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Molecular evidence for the origins of Antarctic fishes: paraphyly of the Bovichtidae and no indication for the monophyly of the Notothenioidei (Teleostei)

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Abstract The notothenioids are an Antarctic suborder of perciform fishes to which increasing interest is being devoted. To investigate their origin, one must address two questions. First, are Bovichtidae (*Bovichtus*, *Cottoperca*, *Pseudaphritis*), the sister-group of the rest of the suborder, monophyletic ? Secondly, what is the sister-group of the Notothenioidei ? These questions were addressed by determining the complete nucleotide sequence of the D2 and D8 domains of 28S rDNA (759 sites, among which 158 informative for parsimony), for 6 notothenioids and a collection of 6 outgroup taxa including the Trachinoidei and Zoarcoidei. Different outgroups (or combinations of outgroups) and different weighting schemes support the inference that *Pseudaphritis* is closer to the rest of the Notothenioidei than *Cottoperca* and *Bovichtus* are. Relationships of *Cottoperca* and *Bovichtus* remain unclear with respect to outgroups. Our molecular data therefore clearly show that the Bovichtidae are paraphyletic, but their relationships are not those suggested by Balushkin in 1992. Our data provide no indication of the monophyly of the Notothenioidei in its classical sense. Most of the homoplasy is due to outgroup sequences and interrelationships of outgroups are unresolved. Some morphological synapomorphies shared by *Pseudaphritis* and the rest of the non-bovichtid Notothenioidei are proposed, including some that were identified by Voskoboynikova in 1993.

Introduction

Notothenioids comprise six families of Antarctic or sub-Antarctic fishes displaying considerable morphological and ecological diversity. This suborder dominates the fauna of the Antarctic shelf and peri-Antarctic shelves and banks. Most notothenioids are bottom fishes confined to waters less than 1,000 m deep and have the ability to live at subzero temperatures (Eastman 1993). This is one of the reasons why their physiology and biochemical adaptations are extensively studied. Many members of this suborder possess antifreeze proteins in their blood, and some lack haemoglobin. To understand how these peculiarities have evolved, a precise general phylogenetic picture of the whole group is necessary. The aim of this paper is to clarify the relationships of some of the members of this group and the interrelationships of the suborder with other taxa. Like many other taxa studied during the development of comparative biology, the suborder Notothenioidei has to pass a critical step from a loose definition (a diagnosis, which is a mixture of shared primitive characters and, possibly, shared derived characters) to an hennigian definition (recognized shared derived characters) (Nelson 1970, 1972, 1974; Lecointre 1994). In other words, we have to know if the taxa involved form a monophyletic group. This leads to three problems.

First, we do not know what notothenioids are, as Eastman (1993) stressed:

> There is not a unique osteological character, or any other known character for that matter, that distinguishes the suborder Notothenioidei. In the absence of such synapomorphic (shared derived) characters, the group is diagnosed by the following presumably unique combination of morphological characters... (then follows a list from Eakin, 1981). If suspected notothenioid fossils are eventually discovered, it will be difficult to recognize as a notothenioid any specimen not possessing the entire suite of characters.

As stressed by Patterson (1988) and Nelson (1989), taxa are defined by derived characters. Clearly, for notothenioids, currently used characters are a mixture of primitive characters and highly convergent charac-

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ters among Perciformes (Eakin 1981). Hastings (1993) selected three features unique to the Notothenioidei, but these characters are questionable and are discussed below.

Second, the definition of the Notothenioidei has to be related to its sister-group. There has always been much confusion in identifying the sister-group of the Notothenioidei, due to absence of knowledge of perciform phylogeny and absence of a definition of the Perciformes. This order, in the opinion of many authors, may be polyphyletic (Nelson 1994). To define the Notothenioidei and to study the phylogeny within the suborder, its sister-group must be identified. There are two likely candidates: Trachinoidei (Berg 1947; Bertin and Arambourg 1958; Gosline 1968; Pietsch 1989; Hastings 1993), and Zoarcoidei (Anderson 1984, 1990). The Blennioidei has also been suggested (Eastman 1993), but this may result from a misreading of Gosline (1968). Gosline (1968) included all notothenioid families within the Blennioidei, but this suborder also included zoarcoid and trachinoid families. Gosline (1968) clearly proposed the Trichonotidae, a trachinoid family, as the sister-group of the Notothenioidei. Later on, Gosline's Blennioidei was split into several parts (Springer and Freihofer 1976; but see also Springer 1993). The Pinguipedidae, a family included at least by Pietsch (1989) and Nelson (1994) within the Trachinoidei, has also been suggested as the possible notothenioid sister-group (Anderson 1990). Hastings (1993) used the Trichonotidae and the Pinguipedidae as notothenioid outgroups (and treated the Bovichtidae as a monophyletic family). All these previously suggested sister-groups, however, are based on highly convergent characters among Perciformes, a difficulty already stressed by Eakin (1981). Eastman (1993) correctly summarized the situation: 'Since a sister group of the suborder has not been identified, characters may be polarized relative to the Bovichtidae, and this family is then used as the "functional outgroup"'. This procedure was used but not discussed by morphologists (Iwami 1985), as well as molecular biologists (Bargelloni et al. 1994: Fig. 3). Consequently, neither the monophyly of the Notothenioidei nor that of the Bovichtidae has been seriously tested.

Thirdly, the earliest family of the notothenioid cladogram, the Bovichtidae (Regan 1914), has never been defined. Unlike most of the Notothenioidei, which are Antarctic, bovichtids are largely non-Antarctic, having a distribution that includes southern South America, southeastern Australia, New Zealand, and a few isolated islands of the Subtropical Convergence (Eastman 1993). Miller (1987) correlated this distribution both with phylogenetic and tectonic data, i.e. the earliest divergence of this family from the rest of the Notothenioidei (its phylogenetic position) and the separation of the New Zealand land mass from the Gondwana, some 75 million years ago, and later the Australian plate 56 million years ago. Once the isolation of the bovichtids was established, the family could have remained sub-Antarctic, while its sister-group, including common ancestors of the rest of the Notothenioidei, could have become Antarctic, as the cold conditions developed. However, Hastings (1993) noticed that the Bovichtidae apparently lack any known synapomorphy, and he concluded his analysis on notothenioid phylogeny with this assertion: "The monophyly of the Bovichtidae has not been corroborated". Nevertheless, he presented the Bovichtidae as a monophyletic family in his tree (Fig. 1). Balushkin (1992) proposed that this family could be paraphyletic, with *Pseudaphritis* as the sister-group of the rest of the Notothenioidei (Fig. 2). This point of view will be discussed below. On the basis of visceral skeleton anatomy, Voskoboynikova (1993) suggested that the most closely related bovichtid to the rest of the Notothenioidei could be *Pseudaphritis*, not *Cottoperca* or *Bovichtus*. The inclusion of *Cottoperca* within the Notothenioidei is problematic for two reasons. First, *Cottoperca* is in many ways morphologically closer to non-specialized perciforms like *Perca* (Voskoboynikova 1993), and thus has so many perciform primitive characters that it is very difficult to investigate its relationships. The second reason is the lack of clear synapomorphies of the Notothenioidei.

The difficulties in defining the Notothenioidei and Bovichtidae on the basis of morphology led us to address these questions using molecular data. Previous molecular phylogenies of the suborder Notothenioidei (Bargelloni et al. 1994; 12S and 16S mitochondrial genes) did not test the monophyly of the Bovichtidae, because only one representative of the Bovichtidae and one outgroup (zoarcoids) were sampled. In such a sample, notothenioids appear monophyletic but the addition of other bovichtids and outgroups may change the result. To test both the monophyly of the Bovichtidae and that of the suborder, the three bovichtid genera and more outgroups are needed. The variability of fish 28S rRNA (Lê et al. 1989; 1993; Lecointre et al. 1993; 1994) led us to carry out this investigation with the complete variable domains D2 and D8 (Hassouna et al. 1984). Miller (1987) suggested that the early evolution of notothenioids, the bovichtids in particular, must have been influenced by tectonic plate movements in the Weddellian Province during the Early Tertiary period. A review of the biogeographical and palaeogeographic data (Miller 1987; Eastman 1993) led to the assumption that cladogeneses within the Bovichtidae were probably associated with the fragmentation of Gondwana, 80–55 million years ago, a divergence time that we predicted to be compatible with the level of variability of these molecular domains. Nevertheless, we suspected possible limitations in resolving relationships between outgroups, because their cladogeneses have probably taken place in a very brief period at that time (88–55 million years ago, considering that most of the Perciformes appeared about 55 million years ago; Benton 1993).

Materials and methods

Species sampling

The three genera of the Bovichtidae (Gon and Heemstra 1990) were sampled: *Pseudaphritis urvillii*, *Cottoperca gobio* and *Bovichtus variegatus*. A fourth problematic genus, *Aurion* (Waite 1916), was not available. However, Hardy (1988) synonymized *Aurion effulgens* with *Bovichtus psychrolutes*. To investigate the origins of the Notothenioidei, we concentrated on outgroup sampling rather than the ingroup. The three bovichtids and three more-derived notothenioids represent the ingroup. Within non-bovichtid notothenioids, *Eleginops maclovinus* is the earliest branch (Balushkin 1992), *Dissostichus mawsoni* is a more derived nototheniid, and *Artedidraco loennbergii* represents a more derived taxon (family Artedidraconidae). The choice of these three taxa is based on the reliability of their presumed relative positions within the Notothenioidei, which are agreed by many authors (Eakin 1981; Iwami 1985; Eastman 1993; Hastings 1993). For outgroups, each of the two potential Notothenioidei sister-groups were sampled: *Austrolycus deprecisseps* and *Pholis gunnellus* for Zoarcoidei and *Trachinus draco* for Trachinoidei. Other suborders were sampled, including *Perca fluviatilis* (Percoidei), *Scomber scombrus* (Scombroidei), and *Labrus bergylta* and *Neolamprologus meeli* for the Labroidei. The sequence from a percomorph but not perciform fish, *Trigla lucerna* (Scorpaeniformes) was taken from Lê et al. (1993). Other percomorphs available in the database of Lê et al. (1993) had incomplete D2 and D8 domains and for this reason their sequences were not used. All other sequences were obtained in our laboratory (among which sequences from *Labrus bergylta*, *Neolamprologus meeli*, *Perca fluviatilis*, *Pholis gunnellus*, and *Scomber scombrus* were obtained by Sébastien Lavoué). Sequences from this paper are available from GenBank under accession numbers U87423 to U87448.

Dissections were performed on specimens from the Muséum National d'Histoire Naturelle MNHN 1895-0200 (*Pseudaphritis urvillii*), MNHN 1986-0185 (*Bovichtus variegatus*) and MNHN 1990-0866 (*Cottoperca gobio*).

Sequencing techniques

Most of the tissues were muscle-fixed in 70% ethanol, though some were stored at −80°C. Ethanol-fixed tissues were dried in a vacuum centrifuge before DNA extraction. Tissues were powdered in liquid nitrogen using a mortar and pestle, and then suspended in a CTAB solution at 60°C, following the method of Winnepenninckx et al. (1993). RNase (0.5 units) was added to the second aqueous phase, which was then incubated at 37°C for 30 min to remove RNA. Total genomic DNA was precipitated by the addition of two-thirds of the total volume of isopropanol and then stored at 4°C for 2 h or overnight, depending on the size of the pellet. After centrifugation, the pellet was washed following the method of Winnepenninckx et al. (1993), dried and resuspended in sterile ultrapure water. DNA concentration and quality were evaluated with a spectrophotometer. Polymerase catalyzed chain (PCR) reactions (Mullis and Faloona 1987; Saiki et al. 1988) were performed in a 50-µl volume using 0.3-µg of template DNA and 50 pmol of each of the two primers. Primer sequences are: C′1, 5′ACCCGCTGAATTTAAGCAT3′; D2, 5′TCCGTGTTTCAAGACGGG3′; C′72, 5′GTGCAGATCTTGGTGGTAGT3′; D8, 5′ATTCCCCTGGTCCGCACCAGTT3′. The PCR mix usually contained (final concentrations) 20 mM Tris-HCl, pH 8.55, 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 150 µg/ml BSA, 5% DMSO, 330 µM dNTP each, and 0.3 µl (1.5 units) of Taq polymerase (Bioprobe). Temperature cycles were performed using a Biometra trioblock. Thermal cycling was denaturation 94°C 4 min, annealing temperature (AT) 2 min, extension 72°C 2 min, then 29 × (94°C 1 min, AT 1 min, 72°C 1 min), 72°C 4 min, pause at 20°C. The D2 domain was amplified with the primers C′1 and D2 (AT at 55 to 60°C depending on the species). The D8 domain was amplified with the primers C′72 and D8 (AT also between 55 and 60°C depending on the species). PCR products, which were always opened in a separate room under a special hood, were checked by electrophoresis in 1% agarose-BET and TBE buffer (Sambrook et al. 1989), and visualized with the molecular weight marker VI of Boehringer Mannheim. PCR products were cloned in the phagemidic PCR-script TM SK(+) vector using the PCR-script TM SK (+) cloning kit (Stratagene) following the procedure recommended by the manufacturer. This kit has a unique SrfI site in the MCS of the vector. The ligation is performed in the presence of SrfI and ligase; SrfI reopens religated vectors, and then maintains a high steady-state concentration of opened vector DNA, consequently increasing the ligation efficiency. A classical white/blue selection (Sambrook et al. 1989) was used for screening recombinant clones. Four white colonies per cloning were picked and grown overnight in L-broth at 37°C. The phagemidic DNA was then extracted (Sambrook et al. 1989). For each colony, the size of the insert was checked by digestion of the recombinant phagemidic DNA with BssHII and electrophoresed in 1% agarose gel (as described above). Sequencing on microplates was performed with the T7 sequencing kit from Pharmacia, using the method of terminator dideoxynucleotides (Sanger et al. 1977). Each colony was sequenced with external vector primers KS and T3, and at least two colonies per cloning were sequenced. To get the complete variable domains D2 and D8 of the 28S gene, two internal primers were used for sequencing, C′2: 5′GAAAAGAACTTTGRARAGAGAGT3′ and C′8: 5′AACTTCGG GATAAGGATTGGCTC3′; respectively.

Data analysis

Sequences were read and entered twice using the computer package MUST (Philippe 1993), and aligned using the facilities of ED, within MUST. Insertions and deletions were analysed as such (one indel counting as one character whatever indels in the neighbouring sites). Indels were also recoded to count contiguous indels as a single event, using the technique of Barriel (1994). Both techniques yielded the same results. Analyses were carried out when deleting from the data-specific positions involving question marks (see the end of the Appendix), to check their impact. Deleting these positions did not change the results. Relative transitional saturation was examined using the COMP-MAT program of MUST, by plotting pairwise transitional differences against pairwise transversional differences. MUST includes the Neighbor-Joining method (Saitou and Nei 1987) and allows very fast bootstrap analyses with this tree-construction method using the NJBOOT program. These methods were used to check identity of results between NJ and parsimony methods, and no topological differences were detected. Bootstrap proportions from NJBOOT are generally slightly higher than those obtained from a bootstrap-parsimony approach (data not shown). MUST generates an output file in the NEXUS format of PAUP. The main phylogenetic analyses were performed with PAUP 3.1.1 (Swofford 1993). Characters were unordered. Heuristic searches were performed with the whole species set (14 species) with various weighting schemes (transversions/transitions = 1, 2, 5, 10, no transitions). In this case, all non-notothenioid species were a priori declared as outgroups. To reduce computing time, Branch and Bound was used only when no weighting schemes were applied. Exhaustive searches were performed when a single outgroup species was used. This was done with each outgroup. Bootstrap analyses (Felsenstein 1985) were performed with PAUP, using heuristic searches and 1,000 iterations, for TV/TS = 1 and TV/TS = 2.

Absolute saturation tests were performed using COMP-MAT of MUST and PAUP. Pairwise number of differences were plotted against pairwise number of inferred substitutions in the most parsimonious tree, for transitions and transversions separately. This allowed a check on absolute transversional saturation that would not have been detected by the relative saturation test de-

scribed above. To perform this, the most parsimonious tree from PAUP is saved with its branch lengths. This tree is recognized by the AF_PAUP3 program of MUST, which generates the corresponding patristic distance matrix that can be compared to a percent difference matrix using COMP-MAT. This was done using, in PAUP, ACCTRAN and DELTRAN optimizations in order to check the impact of optimization on the plot. This impact has a null or negligible impact on the correlation of the two matrices.

Results

The complete D2 domain has a size of about 440 base pairs (bp); the D8 has about 350 bp. The alignment provided 759 sites among which 158 were informative for parsimony (see Appendix). On the complete D2 and D8 domains of the 14 species, no, or very little, transitional saturation was detected. The correlation coefficient calculated from plotting pairwise transversional differences against pairwise transitional differences was 0.81. The correlation coefficient between pairwise number of differences and pairwise number of inferred substitutions was 0.71 for transitions only and 0.88 for transversions only (graphical plots are available on request: they all show a linear relationship). No significant saturation was therefore detected, and thus no weighting scheme is absolutely necessary.

A Branch and Bound search on the 14 species yielded six most-parsimonious trees (strict consensus shown Fig. 3), each having 664 steps, a consistency index (C.I.) of 0.681 and retention index (R.I.) of 0.448. Differences between the six trees concerned only interrelationships between outgroups and the position of *Cottoperca*. *Pseudaphritis* was always found to be the sister-group of the rest of the Notothenioidei, excluding *Cottoperca* and *Bovichtus*. *Bovichtus* was always the sister-group of node A. However, *Cottoperca* must be included within the outgroup multifurcation: notothenioids were therefore not found to be monophyletic. When a higher weight was given to transversions (TV/TS = 2), the two most parsimonious trees were the same concerning relationships within node A, but different concerning outgroups. *Bovichtus* was clustered within the outgroup multifurcation. Giving higher weights to transversions (5, 10, etc.) changed the outgroups-*Cottoperca*-*Bovichtus* interrelationships but did not change anything within the rest of the notothenioids (nodes A, B, C). However, whatever the weighting scheme, the robustness of the nodes must be considered.

Two ways to consider the reliability of the nodes are branch length (number of changes present at a node) and bootstrap proportion. These two criteria are not equivalent, as stressed by Darlu Pierre Darlu (unpublished work). When no weighting scheme was used (but also when TV/TS = 2), the position of *Pseudaphritis* was supported by a long branch length. In one of the four equiparsimonious trees (the one found by the heuristic search, Fig. 4), the length of branch A was 14 to 23 changes according to the optimization chosen, 19 under ACCTRAN, 4 of them being characters that change only once (unreversed synapomorphies). This tree is shown to illustrate branch lengths. The length was of the same range whatever the 664-step tree among the 6: branch A, uniting *Pseudaphritis* to the rest of the non-bovichtid notothenioids, was always one of the longest internal branches (13–23 steps). Positions of *Eleginops*, *Artedidraco* and *Dissostichus* were also supported by similar values: 12–33 steps for node B and 13–28 steps for node C. Bootstrap proportions are given in

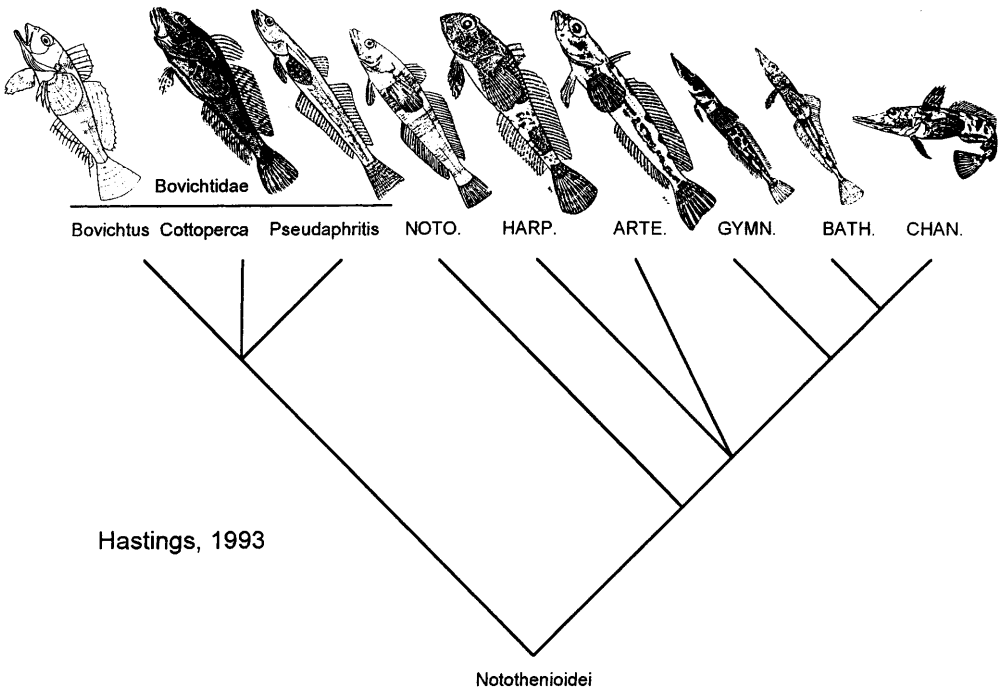

Fig. 1 Cladogram of the notothenioid families proposed by Hastings (1993). The Bovichtidae are presented as monophyletic, although no synapomorphies were proposed. *NOTO*. Nototheniidae, *HARP*. Harpagiferidae, *ARTE*. Artedidraconidae, *GYMN*. Gymnodraconinae, *BATH*. Bathydraconinae, *CHAN*. Channichthyidae. Fish illustrations taken from Gon and Heemstra (1990), Miller (1993) and Eastman (1993)

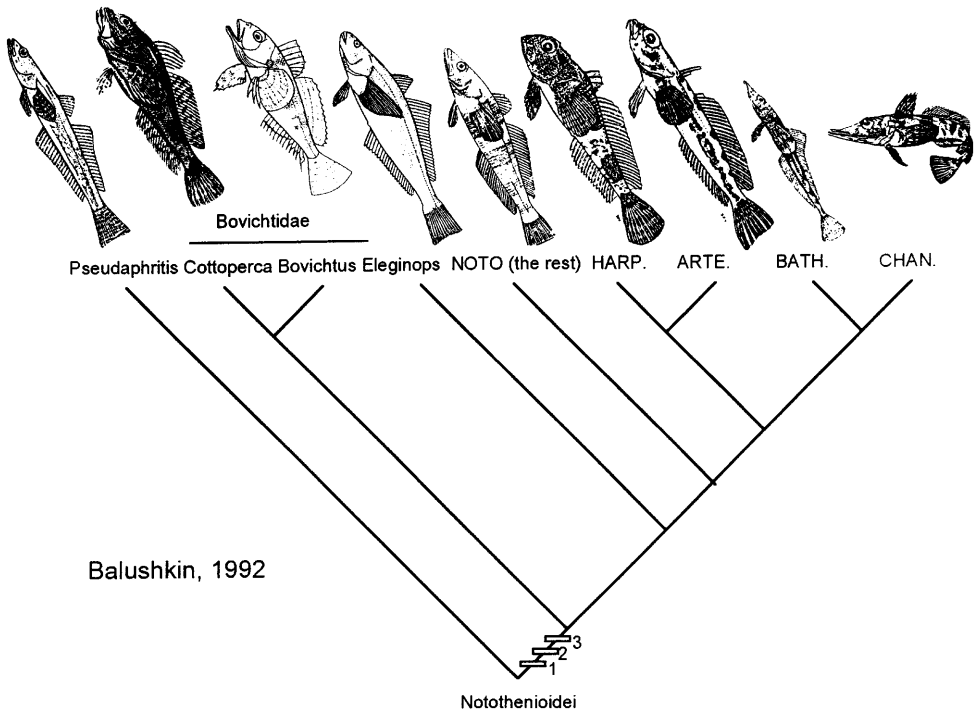

Fig. 2 Cladogram of the notothenioids proposed by Balushkin (1992). *Pseudaphritis* is excluded from the Bovichtidae, and classified in a separate family, the Pseudaphritidae. The Bovichtidae therefore contains only *Cottoperca* and *Bovichtus*. *Eleginops* is classified in a separate family, the Eleginopsidae. See Fig. 1 for abbreviations. Synapomorphies defined by Balushkin for the clade uniting new Bovichtidae and the rest: (1) absence of predorsal bone, (2) absence of teeth on the ectopterygoid, (3) absence of spinous rays in anal fin. They are discussed in the text. Fish illustrations taken from Gon and Heemstra (1990), Miller (1993) and Eastman (1993)

Fig. 3. It should be stressed here that we consider reliable a node whose bootstrap proportion is 70% or higher, as independently shown by Zharkikh and Li (1992), Hillis and Bull (1993) and Lecointre et al. (1994). Relationships of *Bovichtus*and *Cottoperca* were unresolved (bootstrap proportions respectively of 51% and less than 50%), while those of *Pseudaphritis*, *Eleginops*, *Artedidraco* and *Dissostichus* were robust, with bootstrap proportions above 70%, and even above 95%. Relationships of these four taxa are reliable through the two robustness criteria, while relationships within outgroups are not supported by the bootstrap test, in spite of some long internal branches (for example, the branch uniting *Perca*, labroids and zoarcoids).

In the search for the most parsimonious tree, constraining monophyletic notothenioids requires no more steps than in the present most parsimonious trees since several of the 6 equiparsimonious trees show monophyletic notothenioids, while constraining monophyletic bovichtids requires 681 steps (17 extra steps). Exhaustive searches and bootstrap analyses were also performed using a single outgroup, sequentially for each outgroup. The g1 statistic, the CI and the RI of the most parsimonious trees (each constantly showing clades A, B, C) are given in Table 1. Bootstrap proportions of nodes A, B, and C (Fig. 3) are also given in each case. One can note in Table 1 that CIs and RIs are much higher when a single outgroup is taken. This shows that most of the homoplasy contained in this data set is due to outgroup interrelationships. This is also clear with the strict consensus tree and the bootstrap proportions obtained, which are always less than 50% for outgroup nodes (Fig. 3). Table 1 clearly shows that the position of *Pseudaphritis* was well supported and did not depend on the outgroup, while positions of *Bovichtus* and *Cottoperca* were poorly supported and slightly sensitive to outgroup sampling. Our structured data reliably lead to the conclusion that *Pseudaphritis* is more closely related to the rest of the Notothenioidei than are *Cottoperca* or *Bovichtus*. Moreover, our data clearly show (Figs. 3, 4) that the Nototheniidae is a paraphyletic family, *Eleginops* being the sister-group of the clade *Dissostichus* (another nototheniid) + *Artedidraco*, as already suggested by Balushkin (Fig. 2).

In summary, whatever the options chosen (recoding indels or not, under various weighting schemes, etc.), relationships between outgroup taxa remain unstable with low bootstrap values, while nodes A, B, and C are always robust whatever the parameters, and congruent with the results of Bargelloni et al. (1994). We have shown that most of the homoplasy in these data is contained in the outgroup sequences.

Discussion

Are the Notothenioidei monophyletic ?

Eakin (1981) listed five possible synapomorphies for the notothenioids, but recognized for most of them several appearances in other perciforms. Hastings (1993) retained only three synapomorphies for the clade Notothenioidei: (1) posterior pleural ribs floating, (2) presence of a nasal accessory organ, and (3) three

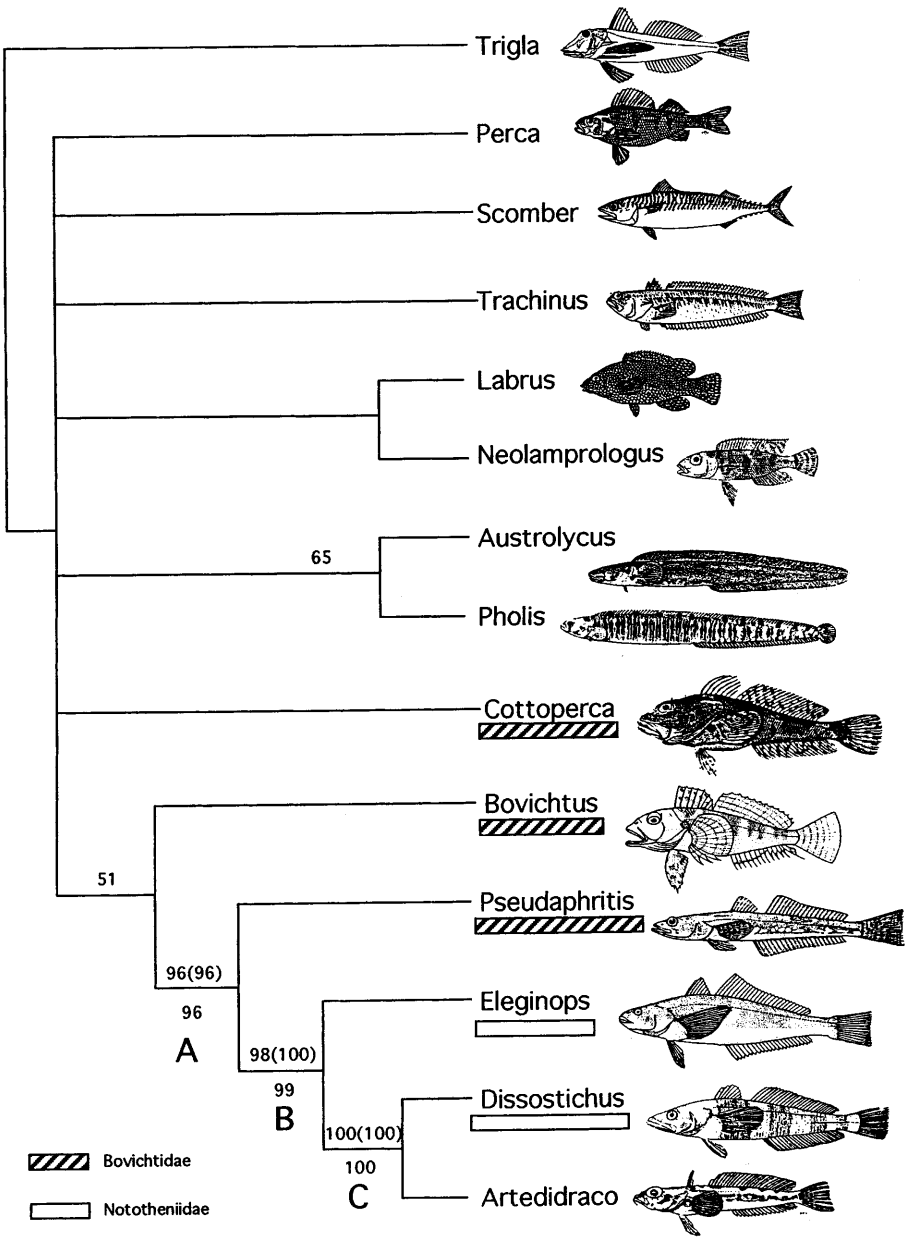

Fig. 3 Strict consensus tree of the six equiparsimonious trees obtained from a branch and bound search (PAUP 3.1.1) on the 759 sites of the complete D2 and D8 domains of the 28S rDNA. Each tree has a length of 664 steps, C.I. of 0.681 and R.I. of 0.448. *Numbers above branches* refer to the bootstrap proportion obtained with heuristic searches, 1,000 replicates, from two weighting options TV/TS = 1 (TV/TS = 2). Absence of bootstrap proportion means that the corresponding node was absent in the corresponding bootstrap-consensus tree or had a bootstrap proportion inferior to 50%. The *number below branches* refers to bootstrap proportions found when the 5′ region incomplete for *Trigla* and *Perca* was deleted from the analysis (region 1–254 deleted, see Appendix 1). Nodes A, B and C were always found whatever the above options or whatever the outgroup chosen, with bootstrap proportions indicated in Table 1. Fish illustrations taken from Whitehead et al. (1984), Gon and Heemstra (1990), Miller (1993) and Eastman (1993)

platelike pectoral radials, the uppermost free one being homologous to radial number 2 of the other Perciformes (which have four rodlike pectoral radials). The first character is of doubtful value because, as stressed by Eakin, it may be correlated with a sluggish, benthic existence, since it is also found in cottids, platycephaliforms, uranoscopoids (see Pietsch 1989: 281) and gobiesocids. The second character is uncertain for the moment because it has not yet been surveyed in taxa with a single nostril (e.g. the Zoarcoidei). The third character is the most interesting because it is less likely to be convergent in other Perciformes. Eakin (1981) reported that callionymids, *Melanostigma*, and *Scorpaenichthys* have three pectoral radials. However, none of these, except the callionymids, shows the arrangement of flat, platelike radials seen in notothenioids. This precise arrangement is not found in the two best sister-group candidates, the Trachinoidei (which all have four pectoral radials; Pietsch 1989) and the Zoarcoidei [which have four pectoral radials (see Arnulf et al. 1987; Anderson 1982) except *Melanostigma* (Yarberry 1965)]. Moreover, polarization of this character is supported by ontogenetic data. Hastings (1993) reported that Andriyashev observed a first (upper) pectoral radial in juveniles of the Bovichtidae, a bone absent in adult bovichtids. The presence of four free radials has also been shown in larvae of some nototheniids and bathydraconids (Voskoboynikova and Tereshchuk 1991; Voskoboynikova et al. 1994), whereas the free first radial is not present in adults. The first (upper) radial becomes fused with the scapula during growth in bovichtids, an ontogenetic sequence

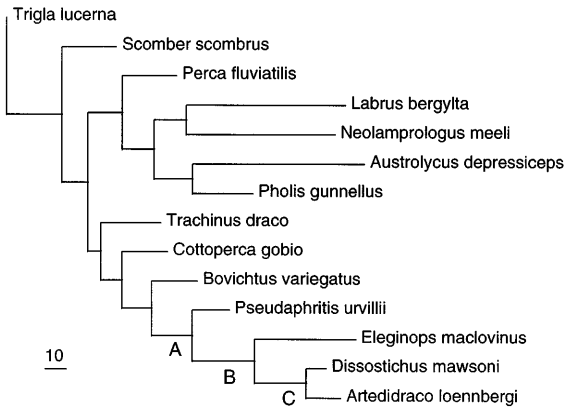

Fig. 4 The most parsimonious tree obtained by the heuristic search of PAUP 3.1.1., under ACCTRAN optimization, shown here for branch lengths (it corresponds to 1 of the 6 equiparsimonious trees obtained through the branch and bound procedure); 664 steps, C.I. = 0.681, R.I. = 0.448

that may reflect the phylogenetic sequence (Hastings 1993). In summary, one must admit that morphological evidence for the monophyly of the suborder is rather weak, since it rests on a single synapomorphy. What about the present molecular data ?

Under the bootstrap test, our data do not support the monophyly of the Notothenioidei. There are two possible non-exclusive hypotheses to explain this. First, Notothenioidei are not monophyletic. Secondly, Notothenioidei may be monophyletic, but the early cladogeneses of the group (leading to *Cottoperca*, *Bovichtus*) were simultaneous with the explosive perciform diversification, explaining the absence of resolution of outgroup nodes.

The first hypothesis would not be surprising given the lack of unambiguous morphological synapomorphies for the diagnosis of the suborder (Eastman 1993). But the results presented in this paper do not clearly support this conclusion either. A robust demonstration of the paraphyly of this suborder would have occurred if one of its members (here *Cottoperca* or *Bovichtus*) was the sister-group of one of the outgroups with good statistical support. But this is not the case: the positions of *Cottoperca* and *Bovichtus* are unreliable. We therefore cannot conclude that the suborder is paraphyletic.

The second hypothesis is more complex. Disregarding whether the Notothenioidei are monophyletic or not, and whatever the relationships of *Cottoperca* and *Bovichtus*, it is clear that the emergence of the earliest notothenioid lineages dates back to the time of perciform diversification. Given this, two interpretations are possible.

First, one possibility is that the observed lack of resolution within outgroups corresponds to an explosive radiation of perciforms (multiple cladogeneses in a short time span). This is corroborated by the fossil record (Benton 1993), which shows that the great majority of perciform families are known from the Lower Eocene (Ypresian/Lutetian) period, between 55 and 45 million years ago, except two *incertae sedis* Perciformes known from the Upper Cretaceous, and the family Apogonidae, also known from the Coniacian and the Campanian. Some isolated perciform families (Menidae, Gempylidae, Scombridae) are known from the Danian (65 million years ago). Simultaneous appearances in the fossil record of more than 60 perciform families means that it will probably be difficult to investigate their relationships, because only a very short time span was available for putative common ancestors to accumulate molecular synapomorphies. This problem has been discussed elsewhere (Lê et al. 1993; Philippe et al. 1994). Here, the result would be an insufficient number of positions discriminating clades within the outgroup taxa, homoplasy apart.

A second possibility is that this absence of resolution within outgroups corresponds to mutational saturation of the molecular domains under investigation. But this possibility can be reasonably rejected: although ho-

Table 1 Bootstrap proportions (BP) obtained for nodes A (third column), B (fourth column), and C (fifth column) of Fig. 3, when a single outgroup was used. The outgroup is indicated in the first column. Exhaustive searches were performed on each data set of seven species, and always gave the same topology except the branching points of *Cottoperca* and *Bovichtus*, indicated in the second column. The C.I. (sixth column), R.I. (seventh column), and the g1 statistic (eighth column) are given in each case, showing that our tree-lengths distribution is left-skewed, and therefore that our data were significantly structured (Hillis 1991; Huelsenbeck 1991; Hillis and Huelsenbeck 1992). Relationships of *Pseudaphritis*, *Eleginops*, *Dissostichus* and *Artedidraco* did not depend on the outgroup, while those of *Cottoperca* and *Bovichtus* did (they were different in two cases, with *Labrus* or *Trigla* as the outgroup), i.e. *Pseudaphritis* was always found to be the closest taxon to the rest of the non-bovichtid notothenioids. Various combinations of outgroups were also tried (not shown), which gave the same conclusion

Outgroup	*Bovichtus*/*Cottoperca*/The rest	BP of node A	BP of node B	BP of node C	Tree length	C.I.	R.I.	g1
Trigla	B (C, rest): 85%	99%	99%	100%	249	0.859	0.682	−1,015
Perca	C (B, rest): 84%	99%	97%	100%	256	0.855	0.670	−0,92
Scomber	C (B, rest): 57%	96%	99%	100%	252	0.845	0.658	−1,058
Labrus	Unresolved	97%	99%	100%	309	0.832	0.584	−0,956
Neolamprologus	C (B, rest): 63%	87%	97%	99%	309	0.828	0.562	−0,914
Austrolycus	C (B, rest): 63%	93%	97%	100%	323	0.864	0.621	−0,986
Pholis	C (B, rest): 63%	96%	99%	100%	271	0.849	0.631	−0,973
Trachinus	C (B, rest): 67%	99%	99%	100%	255	0.875	0.704	−0,985

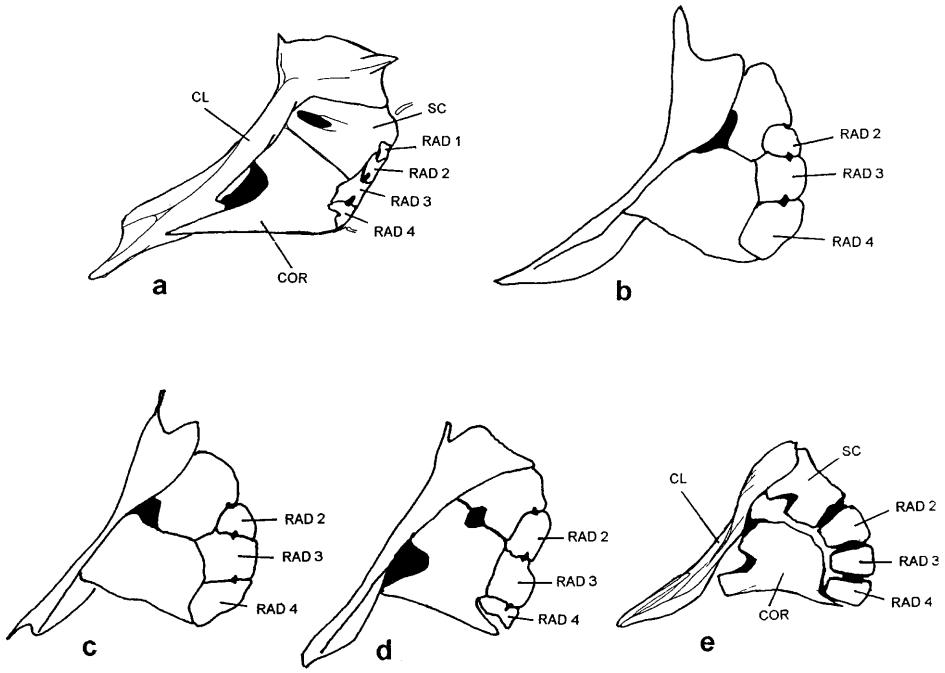

Fig. 5a–e Pectoral girdles of one trachinoid and four notothenioids. **a** *Ichthyscopus insperatus* (Trachinoidei, Uranoscopidae), after Pietsch (1989); **b** *Bovichtus variegatus* (Notothenioidei, Bovichtidae, MNHN 1986-0185); **c** *Cottoperca gobio*(MNHN 1990-0866); **d** *Pseudaphritis urvillii* (MNHN 1895-0200); **e** *Champsocephalus gunnari* (Notothenioidei, Channichthyidae), after Iwami (1985) *CL* cleithrum; *COR* coracoid; *SC* scapula; *RAD 1–4* radial number 1–4. In **b**, **c**, **d**, and **e**, RAD1 is fused with the scapula. In **d** and **e**, RAD2 has a connection both with the scapula and the coracoid

moplasy is present, neither important absolute transitional saturation nor absolute transversional saturation were detected in the data set. The domains D2 and D8 of the 28S rDNA have the appropriate evolutionary rate to investigate the relationships within notothenioids (see Table 1 when only one outgroup is chosen), but limitations appear in the search for sister-groups of the notothenioids and the relationships of the bovichtids: it is clear that most of the homoplasy contained in the present data concerns outgroup taxa. It is possible that outgroups' interrelationships are the limit of the phylogenetic resolution offered by the D2 and D8 domains, but this limit is not enough exceeded to detect saturation.

The absence of resolution within outgroups may due to a perciform "explosive radiation", i.e. a lack of positions informative for outgroup interrelationships, which is supported by the fossil record. But our data did not demonstrate this: explosive radiation or not, the variable domains of the 28S gene studied here may have also reached their limit of phylogenetic resolution, even without mutational saturation. These two possibilities are not incompatible with each other. Notothenioids may or may not be monophyletic; lack of resolution between outgroups may not allow this to be answered precisely. In addition, our data clearly indicate that the times of emergence of *Cottoperca* and *Bovichtus* are close to those of perciform suborders.

What is wrong with bovichtid morphological characters ?

There are three opinions about relationships within bovichtids. First, the family is generally considered as a clade, but no hennigian definition (no synapomorphy) has been proposed (Hastings 1993). Secondly, Balushkin (1992) proposed that this family could be paraphyletic (Fig. 2), with *Pseudaphritis* as the sister-group of the rest of the notothenioids. This point of view is based on three morphological characters that will be discussed below. Thirdly, Voskoboynikova (1993) considers *Pseudaphritis* as more closely related to the rest of the Notothenioidei than are *Cottoperca* and *Bovichtus*. This is discussed in the next section.

Balushkin (1992) based his argument on three characters:

Character 1, Predorsal bone present in *Pseudaphritis* (plesiomorph state), absent in *Cottoperca*, *Bovichtus* and the rest of the Notothenioidei (apomorph state);
Character 2, Teeth present on the ectopterygoid in *Pseudaphritis*, absent in *Cottoperca*, *Bovichtus* and the rest of the Notothenioidei ;
Character 3, Spinous rays present in anal fin in *Pseudaphritis*, absent in *Cottoperca*, *Bovichtus* and the rest of the Notothenioidei.

Unfortunately, Balushkin did not sufficiently describe the state of these characters in the two best outgroup candidates to the Notothenioidei, the Trachiniodei and the Zoarcoidei. The state of these characters in the outgroup is very important to determine correctly their polarity within notothenioids. We show here that Balushkin's characters were not correctly polarized.

Character 1

Predorsal bones are pterygiophores anterior to the dorsal fin that have lost their associated spines or rays

(Pietsch 1989). Balushkin (1992) shows a predorsal bone in *Pseudaphritis* and no predorsal in *Bovichtus*. He argues that the "position of the predorsal bone and the first support of ID in this species is similar to that in *Embolichthys mitsukurii* from the family Ammodytidae...", suggesting in this way that this could represent the plesiomorphic state for notothenioids. But it is clear that the presence or absence of predorsal bones, and their number and positions are highly variable among trachinoid families, as shown by Pietsch (1989: 256–257). It is therefore very difficult to decide what is the plesiomorphic state for notothenioids. Within zoarcoids, the character is also variable. Many taxa have no predorsal bone, like *Melanostigma* (Yarberry 1965), *Thermarces cerberus*, and *Pachycara thermophilum* (personal observation). Anderson (1982) recorded in *Gymnelus viridis* an isolated bone interpreted as "anomalous fused first and second pterygiophores" difficult to homologize with predorsals.

Character 2

Supposing that absence of teeth on the ectopterygoid is apomorphic within notothenioids implies that their presence is plesiomorphic, and therefore found in the outgroup(s). This is not the case. Zoarcoids we have investigated do not show such teeth. For instance, the Zoarcidae *Maynea*, *Eucryphycus* (Anderson 1988a), *Plesienchelys* (Anderson 1988b), *Melanostigma* (Yarberry 1965), *Thermarces* (Arnulf et al. 1987), and *Derepodichthys* (Anderson and Hubbs 1981) all have no teeth on the ectopterygoid. It is the same in the Anarrhichadidae (Le Cabellec et al. 1978). There is no need to investigate all the zoarcoid families: in the best case, the character is variable within the Zoarcoidei, and in the worse case, none of the Zoarcoidei have teeth on the ectopterygoid. Absence of these teeth in just one or two zoarcoid families is sufficient to invalidate the plesiomorphic interpretation of this character state within Notothenioidei. It is exactly the same for the trachinoid suborder. Pietsch (1989) recorded no teeth on the ectopterygoid of uranoscopids.

Character 3

No spinous rays are found in the anal fin of most of the zoarcoids, except in the Zaproridae and Stichaeidae. Polarization is, at best, ambiguous. Concerning the Trachinoidei, the work of Pietsch (1989: 256–257) clearly shows that the presence and the number of spinous rays in the anal fin are highly variable between and within families.

Other characters supporting the present position of *Pseudaphritis*

Voskoboynikova (1993) suggested that *Pseudaphritis* "differed significantly" from *Cottoperca* and *Bovichtus* in having: (1) the ventral process of the hyomandibula shortened; (2) the loss of the connection between the ectopterygoid and the metapterygoid (see also Voskoboynikova 1982); (3) the reduction in size of the ectopterygoid and the mesopterygoid; (4) a decrease in number of branchiostegal rays; and (5) appearance in the opercular bones of connective tissues. These features are not autapomorphies of *Pseudaphritis*. Indeed, Voskoboynikova (1993) considered them as "basic trends in the evolution of the Notothenioidei" and they are clearly found in more derived notothenioid taxa. In other words, these features are not found in *Cottoperca* nor in *Bovichtus*, but they are found in *Pseudaphritis* and the rest of the notothenioids (for characters 2 and 3 see also Voskoboynikova 1982). Voskoboynikova therefore admitted the present position of *Pseudaphritis* (although using a non-cladist terminology), and confirmed her acceptance of this point of view (personal communication). Here we must stress that such an interpretation of "trends" depends on the states of these characters found within potential outgroups. Voskoboynikova (1993) explicitly listed the outgroups on which her interpretation was based: Percidae, Serranidae, Mugiloididae (= Pinguipedidae), Trachinidae, Uranoscopidae, a collection allowing a reliable comparison.

Reduction in number of branchiostegal rays is an ambiguous character in the sense that *Pseudaphritis* has six like most of the notothenioids (*Cottoperca* have seven like many perciforms) but some outgroups like *Parapercis* and the Trachinoidei (Pietsch 1989) also have six and some more derived notothenioids like *Dissostichus* and *Aethotaxis* have seven (Voskoboynikova 1993). This character will be left apart for the moment.

The position of the second platelike radial might be informative. Trachinoids have four pectoral radials (Pietsch 1989). The first and second are in connection only with the scapula (therefore plesiomorphic state for notothenioids, Fig. 5a). The situation is the same in the zoarcoids we have seen (see for instance Anderson 1982; Arnulf et al. 1987), although the number of pectoral radials is unknown in some zoarcid subfamilies (Arnulf et al. 1987), and is probably three in *Derepodichthys alepidotus* (Anderson and Hubbs 1981). In the non-bovichtid notothenioids, the second pectoral radial is connected to both the scapula and the coracoid (Eakin 1981; Iwami 1985), an apomorphic state within notothenioids (Fig. 5e). In adult bovichtids, the first pectoral radial has fused with the scapula (Fig. 5b, c, d), and the ontogenetic sequence cited above allows us to homologize the second radial of the Trachinoidei with the second radial (first free radial) of the notothenioids. Iwami (1985) observed that in *Bovichtus* the second (upper) platelike radial was only in connection with the scapula (Fig. 5b, plesiomorphic state) while the case was ambiguous in *Pseudaphritis* (Iwami 1985). We have dissected the pectoral girdle of three bovichtids: *Pseudaphritis urvillii*, *Bovichtus variegatus*, and *Cottoperca gobio*, and have confirmed the connections of

the second radial as described by Iwami (1985) in *Bovichtus variegatus* (Fig. 5b). In *Cottoperca gobio*, the second radial is also only connected with the scapula (Fig. 5c), but in *Pseudaphritis urvillii*, the second radial is extended further downwards and also has a short contact with the upper angle of the coracoid (Fig. 5d). Consequently, this double connection of the second pectoral radial can be proposed as an apomorphy shared by *Pseudaphritis* and other non-bovichtid notothenioids.

We noticed that the characters listed by Eakin (1981), concerning bovichtids, were based on the observation of *Bovichtus* and *Cottoperca*, but not *Pseudaphritis*. We examined in *Pseudaphritis* the state of each character listed as different between bovichtids and other notothenioids. The aim was to find some characters in which the state in *Pseudaphritis* was different from those in the two other bovichtids, but were similar to the rest of the notothenioids. Like *Cottoperca* and *Bovichtus*, *Pseudaphritis* has a single lateral line, last anal fin ray divided to the base, gill membranes separate and free from ithmus, palatine and vomerine teeth. These characters are symplesiomorphies because they are also found in various outgroups. Only one character retained our attention: the last second dorsal fin ray is not divided to the base in *Pseudaphritis*. This is also found in other notothenioids, but not in *Cottoperca*, *Bovichtus* and the outgroups. This character could also be another apomorphy of node A (Fig. 3).

G. di Prisco (personal communication) corroborated the findings presented here by an electrophoretic comparison of the haemoglobin components of *Pseudaphritis* with those of *Cottoperca* and more derived notothenioids. While *Cottoperca* and *Bovichtus* have two electrophoretic components (Hb1 and Hb2) in equal amounts like other perciforms, *Pseudaphritis* has only one (Hb1) accounting for 95% of the total as in other notothenioids (R. D'Avino, M. Romano, M. Carratore and G. di Prisco personal communication; see also di Prisco et al. 1991).

Conclusion

Our molecular data support the paraphyly of the Bovichtidae, with *Pseudaphritis* as the sister-group of the rest of the Notothenioidei excluding *Cottoperca* and *Bovichtus*, and yield no indication for the monophyly of the Notothenioidei. Currently the sister-group of *Cottoperca* is not known. Clearly, the synapomorphies of Balushkin (1992) were not correctly polarized. Synapomorphies of the new clade *Pseudaphritis* + the rest of the non-bovichtid notothenioids could be: (1) the second (the uppermost free) pectoral radial in connection both with the scapula and the coracoid (the first being fused with the coracoid); (2) last second dorsal fin ray divided to the base; (3) the ventral process of the hyomandibula shortened; (4) the loss of the connection between the ectopterygoid and the metapterygoid; (5) appearance in the opercular bones of connective tissues and; (6) major Hb1 component in the haemoglobin, loss of all haemoglobins being a more derived state. In the future, if *Bovichtus* and *Cottoperca* can be shown to be the sister-group of one of the outgroups with significant supporting data, then these apomorphies could provide a new definition of the Notothenioidei. For the moment, the definition of the Notothenioidei in its classical sense seems to lie on a single synapomorphy, i.e. the presence of three platelike pectoral radials [the first (upper) one being fused with the coracoid], admitting a convergence in the Callionymidae.

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Appendix 1 (Part I-IV)

	1	1111111112	2222222223	3333333334	4444444445	
	1234567890	1234567890	1234567890	1234567890	1234567890	
Trachinus draco	GGGUGGGGUC	CGCGCAGUCU	GCUCGGGGGA	UUCAACUCGG	CGGGACAGGG	50
Labrus bergylta	----------	----------	--C-------	----------	----UAN---	50
Neolamprologus meeli	----------	----------	--C-----*-	----------	----C-C---	49
Scomber scombrus	----------	---------C	--C-------	----------	----UU----	50
Trigla lucerna						0
Perca fluviatilis						0
Pholis gunnellus	----------	----------	-GCG--***-	----------	U----AC---	47
Austrolycus depressiceps	--------C-	----------	-GCG--***-	----------	----UAUU--	45
Cottoperca gobio	----------	----------	--C-------	----------	----U-----	50
Bovichtus variegatus	----------	---------C	--C-------	----------	----UU----	50
Pseudaphritis urvillii	----------	---------C	--C-------	----------	----UU----	50
Eleginops maclovinus	----------	---------C	--C-------	----------	----U-----	50
Artedidraco loennbergi	----------	---------C	--C-------	----------	----U-----	50
Dissostichus mawsoni	----------	---------C	--C-------	----------	----U-----	50
					1111111	
	5555555556	6666666667	7777777888	8889999999	9990000000	
	1234567890	1234567892	3456789012	3490123456	7890123456	
Trachinus draco	*ACGGCCGCU	CUGUGGUGGA	GGAUCCCCUC	GUGGGACCUC	UCCCCGGCGC	99
Labrus bergylta	*---------	-G---UGU-G	----------	--------C-	-----UUGCU	99
Neolamprologus meeli	G-AC-----G	-G---UG---	----------	-C-----U--	C-----CU-U	99
Scomber scombrus	*---------	-G---UG---	----------	----------	----------	99
Trigla lucerna						0
Perca fluviatilis						0
Pholis gunnellus	*--------G	-G---UG---	-----U----	----------	------AA--	96
Austrolycus depressiceps	G----U--UC	GG---CGU-G	----------	----------	-----C-UU-	95
Cottoperca gobio	*---------	-G---CG---	-----U----	----------	-------GUU	99
Bovichtus variegatus	*---------	-G---UG---	-----U----	----------	----------	99
Pseudaphritis urvillii	*---------	-G---UG---	-----U----	-C--------	------A---	99
Eleginops maclovinus	*--------A	-G---CG-*-	-----U----	----------	----------	98
Artedidraco loennbergi	*U--------	-G---UG---	-----U----	----------	-------U--	99
Dissostichus mawsoni	*U--------	-G---UG---	-----U----	----------	----------	99
	1111111111	1111111111	1111111111	1111111111	1111111111	
	0001111111	1112222222	2223333333	3334444444	4455555555	
	7890123456	7890123456	7890123456	7890123457	8901234567	
Trachinus draco	UGGCUGGCCC	UC*GCCGGGC	GCAUUUCCUC	C*GUGGCGGU	GCGCCGCGAC	147
Labrus bergylta	GU--------	GUC-------	------U---	-****-ACC-	----------	145
Neolamprologus meeli	C---------	C-C-------	----------	-*-C------	---U------	148
Scomber scombrus	----------	--*-------	----------	-*-C------	------**--	145
Trigla lucerna						0
Perca fluviatilis	--	CUC-------	----------	-C--R-----	-----N*---	41
Pholis gunnellus	----------	CA*-------	----------	-*--------	----------	144
Austrolycus depressiceps	*---------	CG*-------	--------C-	-*--------	----------	142
Cottoperca gobio	----------	--*-------	----------	-*-A------	----------	147
Bovichtus variegatus	----------	--*-------	----------	-*-A------	----------	147
Pseudaphritis urvillii	----C-----	C-*-----*-	----------	-*-C------	----------	146
Eleginops maclovinus	----C-----	C-*-----*-	----------	-*-C------	------**--	143
Artedidraco loennbergi	----------	C-*-------	---Y------	-*-C------	----GC*---	146
Dissostichus mawsoni	----------	C-*-------	----------	-*-C------	------**--	145
	1111111111	1111111111	1111111111	1111111111	2222222222	
	5566666666	6777777777	7888888888	9999999999	2222333333	
	8901234567	9012345678	9012456789	0123456789	6789012345	
Trachinus draco	CGGCUCUAGG	UCGGCUUGGA	AAGGCUCGGG	GCGAAGGUGG	CUCGC*GGCU	196
Labrus bergylta	----------	----------	----------	----------	-----*--UC	194
Neolamprologus meeli	------CG-U	-----CA---	-G--UCU---	----------	-----*----	197
Scomber scombrus	-------G--	----------	----------	----------	-----*----	194
Trigla lucerna					--	2
Perca fluviatilis	-------G--	----------	----------	A---------	-----*-A--	90
Pholis gunnellus	----------	----------	----------	----------	-----*-A--	193
Austrolycus depressiceps	----------	----------	----------	----------	-----A----	192
Cottoperca gobio	-------G--	----------	----------	----------	-----*----	196
Bovichtus variegatus	------C---	----------	----------	----------	-----*---C	196
Pseudaphritis urvillii	-------G--	----------	----------	----------	-----*---C	195
Eleginops maclovinus	-------G--	----------	----------	----------	-----*----	192
Artedidraco loennbergi	-------G--	-----C----	---S*-----	----------	-----*----	194
Dissostichus mawsoni	-------G--	----------	----------	----------	-----*-A--	194

(Part II)

	2222222222	2222222222	2222222222	2222222222	2222222222	
	3334444444	4555555666	6666666777	7777777888	8888888999	
	6890123478	9012345012	3456789012	3456789012	3456789045	
Trachinus draco	UCGGCGGCGA	GCUUUACAGC	GCCCUCCGUC	U*GGACCUCG	CCG**CUUCC	243
Labrus bergylta	-----C-U--	----------	--U-C---C-	C*--------	G-CGG-----	243
Neolamprologus meeli	C----C----	----------	---UCUU-C-	C*---AU---	---**---A-	244
Scomber scombrus	-----C----	----------	--------G-	CC--------	---**-----	242
Trigla lucerna	C-*--C-A--	*-K-Y-----	---SSS--C-	S*-----K--	---**-----	47
Perca fluviatilis	C----C----	----------	----C-*---	-*--------	---**U----	136
Pholis gunnellus	-----C---U	----------	----C---C-	CC--------	--CG*-----	242
Austrolycus depressiceps	C----C---U	------U--G	------U---	-*--------	---**-----	239
Cottoperca gobio	C----C----	----------	--------C-	C*--------	---**-----	243
Bovichtus variegatus	-----C-U--	----------	----C---C-	C*--------	---**-----	243
Pseudaphritis urvillii	-----C-U--	----------	---UC---C-	C*--------	---**-----	242
Eleginops maclovinus	C----CUG**	----------	--------C-	C*--------	---**-----	237
Artedidraco loennbergi	-----CUG**	----------	--------C-	C*--------	---**-----	239
Dissostichus mawsoni	-----C---U	----------	--------C-	C*--------	---**-----	241
	2222333333	3333333333	3333333333	3333333333	3333344444	
	9999000000	0000111111	1111222222	2233333333	3399000000	
	6789012345	6789012345	6789234567	8901234567	8978901234	
Trachinus draco	AGGGGCCGUG	GACGAA*GUG	CUCCGUGCGC	CCUCUCUCCC	CGGGGGAGGG	292
Labrus bergylta	C---------	---U--*---	---GC----U	-U------GU	----C-----	292
Neolamprologus meeli	C-------C-	---UCU*---	---*UG----	----------	-C--------	292
Scomber scombrus	C---------	---A--*---	----------	----------	GC--------	291
Trigla lucerna	C---------	---U--SS--	---*S-----	----------	--C-------	96
Perca fluviatilis	U---------	---U--*---	---*------	----------	----S-----	184
Pholis gunnellus	C---------	---U--*---	---SS-----	----------	----------	291
Austrolycus depressiceps	----------	--UU--*---	--UGC-----	----------	----------	288
Cottoperca gobio	C---------	---U--*---	---SK-----	----------	-U--------	292
Bovichtus variegatus	C---------	---U--*---	---GC-----	---------U	GC--------	292
Pseudaphritis urvillii	C---------	---U--*---	----------	---------U	GC--------	291
Eleginops maclovinus	C---------	---A--*---	----------	----------	UC--------	286
Artedidraco loennbergi	C---------	---A--*---	----------	-------U--	-C--------	288
Dissostichus mawsoni	C---------	---A--*---	----------	----------	-C--------	290
	4444444444	4444444444	4444444444	4444444444	4444444444	
	0011111111	1222222222	2333333334	4444444455	5555555566	
	8901235678	9012345678	9023456780	1234578901	2345678901	
Trachinus draco	ACGGGGCCCC	UC*GCUCCCG	GUGCGACUGU	CAACCGGGGC	GGACUGUCCU	341
Labrus bergylta	----------	CU*-------	UC--------	-----U----	----------	341
Neolamprologus meeli	----------	CUC-------	-C--------	----------	----------	342
Scomber scombrus	----------	CU*-------	-C--------	-G--------	----------	340
Trigla lucerna	----------	CUU-------	-C--------	-G--------	----------	146
Perca fluviatilis	----------	CU*-------	-C---G----	-G--------	----------	233
Pholis gunnellus	----------	CU*-------	-C--------	-U--------	----------	340
Austrolycus depressiceps	----A-----	CU*-------	-C--------	----------	------C---	337
Cottoperca gobio	----------	CU*-------	----------	-G--------	----------	341
Bovichtus variegatus	----------	CU*-------	----------	-G--------	----------	341
Pseudaphritis urvillii	----------	CU*-------	----------	-G--------	----------	340
Eleginops maclovinus	----------	CU*-------	----------	-G--------	----------	335
Artedidraco loennbergi	----------	CU*-U-----	----------	----------	----------	337
Dissostichus mawsoni	----------	CU*-U-----	----------	----------	----------	339
	4444444444	4444444444	4444555555	5555555555	5555555555	
	6666666777	7777888888	8889111222	2222222333	3333344444	
	2456789234	5679013456	7890789012	3456789012	3478901234	
Trachinus draco	CAGUGCGCCC	CAACCGCGUC	G*UGCGCC**	AGGGCGGGGA	UCGGCUCUCG	388
Labrus bergylta	----------	UG-*------	-*C-UCG-C*	C---------	-------A--	388
Neolamprologus meeli	--------U-	----------	-*C--CG-C*	----------	-----C-A*-	389
Scomber scombrus	----------	----------	-*C-UCG-C*	----------	-----C-A--	388
Trigla lucerna	----------	----------	-*C-UCG-C*	----------	C------A--	194
Perca fluviatilis	----------	----------	-*C-UCG-C*	----------	-------A--	281
Pholis gunnellus	----------	---*------	-GC-UCG-C*	----------	-------A--	388
Austrolycus depressiceps	----------	-G------C-	-*C-U---CC	----------	C------A--	386
Cottoperca gobio	----------	----------	-*--GC--C*	----------	-----C-A--	389
Bovichtus variegatus	----------	----------	-*------C*	----------	C------A--	389
Pseudaphritis urvillii	----------	-U--------	-*---S--C*	----------	-----C-A--	388
Eleginops maclovinus	--------U-	-G--------	-*------C*	*-A-------	-------A--	382
Artedidraco loennbergi	--------U-	-G--------	-*---CGUC*	--A-------	-------A--	385
Dissostichus mawsoni	--------U-	-G--------	-*---CGUC*	--A-------	-------A--	387

	5555555555	5555555555	5555555555	5555555555	5777777777	
	4444455555	5666666666	6777777777	7888888889	9556666666	
	5678905678	9012345678	9012345678	9012347890	1890123456	
Trachinus draco	UAAA**AGGC	GUCAGGGGUC	UGCGGCG*AU	GUCGGCAACC	CUGGGCUCGA	435
Labrus bergylta	--C-AC----	----------	A------*--	----------	----------	437
Neolamprologus meeli	CC--**----	-CA-C-----	-------*--	------U---	----*----C	435
Scomber scombrus	-C--**----	-CA-------	-------*--	----------	---------U	435
Trigla lucerna	----**----	----------	A------*--	----------	---------U	241
Perca fluviatilis	----**----	----------	-------*--	----------	----*-----	327
Pholis gunnellus	----**----	----------	-----GSG--	----------	----------	436
Austrolycus depressiceps	----**----	-----A----	A----U-*--	----------	----------	433
Cottoperca gobio	----**----	-Y--------	-------*--	----------	----*-----	435
Bovichtus variegatus	----**----	----------	-------*--	----------	----------	436
Pseudaphritis urvillii	----**----	-C--------	-------*--	----------	----------	435
Eleginops maclovinus	-C-U**G---	----------	-------*--	----------	----------	429
Artedidraco loennbergi	----**----	----------	-------*--	----------	----------	432
Dissostichus mawsoni	----**----	----------	-------*--	----------	----------	434
	7777777777	7777777777	777777777	7778888888	8888888888	
	6667777777	7778888888	8889999999	9990000000	0001111111	
	7890123456	7890123456	7890123456	7890123456	7890123456	
Trachinus draco	GCCGCGG*CU	GGGGG*AGCA	GUCGCUCCGU	CGCCCU*CCU	CUCUCCGCCG	482
Labrus bergylta	-------*--	-----*----	---------C	-----G*---	----------	484
Neolamprologus meeli	-------*--	----**----	----------	------*---	----------	481
Scomber scombrus	-------*--	-----*----	-----C-U--	------*---	---G--C---	482
Trigla lucerna	-------*--	---NN*----	-----C----	------*---	---C------	288
Perca fluviatilis	-------*--	----**-CG-	--U--C----	------*---	----------	373
Pholis gunnellus	-------*--	----**----	---AUC----	------*---	----------	482
Austrolycus depressiceps	-------*--	-----*--U-	---A-----C	-----A*---	----------	480
Cottoperca gobio	-------*--	----**----	----UC----	------*---	----------	481
Bovichtus variegatus	-------*--	-----*----	----UC----	------*---	----------	483
Pseudaphritis urvillii	-------G--	-----G----	----UC----	------*---	----------	484
Eleginops maclovinus	-------*--	-----*----	----UC---C	------CU--	----------	477
Artedidraco loennbergi	-------*--	-----*----	----UC----	------UGG-	----------	480
Dissostichus mawsoni	-------*--	-----*----	----UC----	------U---	----------	482
	8888888888	8888888888	8888888888	8888888888	8888888888	
	1112222222	2223333333	3334444444	4555555555	5666666667	
	7890123456	7890123456	7890125678	9012345678	9012567890	
Trachinus draco	CUGGAAGCGC	GGUGU**GCG	GCCCGU*CUC	GC****GGGG	CCCA*UGU*C	523
Labrus bergylta	--------UG	-CGU***U--	-----C*---	--****----	-U-CUC--*U	525
Neolamprologus meeli	-C-----*GC	----C**---	---*-C*---	--****----	-U-G*C--*-	520
Scomber scombrus	UC-------U	----C**---	------*---	--****----	--UU*C--*-	523
Trigla lucerna	-C--------	-----**---	------*---	--****----	---U*C--*-	329
Perca fluviatilis	------*---	----C**---	------*---	--****----	-U-U*C--*-	413
Pholis gunnellus	----------	---U-*GU--	------*---	-U****----	--UU*C--*-	524
Austrolycus depressiceps	-C-*--C---	ACGA-GC---	-----A*-C-	U-****----	---U*C--*-	522
Cottoperca gobio	---------U	----C**---	---GC**---	--****----	---U*C--*-	521
Bovichtus variegatus	-C-----G-U	-CA-C****-	------*---	--****----	-U-U*C--**	521
Pseudaphritis urvillii	-C-CGCA-AG	----C**---	------*---	--***G----	---U*C--*-	526
Eleginops maclovinus	-CC--G--UG	-CC-***C--	------C---	CACCGG----	---UCG--*-	523
Artedidraco loennbergi	-----UU---	---CG***A-	------C---	U-C**G----	-U-U*C--G-	524
Dissostichus mawsoni	-C---UU---	---CG***A-	------C---	U-C**G----	-U-U*C--G-	526
	8888888888	8888888888	8888888999	9999999999	9999999999	
	7777777778	8888888999	9999999000	0000000111	1111111222	
	1234567890	1456789012	3456789012	3456789012	3456789012	
Trachinus draco	CGCGGCGCCU	C**GUGCGUC	GCG*UGGCGU	GGGUUUUC**	GCGGGGCGG*	567
Labrus bergylta	U---U-----	-**-------	-***C----G	--*--C--**	U--C--G-UG	567
Neolamprologus meeli	U---------	-**-------	-U**C-----	-------**	-----CG-**	562
Scomber scombrus	--G-------	-**-------	U--*C----G	-------**	---------*	567
Trigla lucerna	----------	-**UG-U---	-UU*G-----	-------**	---------*	373
Perca fluviatilis	G-----A-*-	-****-U---	-UC*GU---G	-------**	---------*	454
Pholis gunnellus	U---------	-*ACG-----	-UU*G----G	----C--U**	---------*	569
Austrolycus depressiceps	-U-A------	-*ACG-----	--U*G----G	-----GC-**	CG-------*	567
Cottoperca gobio	----------	U**CG-----	UA**G----G	-------**	---------*	564
Bovichtus variegatus	--------U-	-***G-----	-UC*G----G	-------**	---------*	564
Pseudaphritis urvillii	----------	-***G-----	---*C----G	----C--UC*	---------*	570
Eleginops maclovinus	--GA----U-	-GCCG----U	C-CGGCCG-G	------CUCG	--CCC-G--G	573
Artedidraco loennbergi	--U-----U-	-***G-U---	---*GU---G	--*-----GG	U-----GU-G	569
Dissostichus mawsoni	U-U-----U-	-***G-U---	---*GU---G	-----C--GG	U-----G-CG	572

(Part IV)

	9999999999 2222222333 3456789012	9999999999 3333333444 3456789012	9999999999 4444445555 3456890123	9999999999 5555556666 4567890123	9999999999 6666667777 4567890123	
Trachinus draco	***UGUCCGU	*CGCCGU*GU	GGAAGGCGGG	CCGGUGGAGG	GGAUCGGGUA	612
Labrus bergylta	G**------G	*-----C*--	C---------	----------	---CU-----	613
Neolamprologus meeli	***------C	*------*--	----------	----C--G--	-*--GU---*	605
Scomber scombrus	***------A	*-----C*--	----------	U----C-G--	--G-------	612
Trigla lucerna	***-------	*------*--	----------	U------G--	----------	418
Perca fluviatilis	***------A	*------*--	----------	A---------	----------	499
Pholis gunnellus	***-------	*------*--	C---------	----------	----------	614
Austrolycus depressiceps	***--AU---	*A-U---*--	C-CC----UA	A---------	---------U	612
Cottoperca gobio	***------A	*-----C*--	----------	----------	----------	609
Bovichtus variegatus	***------A	A------*--	----------	----------	----------	610
Pseudaphritis urvillii	***------A	*-----C*-C	----------	----A-----	***-U--A--	612
Eleginops maclovinus	GCG------A	CGC---CGCC	----------	----A-----	***-U--A--	620
Artedidraco loennbergi	***------A	*G-----*-C	----------	----A-----	***-U--A--	611
Dissostichus mawsoni	G**------A	*G-----*-C	----------	----A-----	***-U--A--	615

	9999999999 7777778888 4567893456	9999999999 8889999999 7890123456	lllllll 9990000000 9990000000 7890123456	lllllllll 0000000000 0001111111 7890123456	lllllllll 0000000000 1112222222 7890123456	
Trachinus draco	CGGCGGUUGG	CGGCGGCGAC	UCUGGACGCG	GCGCCGGGCC	CUUCUCGCGG	662
Labrus bergylta	------A--*	----------	------*CGC	----------	----------	661
Neolamprologus meeli	-------G-C	U---------	------*CGC	----------	----------	654
Scomber scombrus	----A-----	----------	------*CGC	----------	----------	661
Trigla lucerna	-------C--	----------	------*CGC	----------	----------	467
Perca fluviatilis	-------*--	U---------	------*CGC	----------	----------	547
Pholis gunnellus	-------C--	U---------	------*CGC	-----A----	----------	663
Austrolycus depressiceps	A-----A--*	U---------	------*CGC	----------	----------	660
Cottoperca gobio	-------C--	---UU-----	------*CGC	----------	----------	658
Bovichtus variegatus	-------C--	-------A--	------ACGC	-M--------	----------	660
Pseudaphritis urvillii	-------C--	----------	------*CGC	----------	----------	661
Eleginops maclovinus	-------C--	----------	------*CGN	-N--------	----------	669
Artedidraco loennbergi	-------C--	----------	------*CGC	----------	----------	660
Dissostichus mawsoni	-------C--	----------	------*CGC	----------	----------	664

	lllllllllll 0000000000 2223333333 7890123456	lllllllllll 0000000000 3334444445 7890123456	lllllllllll 0000000000 5556666666 7890123489	lllllllllll 0000000000 7777777777 0123456789	lllllllllll 0000000000 8888888899 0123456701	
Trachinus draco	AUCUCCCCAG	CUACGGCC*G	CGCUGGG*CC	CCGUUCGCGC	GGG*GUCC*U	708
Labrus bergylta	----------	--G----GUC	U-UG---G--	------A---	---*----*C	709
Neolamprologus meeli	---A--U---	--G---UGCC	--UC---GU-	--C------G	---*U---CC	703
Scomber scombrus	----------	---GC--**-	--U----*--	------A---	---*----**	705
Trigla lucerna	----------	--NG*****C	S-UC---*--	------A---	--UG----**	509
Perca fluviatilis	----------	--------*-	-------*--	----------	---*----*-	593
Pholis gunnellus	----------	-------GCC	-------*U-	----------	---*C---*-	710
Austrolycus depressiceps	----------	------UGCU	--UC---*U-	----------	---UCG--*-	708
Cottoperca gobio	----------	--------C-	--U----*--	----------	---*----*C	705
Bovichtus variegatus	----------	--G--M***-	--U----*M-	------AM--	---*-W--**	703
Pseudaphritis urvillii	----------	-------G*C	--U----*U-	----------	---GC---**	707
Eleginops maclovinus	----------	-------GCC	--UC---UA-	-------M--	---*CC--**	716
Artedidraco loennbergi	----------	-------G*C	--U----GU-	----------	---CU---**	707
Dissostichus mawsoni	----------	-------GCC	--U----GU-	----------	---*C---*C	712

	lllllllll 0000000001 9999999990 2345678901					
Trachinus draco	GGCGGGUCG					717
Labrus bergylta	U---------					718
Neolamprologus meeli	----------					712
Scomber scombrus	*---------					713
Trigla lucerna	----------					518
Perca fluviatilis	----------					602
Pholis gunnellus	--U-------					719
Austrolycus depressiceps	C-G-C-----					717
Cottoperca gobio	----------					714
Bovichtus variegatus	--M-------					712
Pseudaphritis urvillii	----------					716
Eleginops maclovinus	*---------					724
Artedidraco loennbergi	----------					716
Dissostichus mawsoni	----------					721

Appendix 1

Aligned sequences of the D2 and D8 domains of the 28S rRNA. A *star* indicates a gap and a *dash* indicates that the nucleotide is the same as in the first line. Ambiguous nucleotides are named according to the standard nomenclature. Positions are numbered according to the absolute nomenclature of our aligned fish 28S database. Consequently, two neighbour positions can have non-neighbour numbers. This does not mean that positions have been deleted from this analysis, but that some other hidden species (that has nothing to do with the present study) has an insertion at this point. This does not alter the continuity of the sequences analysed here. Two stretches of numbered positions have been deleted: (1) the stretch 592–757: a zone including the end of the D2 domain and the beginning of the D8, where many species have missing sequences; and (2) a stretch of 13 positions (340–352) including an insertion in *Eleginops*

Note that *Trigla* and *Perca* have missing sequences in the region 1–233. Parsimony and bootstrap-parsimony analyses have been carried out using: (1) the complete present data set (759 positions, among which 332 variable and 158 informative for parsimony), and (2) with region 1–254 deleted (544 positions, among which 250 variable and 119 informative for parsimony), to check the impact of the inconsistent region of question marks. The results were exactly the same, with the same tree within the ingroup and the same irresolution between outgroups