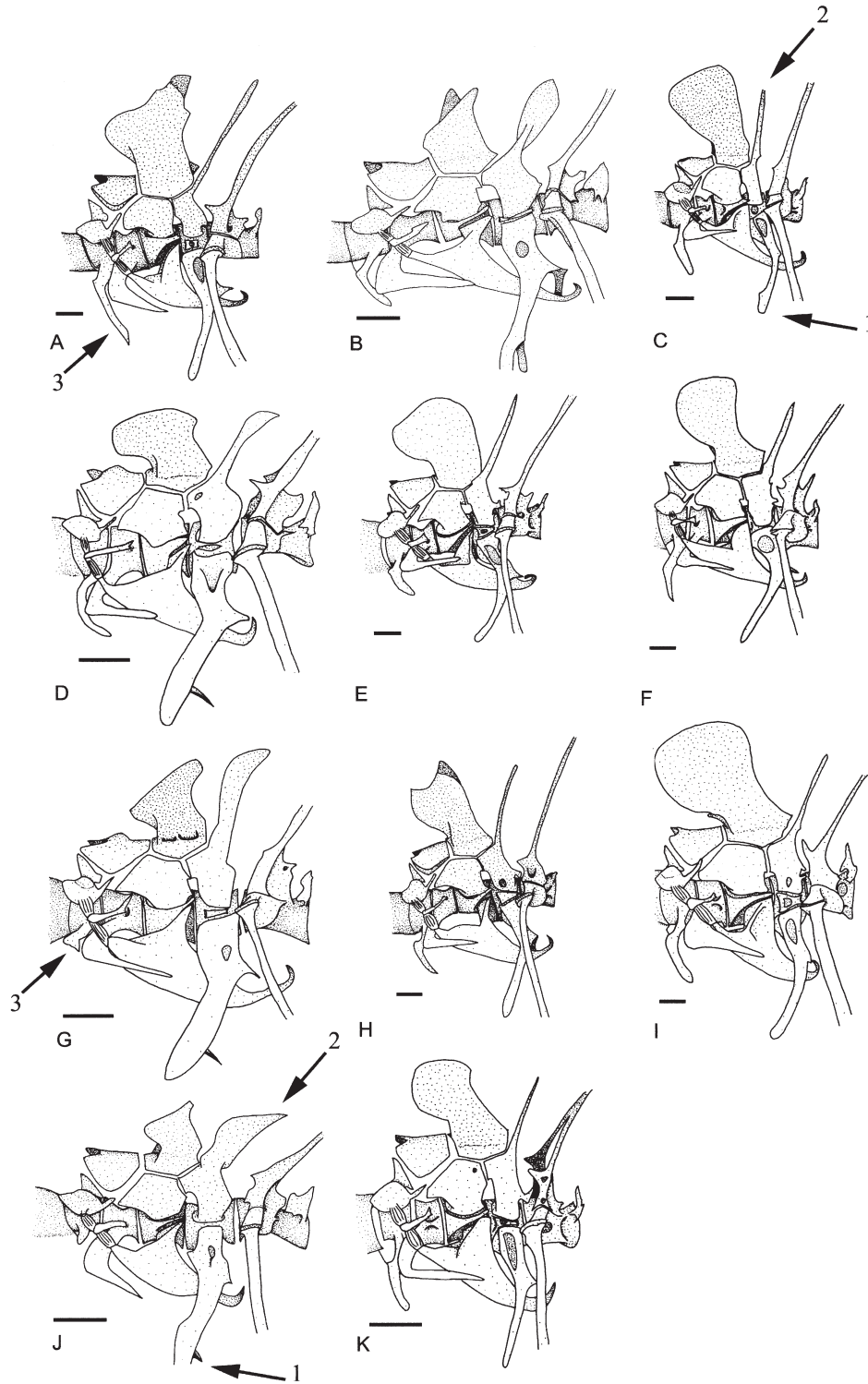


Figure 3. The Weberian apparatus and anterior vertebrae of *Danio*. Camera lucida drawings of A, *D. aequipinnatus* (AMNH 15761) note relative size of first lateral process (arrow 3), compare to G; B, *D. albolineatus* (CU 82547); C, *D. devario* (CU 82548) note absence of flange of os suspensorium (arrow 1), compare to J; D, *D. kerri* (CU 82554); E, *D. malabaricus* (MCZ 52399); F, *D. pathirana* (CU 85509); G, *D. pulcher* (CU 77840) note relative size of first lateral process (arrow 3), compare to A; H, *D. quangbinhensis* (AMNH 227913); I, *D. regina* (CU 82550); J, *D. rerio* (CU 82546) note presence of flange on os suspensorium (arrow 1), compare to C; K, *D. browni* (CU 77893). Scale bars = 0.5 mm.

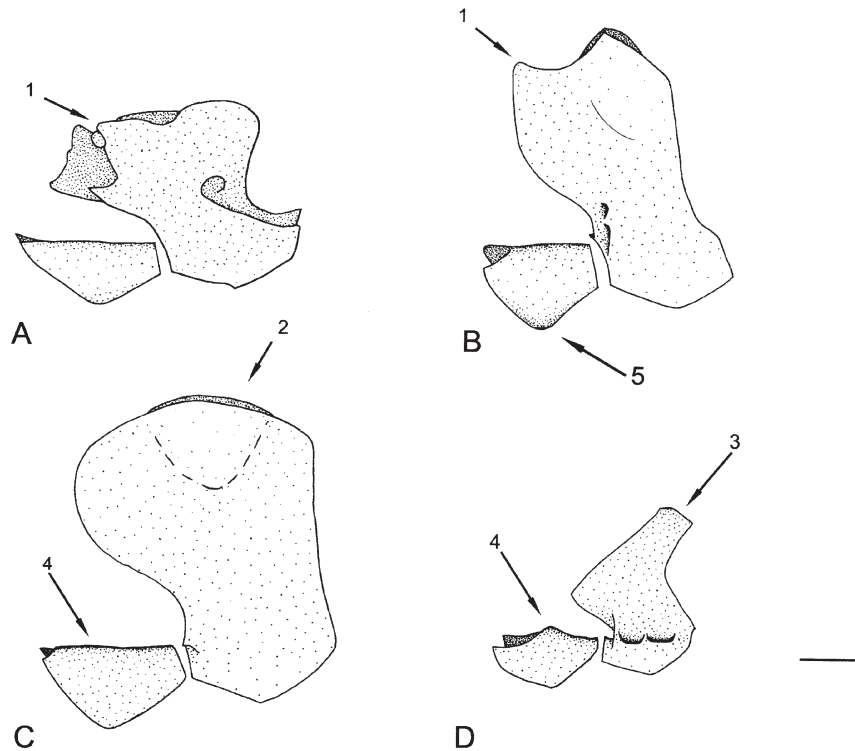


Figure 4. Variation in the supraneurals. A, *Opsariichthys unicrostris* (MCZ 32375); B, *D. quangbinhensis* (AMNH 227913); C, *D. malabaricus* (MCZ 52399); and D, *D. pulcher* (CU 77840). Arrow 1 shows the fusion of the anterior margin of the supraneural seen in all danios (B) relative to the outgroup *Opsariichthys* (A). Arrow 2 indicates the bowl shaped depression (C) in the dorsal surface of the supraneural as seen in *D. alabaricus*, *D. pathirana* and *D. regina*. Arrow 3 indicates the fused, thin dorsal margin of the second supraneural (D) characteristic of *D. kerri*, *D. pulcher* and *D. rerio*. Arrow 4 indicates the saddle-like medial ridge of the anterior supraneural as seen in *D. kerri* and *D. pulcher*. Arrow 5 points out the medial curving of the anterior supraneural (B) as seen in *D. quangbinhensis* and *D. aequipinnatus*. Scale bar = 0.5 mm.

pronounced saddle-like medial ridge to this supraneural (Fig. 4d, arrow 4; character 8, Table 2).

Tripus

The tripus, the most posterior of the Weberian ossicles, is derived from the parapophyses and rib of the third centrum (Rosen & Greenwood, 1970) and connects to the gas bladder through ligaments connected to the transformer process. The tripus of *Danio* occurs in two general forms. The first has a distinct ridge running perpendicular to the vertebral column often creating a depression anteriorly and sometimes posteriorly as well (Fig. 5a; character 9, Table 2). This is found in *D. aequipinnatus*, *D. devario*, *D. malabaricus*, *D. pathirana*, *D. quangbinhensis*, *D. browni* and *D. regina*. The other form of the tripus lacks this ridge and has a smooth surface (Fig. 5b). The transformer process of the tripus varies in length

and thickness within species. In some individuals, it is long, thin, and ribbon-like while in others it ends in a blunt point after turning medially.

Intercalarium

In Cypriniformes, the intercalarium consists of three processes: an ascending process, a horizontal process, which serves as a ligament attachment, and a small articulating process. The intercalarium is derived from the second neural arch and articulates with a small depression in the second centrum (Rosen & Greenwood, 1970). In all species of *Danio* examined the ascending process is absent or has become highly reduced compared to those of *Opsariichthys* and *Zacco* (Fig. 6; character 10, Table 2). The shape and size of the horizontal process is highly variable within species of *Danio* ranging from large and bulbous and divided into two separate heads to nearly absent.

Scaphium and claustrum

The scaphium and claustrum, the most anterior Weberian ossicles, are derived from the first neural arch and form the anterior opening to the neural canal (Rosen & Greenwood, 1970; Harder, 1975). Within *Danio*, these bones show a higher degree of intraspecific variation than interspecific variation. Within each species, some individuals have the ligamentous attachment of the scaphium enlarged and extended posteriorly. This is most exaggerated in older individuals of *D. kerri*. The posterior process of the claustrum varies in size within species but is generally larger in *D. devario*, *D. malabaricus* and *D. pathirana*. Intraspecific variation in shape and size of these elements may be a function of growth (see below).

Anterior vertebrae and their processes

As in all ostariophysans, the first four vertebral centra of *Danio* are foreshortened relative to more posterior vertebrae (Fink & Fink, 1981, 1996). The first centrum is usually the shortest but in some individuals it may

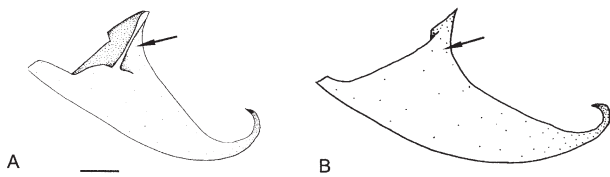


Figure 5. Variation in the tripus. A, *D. malabaricus* (MCZ 52394); B, *D. pulcher* (CU 77840). Note the ridge on the lateral face (arrow) of the tripus of *D. malabaricus*. Scale bars = 0.5 mm.

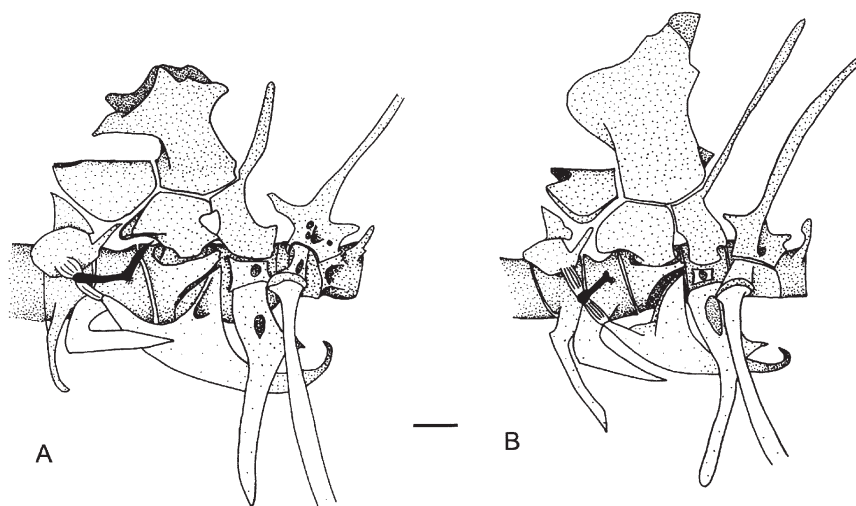


Figure 6. Reduction of the ascending process of the intercalarium. A, *Zacco temminicki* (CU 37570); B, *D. aequipinnatus* (AMNH 15761) with reduced ascending process of the intercalarium (shaded black) exemplifies the condition in *Danio* relative to the outgroups *Zacco* (A) and *Opsariichthys*. Scale bar = 0.5 mm.

be about the same size or slightly larger than the second. In all individuals the third centrum is the least reduced, probably to accommodate the articulation of the tripus and large neural arch. The second and third vertebral centra of *Danio* are not fused but notable calcifications were seen in one individual of *D. browni*. The lateral processes of the first two centra in *D. pulcher* and *D. rerio* (Fig. 3g,j) are quite reduced relative to their usual elongation in other species (compare Fig. 3a and g, arrow 3; character 11, Table 2). The lateral process of the second centrum generally extends downwards and outwards and then curves posteriorly over the tripus.

VARIATION WITHIN THE TRUNK VERTEBRAE

In contrast with vertebral centra 1–4, the trunk vertebrae of danios are unreduced in length and retain parapophyses and pleural ribs. Parapophyses and the dorso-posterior processes of the fifth vertebrae show a high degree of intraspecific variation (see discussion) while this flange is missing from other trunk and caudal vertebrae. There are typically 8–10 trunk vertebrae and 19–21 caudal vertebrae within *Danio*. All specimens of *Danio* examined have 38 or fewer total vertebrae compared to 40–44 vertebrae in *Opsariichthys* and *Zacco* (character 12, Table 2). Meyer *et al.* (1995) reported that species in the slender-bodied subclade of *Danio* typically have fewer total vertebrae (30–34) than species in the deep-bodied subclade (35–37 total vertebrae). Vertebral counts for our sample of 57 *Danio* specimens are consistent with this distinction (slender-bodied species 30–33; deep-bodied species 34–38) so we included high and

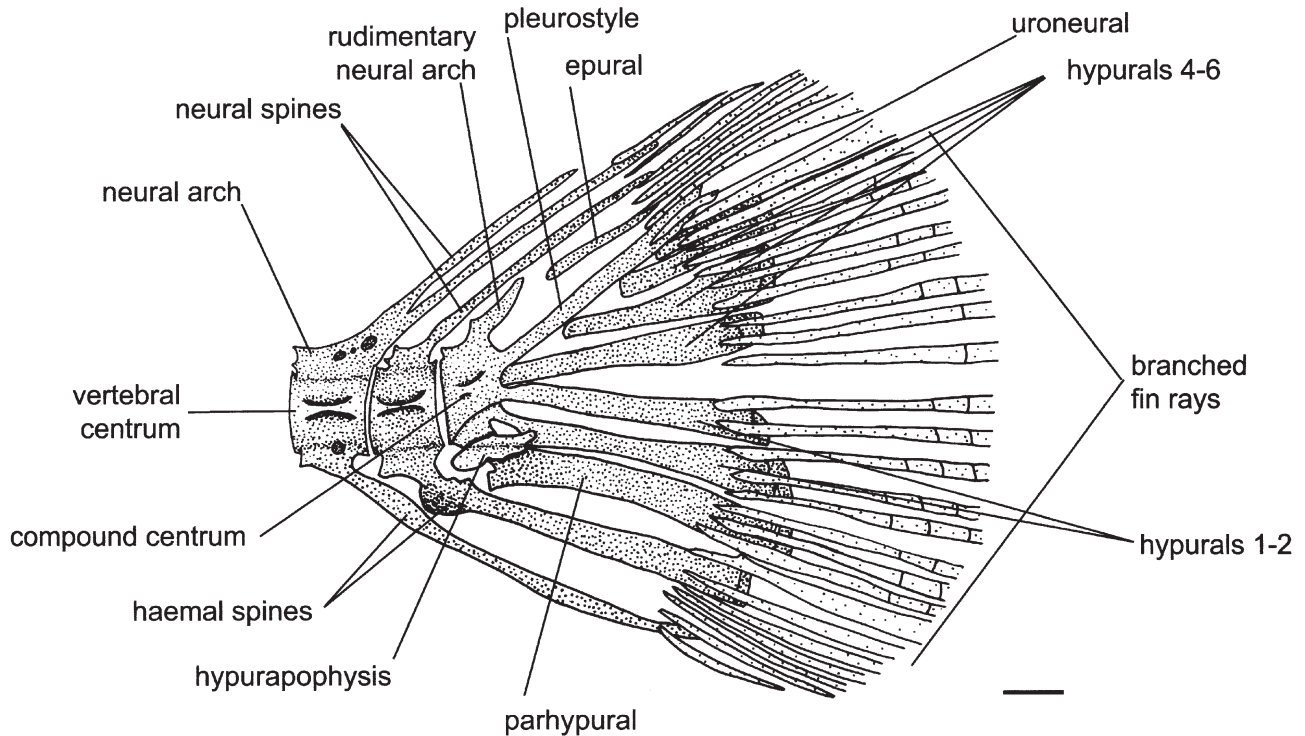


Figure 7. The caudal skeleton of *D. quangbinhensis* (AMNH 227913), left lateral view. Scale bar = 0.5 mm.

low vertebral counts as characters in our analysis (character 12, Table 2). We note that these groupings by vertebral counts are corroborated by other characters (see phylogenetic analysis).

THE CAUDAL SKELETON

The caudal skeleton of *Danio* (Fig. 7) exemplifies the otophysan synapomorphy of a compound terminal centrum and the cypriniform synapomorphy of having two or fewer epurals (Fink & Fink, 1981, 1996).

Neural arches, neural spines, and haemal spines

The neural arches of the three posterior most vertebrae exhibit little variation. Rarely, the neural arch may be slightly enlarged. Three specimens showed a doubling of neural arches on the preural vertebrae and on the compound centrum. The rudimentary neural arch of the compound centrum is highly variable within all species examined. This neural arch, which no longer supports a neural spine may be short and stout or narrow and elongated. It is also common for this neural arch to curve anteriorly near its tip.

Doubling of the neural spines is common in other cyprinids (Eastman, 1980) as well as *Danio*. Nearly all species examined showed auxiliary neural spines originating from the medial face of the associated

neural arch near its base (see Fig. 8e, arrow 1). These auxiliary spines were both paired and unpaired with a spine from the opposite face of the neural arch. One specimen of *D. pathirana* also showed doubling of the haemal spine of preural vertebrae two.

Hypurals and the parhypural

The presence of four hypurals in the upper lobe of the caudal fin is typical of Cyprinidae (Buhan, 1972) though individuals with three hypurals can often be found in natural populations (Buhan, 1972; Eastman, 1980). In these rare individuals, it is most commonly the sixth, the smallest and most dorsal, that is reduced or fused to the fifth hypural (Buhan, 1972). In four species of *Danio*, *D. albolineatus*, *D. kerri*, *D. pulcher* and *D. rerio* it appears that the presence of three hypurals in the upper lobe has become fixed (compare Fig. 8d and e; character 13, Table 2). For the 17 specimens examined of these four species, only one specimen of *D. albolineatus* had a small slip of bone remaining where the sixth hypural is found. The parhypural and other hypurals show little variability except in their width. It is common to find hypurals abutting one another. Rarely, hypurals are even fused together at their tips or along the length of adjacent margins. The hypurapophysis of the parhypural is also highly variable within species. The hypurapophysis

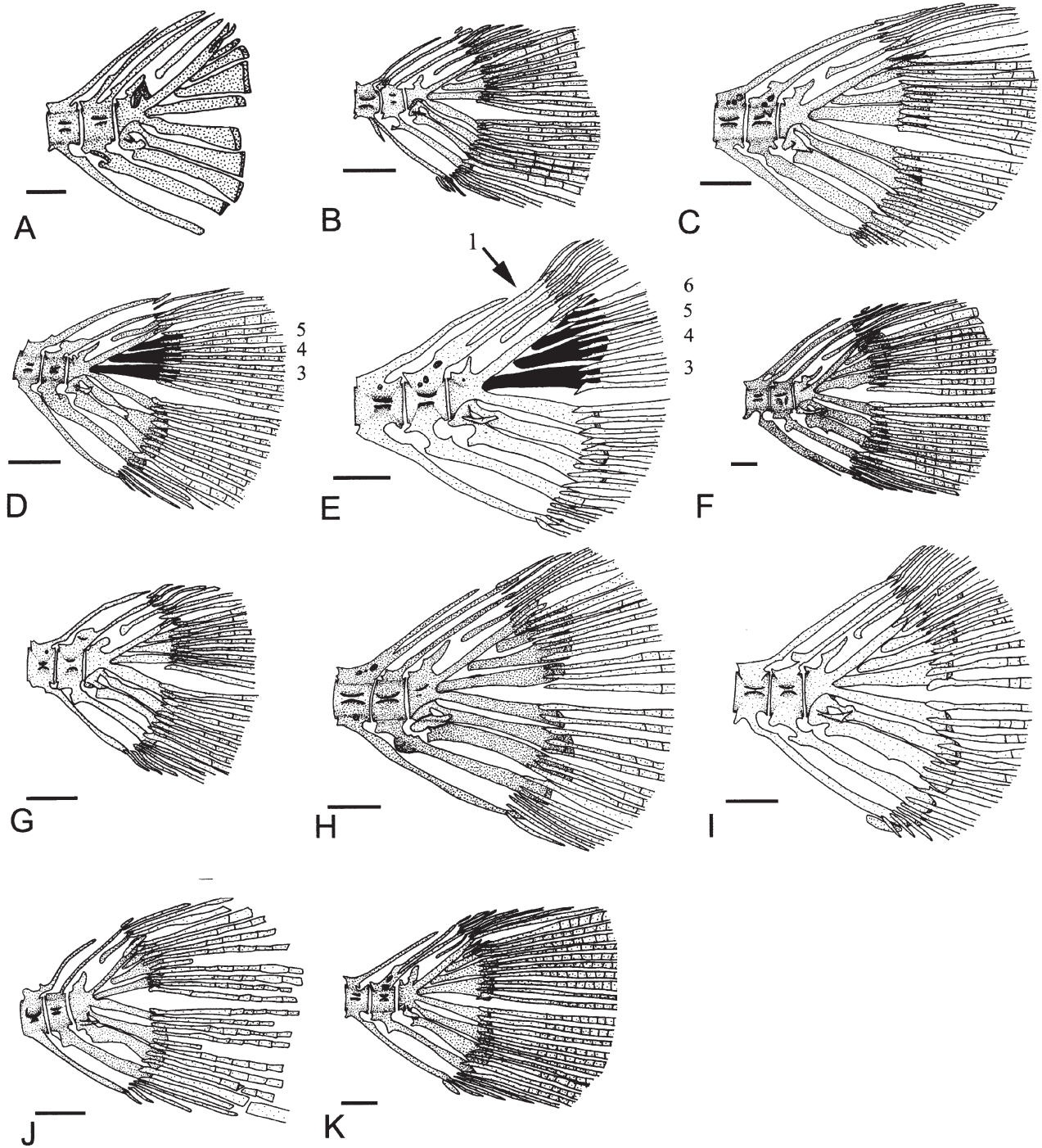


Figure 8. Variation in the caudal skeleton of *Danio*. A, *D. aequipinnatus* (AMNH 15761); B, *D. albolineatus* (CU 77841); C, *D. devario* (CU 82548); D, *D. kerri* (CU 82554) note absence of the sixth hypural; E, *D. malabaricus* (MCZ 52399) note doubling of neural spines (arrow 1) and presence of the sixth hypural; F, *D. pathirana* (CU 85509); G, *D. pulcher* (CU 77840); H, *D. quangbinhensis* (AMNH 227913); I, *D. regina* (CU 82550); J, *D. rerio* (CU 82546); K, *D. browni* (CU 82553). Scale bars = 1 mm.

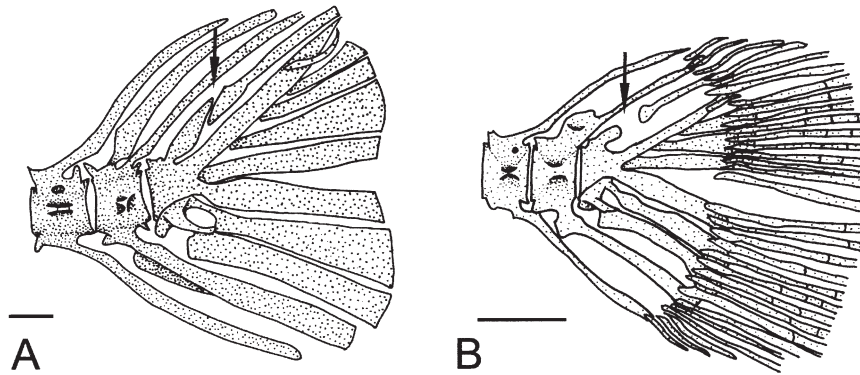


Figure 9. Separation of the epural from the neural arch. A, *Opsariichthys* (MCZ 32375) in which the epural abuts the rudimentary neural arch (arrow); B, *D. pulcher* (CU 77840) illustrates the separation of the neural arch and epural found in all *Danio* examined. Scale bars = 1 mm.

may comprise both an anterior and posterior process, or only one, usually the posterior, or both processes may be reduced to a small projection on the face of the bone.

Epurals and uroneurals

Epurals are homologous to neural spines while uroneurals are homologous to neural arches of more basal teleosts (Patterson, 1968). In Otophysans the first uroneural has fused with the compound centrum (Fink & Fink, 1981, 1996). The remaining uroneural shows little variability within *Danio* and remains an elongated thin slip of bone along the side of the pleurostyle (Fig. 7). Unlike the epurals of *Opsariichthys* and *Zacco*, which nearly abut the rudimentary neural arch, the epural of *Danio* begins above this point leaving a gap between the two bones (Fig. 9; character 14, Table 2).

A COMMENT ON LATE ONTOGENETIC VARIATION IN *DANIO* OSTEOLOGY

Osteological studies often rely on only a few specimens of cleared and stained material for each species of interest, making it difficult to observe intraspecific variation or ontogenetic changes in osteology. Furthermore, the emphasis in ontogenetic studies is generally on early ontogenetic stages. However, late ontogenetic changes have been observed in paddlefish (Grande & Bemis, 1991) and some cyprinids (Cavender & Coburn, 1992). The lack of attention paid to late ontogenetic stages has, in some cases, been the source of apparent conflicts in the literature (see Grande & Bemis, 1991). In older individuals of *Danio* we have also observed a higher frequency of growths and reductions. For example, the posterior processes of the fifth centrum in *D. kerri* seem to undergo

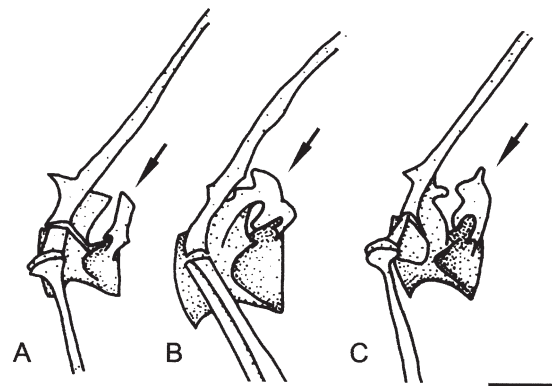


Figure 10. Late ontogenetic variation in the posterior process of the fifth vertebra of *D. kerri*. A, a 'young' individual (CU 82554) of standard length (SL) = 27.6 mm; This individual is known to be less than five years old as it was an F1 from parents wild-caught in 1995. B, a larger individual (SL = 38.7 mm, CU 82551) and C, even larger individual (SL = 43.3 mm, CU 82551). B and C are known to be more than 5 years old; they were caught as adults in 1995 and maintained in captivity for 5 years. Scale bar = 0.5 mm.

changes with growth (Fig. 10). This is exemplified in one individual where this process has fused with the posterior flange of the neural arch (Fig. 10b). The ligamentous attachment on the scaphium also seems to become elongated in older specimens of *D. kerri*, though this observation is based on only three to four individuals of each tentative age class (Fig. 11). Older individuals of *D. kerri* and *D. browni* also commonly exhibit a thinning or complete loss of bone at the articulation of the scaphium and claustrum (Fig. 11b,c). Fusions of vertebrae two and three are common within Cypriniformes (Fink & Fink, 1981, 1996; Cavender & Coburn, 1992) but the actual distribution of this character is uncertain (Cavender & Coburn,

1992). Cavender & Coburn (1992), noting that a better understanding of this fusion's ontogeny is needed, suggest that this fusion may occur earlier in ontogeny in the subfamily Cyprininae than in the subfamily Leuciscinae. In one older individual of *D. browni* there are notable calcifications between vertebral centra two and three while there are no fusions or calcifications in any other individuals of this species examined.

PHYLOGENETIC ANALYSIS

Morphological analysis

Study of the axial skeleton in 11 species of *Danio* yields 14 characters that are potentially useful in phylogenetic analysis. Two pigmentation characters were also incorporated from McClure (1998, 1999). Characters, as described in the preceding text, are summarized in Table 2. Table 3 gives the character

matrix used for phylogenetic analysis. The consensus phylogeny derived from two equally-parsimonious tree is illustrated in Figure 12. According to our analysis, four morphological characters support the monophyly of *Danio*. These characters include: the lack of an anterior groove in the large supraneural (character 3), reduction of the ascending process of the intercalarium (character 10), having 38 or fewer vertebrae (character 12), and presence of a space between the epural and rudimentary neural arch of the compound centrum (character 14). Study of additional *Danio*, rasborin and cyprinid species is desirable, however, to confirm these characters. Our analysis also shows strong support for the basal division of *Danio* into deep-bodied and shallow-bodied subclades, though resolution within these subclades is low. The slender-bodied subclade is supported by six synapomorphies: having a total vertebrae count of 31–33 (character 12),

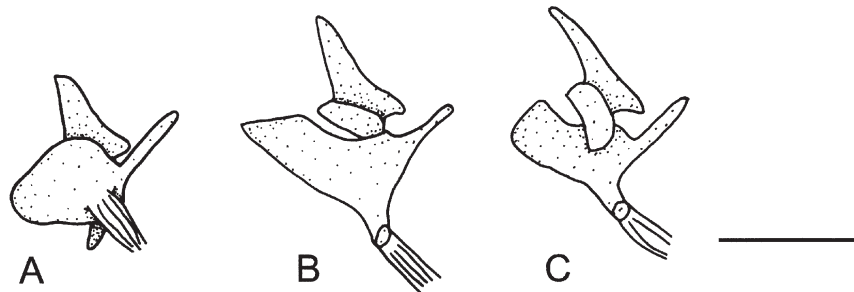


Figure 11. Intraspecific variation of the scaphium in *D. kerri*. A, 'young' individual of standard length (SL) = 27.6 mm (CU 82554); this individual is known to be less than five years old as it was an F1 from parents wild-caught in 1995. B, larger individual (SL = 38.7 mm, CU 82551); C, an even larger individual (SL = 43.3 mm, CU 82551). B and C are known to be more than 5 years old; they were caught as adults in 1995 and maintained in captivity for 5 years. Scale bar = 0.5 mm.

Table 3. Character and state matrix of morphological characters used in phylogenetic analysis

	Character number															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
<i>D. aequipinnatus</i>	0	0	1	0	0	0	1	0	1	1	0	1	0	1	1	1
<i>D. albolineatus</i>	1	1	1	0	0	1	0	0	0	1	0	0	1	1	0	0
<i>D. devario</i>	0	0	1	0	0	0	0	0	1	1	0	1	0	1	1	1
<i>D. kerri</i>	1	1	1	0	1	1	0	1	0	1	0	0	1	1	0	0
<i>D. malabaricus</i>	0	0	1	1	0	0	0	0	1	1	0	1	0	1	1	1
<i>D. pathirana</i>	0	0	1	1	0	0	0	0	1	1	0	1	0	1	3	1
<i>D. pulcher</i>	1	1	1	0	1	1	0	1	0	1	1	0	1	1	0	0
<i>D. quangbinhensis</i>	0	0	1	0	0	0	1	0	1	1	0	1	0	1	1	1
<i>D. regina</i>	0	0	1	1	0	0	0	0	1	1	0	1	0	1	1	1
<i>D. rerio</i>	1	1	1	0	1	1	0	0	0	1	1	0	1	1	0	0
<i>D. browni</i>	0	0	1	0	0	0	0	0	1	1	0	1	0	1	1	1
<i>Opsariichthys uncirostris</i>	0	0	0	0	0	0	0	0	1	0	0	3	0	0	0	0
<i>Zacco temminicki</i>	0	0	0	0	0	0	0	0	1	0	0	3	0	0	0	0

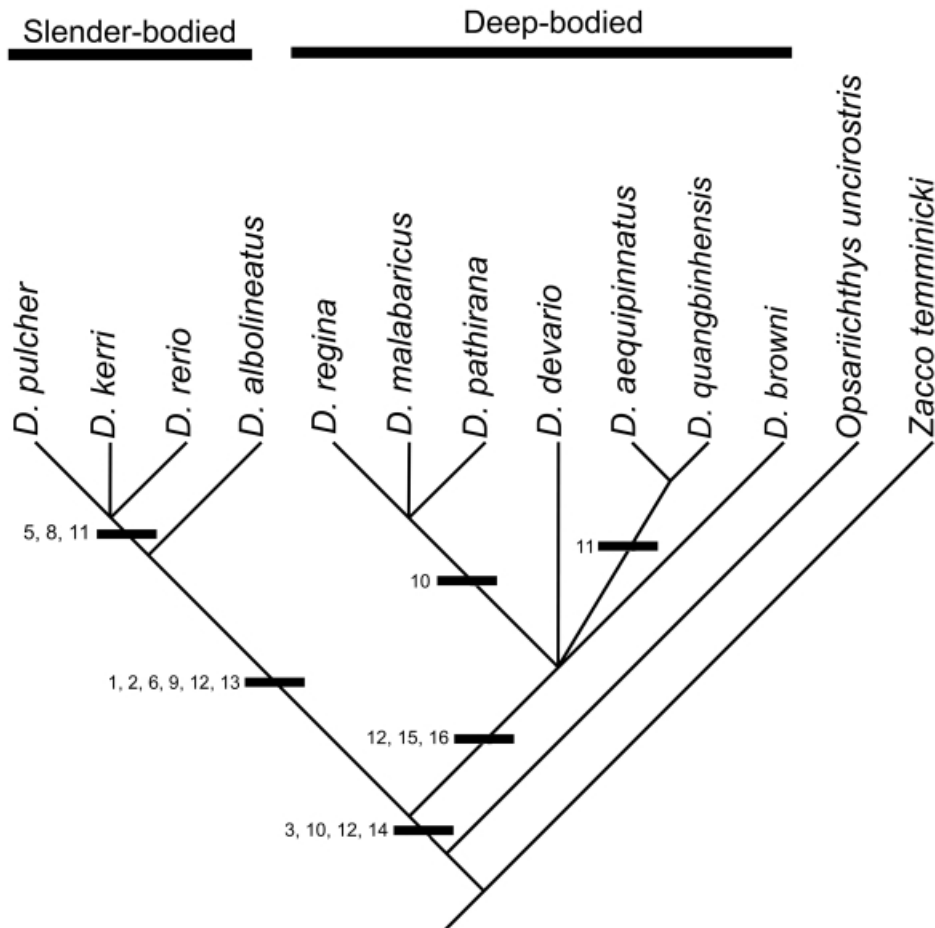


Figure 12. Phylogeny of *Danio* according to morphological data. This phylogeny is the strict consensus tree derived from 16 morphological characters (20 steps, CI = 0.90). Character support for each node is given along side each branch. Numbers refer to characters listed in Table 2.

loss of the sixth hypural (character 13), presence of a medial flange on the os suspensorium (character 1), blade-like fourth neural spine (character 2), lack of a distal ridge on the tripus (character 9), and a fore-shortened large supraneural (character 6). Three characters support the deep-bodied subclade: having a total vertebrae count of 34–38 (character 12), reticulations in melanophore patterning (character 15), and presence of a postopercular spot (character 16).

Molecular analysis

Combined sequences from five genes comprising 2189 total base pairs yields 246 potentially informative sites. Table 4 gives partitions of the total variable sites and informative sites by gene. Molecular data also strongly support the monophyly of *Danio* as well as the basal division into slender and deep-bodied subclades. The bootstrap consensus molecular phylogeny derived from two equally-parsimonious phylogenies is illustrated in Figure 13(a). The two equally-

parsimonious trees differ only in the relationship of *D. malabaricus* to *D. pathirana* and *D. devario*. Though the molecular analysis agrees with the morphological analysis in general topology, they differ in the resolution within the slender-bodied subclade and the position of *D. malabaricus*.

Combined analysis of morphology and molecules

A combined analysis of 16 morphological characters and 246 variable sites in the DNA sequences from five genes yields 135 equally-parsimonious trees. The bootstrap consensus tree is shown in Figure 13(b). The combined analysis strongly supports the monophyly of *Danio* and its basal division into deep-bodied and slender-bodied subclades but it fails to resolve fully the relationships within the deep-bodied clade. The topology of the shallow-bodied clade is identical to that found in our molecular analysis (Fig. 13a) and previous molecular studies (Meyer *et al.*, 1995; Zardoya *et al.*, 1996), but differs from the topology derived only

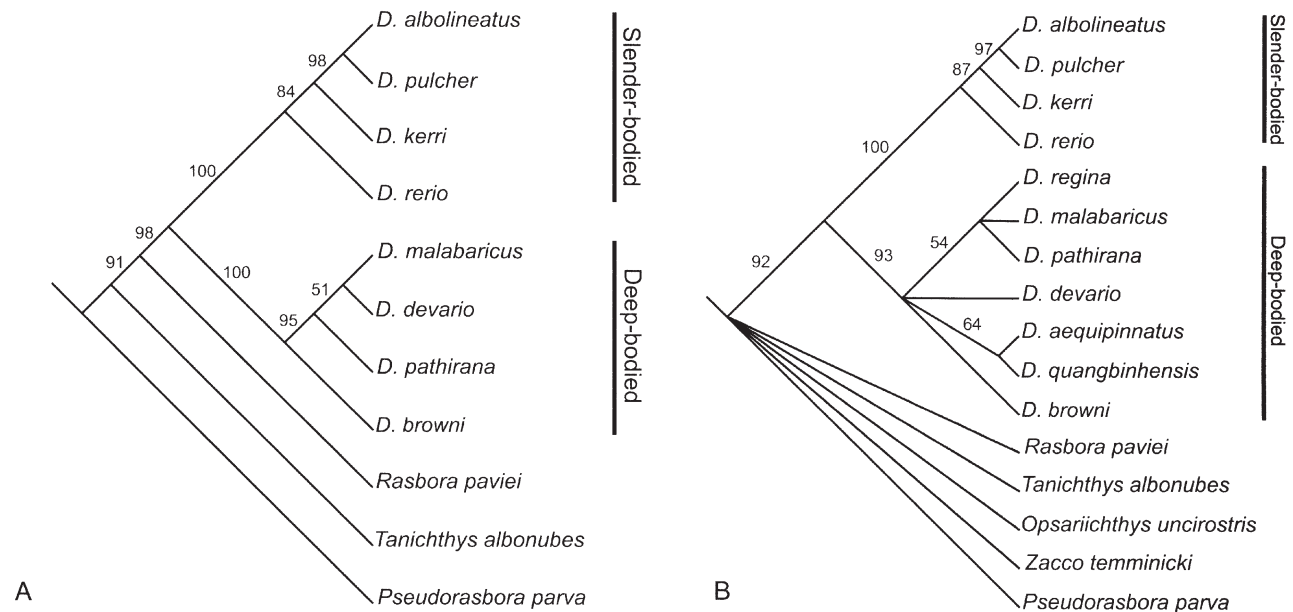


Figure 13. Comparison of phylogenies derived from morphological and combined data sets. A, Strict consensus for molecular data (581 steps; CI = 0.801); B, Strict consensus tree for combined morphological and molecular data sets (611 steps, CI = 0.861). Bootstrap values are given above each internode. Note that the relationships within the slender-bodied clade are identical in the two trees, but relationships within the deep-bodied clade differ in the two trees.

Table 4. DNA sequence data partitioned by gene

	Total base pairs	Variable base pairs	Informative base pairs
16S ribosomal gene	525	156	119
12S ribosomal gene	183	48	31
<i>Sonic hedgehog</i>			
Exon 1	190	10	1
Exon 2	175	22	12
<i>Hoxd 10</i>			
Exon 1	677	111	47
Exon 2	264	37	17
<i>Hedgehog</i>	175	49	19
Total	2189	433	246

from morphological data. The topology of the deep-bodied subclade derived from combined data is identical to that obtained when morphological data are analysed alone.

DISCUSSION

As described above, the consensus tree derived from combined molecular and morphological data retains elements of phylogenies generated by either molecular or morphological data alone. Within the deep-bodied clade, morphological data appear to determine the topological structure, identical in the morphological

and combined analyses. In the shallow-bodied clade, molecular data determine the topological structure, identical in the molecular and combined analysis. Variation in *Hoxd 10*, the 16S and 12S ribosomal genes are responsible for resolution within the slender-bodied clade (Meyer *et al.*, 1995; Zardoya *et al.*, 1996) whereas *Hedgehog* and *Sonic Hedgehog* give support only to the subclade as a whole (Zardoya *et al.*, 1996). Optimization of morphological characters on this combined tree adds two new morphological synapomorphies for *D. rerio*, *D. kerri* and *D. pulcher*. In these three species, the posterior supraneural is reduced to a thin slip of bone (character 5) and the lateral process of the first vertebra is reduced (character 11). PAUP* also interprets the presence of a saddle-like anterior supraneural (character 8) as a synapomorphy for all slender-bodied species except *D. rerio*. However, this optimization forces these three characters to revert to the primitive condition in *D. albolineatus*. Under the same optimization, two independent appearances of the medial ridge on the anterior supraneural is equally parsimonious as the interpretation of the medial ridges as a synapomorphy of *D. kerri* and *D. pulcher*.

Relationships within the deep-bodied subclade are less well resolved. While the molecular data strongly support the position of *D. browni* as the sister taxon to the group *D. devario*, *D. malabaricus* and *D. pathirana*, the relationship between *D. devario* and *D. malabaricus* is weakly supported (Fig. 13a). Relationships among *D. pathirana*, *D. devario* and *D.*

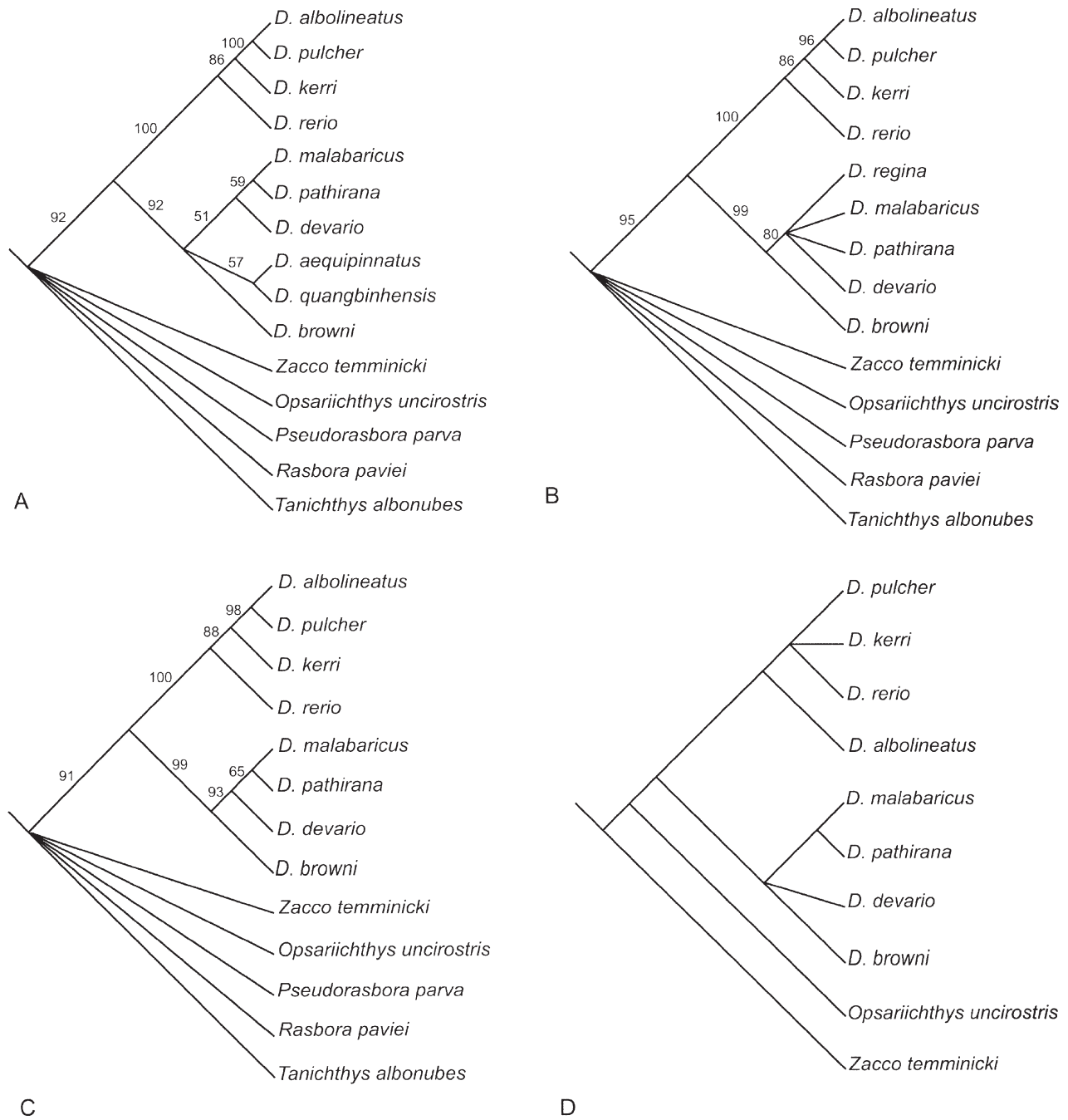


Figure 14. Consensus phylogenies resolved through the systematic deletion of three species. A, deletion of *D. regina* from the combined analysis (621 steps, CI = 0.900); B, deletion of *D. quangbinhensis* and *D. aequipinnatus* from the combined analysis (623 steps, CI = 0.843); C, deletion of *D. regina*, *D. quangbinhensis* and *D. aequipinnatus* from the combined analysis (620 steps; CI = 0.847); D, deletion of *D. regina*, *D. quangbinhensis* and *D. browni* from the morphological analysis (18 steps, CI = 0.921).

malabaricus in the molecular tree are only supported by *Sonic Hedgehog* (Fig. 13a); other genes and the morphological data support different resolutions within this group (Fig. 12; Meyer *et al.*, 1995; Zardoya *et al.*, 1996; McClure, 1998). While one might expect a

combined analysis to resolve this discrepancy, the combined analysis yields a topology identical to that of the morphological analysis. Though there is little resolution within this clade, morphological data places *D. malabaricus* closer to *D. pathirana* than to *D. devario*.

Lack of resolution in the deep-bodied clade may be attributed to interactions between incomplete data sets (Anderson, 2001; Wiens & Reeder, 1995; Wiens, 1998). Within this clade, DNA sequence data are not available for three taxa, *D. regina*, *D. aequipinnatus* and *D. quangbinhensis*. Using the phylogenetic trunk method outlined by Anderson (2001), we systematically removed each of these taxa from the analysis to test their effect on tree topology (Fig. 14). Removal of *D. quangbinhensis* or *D. aequipinnatus* from the analysis has no effect on tree topology. However, deletion of both these taxa simultaneously removes *D. browni* from the polytomy to a position of sister taxa to the remaining taxa (Fig. 18b). Deletion of *D. regina* adds resolution to the other taxa in the deep-bodied subclade by repositioning *D. devario* as sister taxon to *D. malabaricus* and *D. pathirana* (Fig. 14a). When we deleted all three taxa from our analysis, we obtained a fully resolved phylogeny, nearly identical to that generated by molecular data alone (Fig. 14c). In this case, *D. devario* is shown to be sister taxon to *D. malabaricus* and *D. pathirana*, and that *D. browni* is sister taxon to *D. devario*, *D. malabaricus* and *D. pathirana*. When we delete the same three taxa from the morphological analysis *D. malabaricus* remains the sister taxon to *D. pathirana*, not *D. devario* as resolved with molecular data. In the combined tree, morphological data support the close relationship between *D. malabaricus* and *D. pathirana* as well as between *D. aequipinnatus* and *D. quangbinhensis*. Further resolution within the deep-bodied clade awaits broader taxonomic sampling and the addition of gene sequence data for *D. regina*, *D. aequipinnatus* and *D. quangbinhensis*.

IMPLICATIONS FOR *DANIO* TAXONOMY AND RELATIONSHIPS

The taxonomy of *Danio* is problematic and in need of revision (see Fang, 1997; Kullander, 2001). Ten genera and subgenera, including *Brachydanio* Weber & de Beaufort (1916), have been synonymized with *Danio* (Barman, 1991; Eschmeyer, 1998; Fang, 2000b). Only one morphological synapomorphy, the 'danionin' notch in the dentary, has been previously suggested for *Danio* (Howes, 1979; Fang, 2000b; Kullander, 2001; but see Roberts, 1986). In the case of *Brachydanio* (*sensu* Talwar & Jhingran, 1991), no diagnostic characters have been described (Barman, 1991; Fang, 2000b), although the name continues to be used occasionally in the nonsystematic literature. As discussed above, and in other studies done concurrently (Kullander, 2001; Parichy & Johnson, 2001), there is ample support for the slender-bodied and deep-bodied clades. However, data are conflicting regarding the monophyly of *Danio*. Based on rather limited taxo-

nomic samples (8–11 species), all molecular studies (Meyer *et al.*, 1995; Parichy & Johnson, 2001) and our morphological study support the monophyly *Danio*. In contrast, in a recently completed morphological analysis of 13 *Danio* species plus eight closely related genera (Kullander, 2001) *Danio* was not monophyletic in the most-parsimonious tree. In the latter, several non-*Danio* genera (taxa not included in either our study or the molecular studies discussed above) were nested within *Danio*, some most closely related to the slender subclade and others allied with the deep-bodied species. Support for the relationship between these additional genera and the slender-bodied clade was based on a single character, while support for two other genera with the deep-bodied clade is stronger. Given the strong support in all studies for the deep- and slender-bodied clades, it may be appropriate to assign distinct generic names to these two subclades. Whether this is appropriate can only be determined by a far more comprehensive study of both additional taxa and additional characters.

CONCLUSION

Study of the axial skeleton of *Danio* reveals 14 phylogenetically informative characters at the species level. Morphological and molecular data, analysed both separately and together, support the monophyly of *Danio* as well as a basal division of the genus into slender-bodied and deep-bodied subclades. Morphological and molecular analyses resolve slightly different phylogenies within these subclades. The combined analysis retains the result given by molecular data for resolution of the slender-bodied clade and the result given by morphological data for the deep-bodied clade.

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