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Phylogeny of Antarctic dragonfishes (Bathydraconidae, Notothenioidei, Teleostei) and related families based on their anatomy and two mitochondrial genes

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Abstract

Although Antarctic teleosts of the suborder Notothenioidei are well studied, the status of some families remains unclear because of limited taxonomic sampling and sometimes poor statistical support from molecular phylogenies. It is true for the Bathydraconidae, the sister-family of the famous haemoglobin-less icefishes, the Channichthyidae. The present study is aimed at clarifying bathydraconid phylogeny and the interrelationships of higher notothenioid families, taking nototheniids as the outgroup. For this purpose, about 300 positions in the mitochondrial control region, 750 positions in the cytochrome *b*, and a matrix of morphological characters were employed for separate and simultaneous phylogenetic analyses. We conclude that (1) molecular data strongly support the split of bathydraconids into three clades, here called the Bathydraconinae (*Bathydraco*, *Prionodraco*, *Racovitzia*), the Gymnodraconinae (*Gymnodraco*, *Psilodraco*, *Acanthodraco*), and the Cygnodraconinae (*Cygnodraco*, *Gerlachea*, *Parachaenichthys*). Interrelationships between these three and the Channichthyidae remain unclear. Molecular data support neither paraphyly nor monophyly of the bathydraconids, while morphology leads to the monophyly of the family based on the synapomorphic loss of the spinous dorsal fin; (2) The Channichthyidae, the Harpagiferidae, and the Artedidraconidae are monophyletic families; (3) the phylogeny of the haemoglobin-less channichthyids is completely resolved and congruent with the conclusions of Iwami (1985) based on anatomical characters; (4) The present molecular results as well as other molecular studies favour the hypothesis that harpagiferids are the sister-group of artedidraconids, though our morphological matrix puts harpagiferids as the sister-group of all other families on the basis of a single character. With regard to harpagiferid relationships, it is interesting to notice that, when analysed simultaneously, morphological characters are not automatically ''swamped'' within molecular ones: in the tree based on the simultaneous analysis of all available data, morphological characters impose their topology on molecules.

Keywords: Artedidraconidae; Bathydraconidae; Channichthyidae; Dragonfish; Icefish; Incongruence; Harpagiferidae; Notothenioidei

1. Introduction

Over the last 7 years, molecular phylogenetics of the Notothenioidei has made considerable progress. The interest in Antarctic perciform fishes is partly due to their physiological and anatomical adaptations to life in cold waters, and to the loss of haemoglobins in the icefishes, the Channichthyidae. These features place the notothenioids among the most studied marine fishes (di Prisco et al., 1998; Eastman, 1993). DNA sequences globally offered the same picture of interrelationships for these families and confirmed previous cladograms based on anatomical characters (Hastings, 1993; Iwami, 1985). Among the six traditional families composing the suborder, two were clearly found to be paraphyletic on the basis of sequence data (Fig. 1): the Bovichtidae (Lecointre et al., 1997; Ritchie et al., 1997) and the Nototheniidae (Bargelloni et al., 1994, 2000; Ritchie et al., 1997). Two were found to be monophyletic, however, from an incomplete taxonomic sample: the Artedidraconidae and the Harpagiferidae (Bargelloni et al., 1994, 2000; Ritchie et al., 1997). The complete

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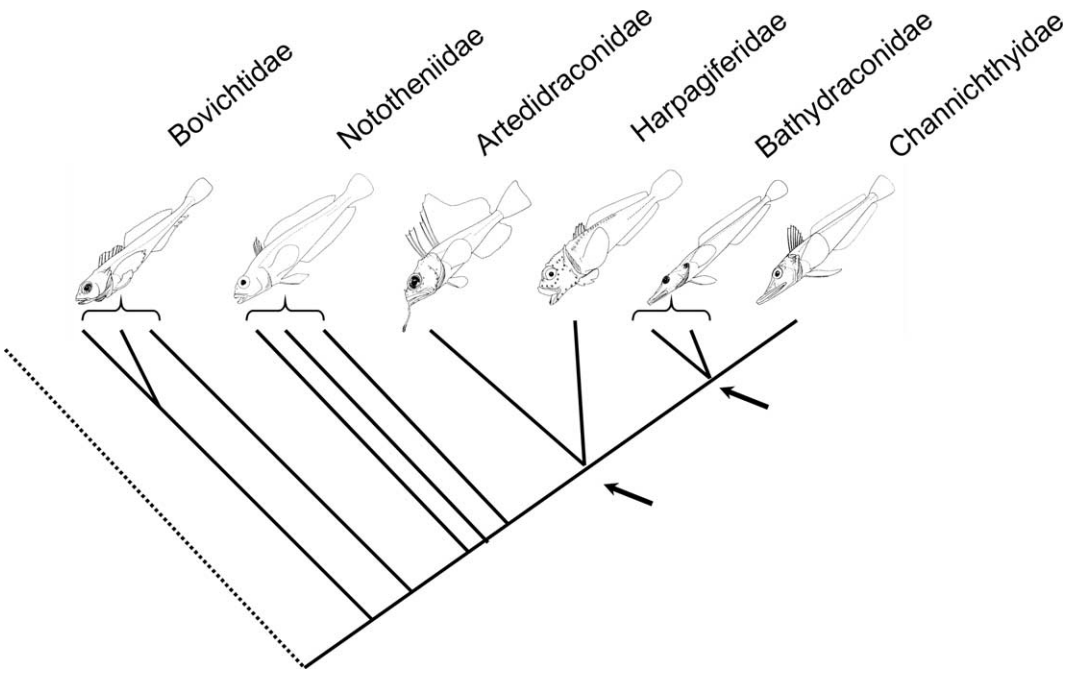

Fig. 1. The bathydraconid problem in the notothenioid tree. Bovichtids and Nototheniids have both been shown to be paraphyletic (Bargelloni et al., 2000; Lecointre et al., 1997; Ritchie et al., 1997). Artedidraconids are most likely the sister-group of harpagiferids (Bargelloni et al., 2000; Eakin, 1981) but with some ambiguities. Bathydraconids may not be monophyletic, but there is some disagreement in the literature. Arrows indicate the multifurcations addressed here.

phylogeny of the Channichthyidae was established (Chen et al., 1998) and appeared as completely congruent with previous anatomical studies (Iwami, 1985). However, the monophyly of the family was not tested by Chen et al. (1998) because a single outgroup was used, *Gymnodraco acuticeps*. In other studies (Bargelloni et al., 2000; Ritchie et al., 1997), the monophyly of the Channichthyidae was established with a sufficient coverage of outgroups but, in turn, the number of channichthyid species was only 4 or 5 out of 15. Finally, neither the monophyly nor the paraphyly of the Bathydraconidae was clearly established from molecular data, both because of insufficient taxonomic sampling and lack of statistical support for the corresponding clades (Bargelloni et al., 1994, 2000; Ritchie et al., 1997; Stam et al., 1997).

As some families were shown to be paraphyletic from studies based on molecular data, some genera have to be excluded from their initial families if one wants to get an up-to-date picture of notothenioid classification. The paraphyletic bovichtids (three genera) are the most basal (Bargelloni et al., 1994, 2000; Stam et al., 1997). Both nuclear and mitochondrial ribosomal genes showed that *Pseudaphritis* is more closely related to the rest of the Notothenioidei than are *Bovichtus* and *Cottoperca* (Lecointre et al., 1997; Ritchie et al., 1997). Then emerge the paraphyletic nototheniids, with the first lineage containing *Eleginops maclovinus*, as the sister-group of the rest of the notothenioids on the basis of mitochondrial ribosomal sequences (Bargelloni and Lecointre, 1998; Bargelloni et al., 2000). The same data show the remaining nototheniids to be composed of four main clades collapsed within a multifurcation, two of them being *Notothenia* and *Dissostichus*. Once again, the paraphyly of the nototheniids is shown because *Pleuragramma antarcticum* appears more closely related to other families than to the nototheniid clade comprising *Trematomus*, *Pagothenia*, *Lepidonotothen*, and *Patagonotothen*. In all molecular phylogenies, harpagiferids appear as the sister-group of artedidraconids; however, the taxonomic sampling of both families is generally minimal and the clade containing the two families is statistically poorly supported (Bargelloni and Lecointre, 1998; Ritchie et al., 1997), though better supported in Bargelloni et al. (2000). Nevertheless, this corroborates the close relationships between harpagiferids and artedidraconids already suspected from anatomical characters (Eakin, 1981; Hastings, 1993; Iwami, 1985). Sequence data indicate bathydraconids as closely related to channichthyids, but here again from incomplete taxonomic samples and with low statistical support (Bargelloni and Lecointre, 1998; Bargelloni et al., 2000). Some confusion remains on that point. The present paper focused on the phylogeny of the higher notothenioids Harpagiferidae, Artedidraconidae, Bathydraconidae, and Channichthyidae, with a special focus on the most problematic family, the Bathydraconidae.

From the morphological point of view, the bathydraconids are closer to the channichthyids than to any other notothenioid family: they share with the chann-

ichthyids the non-protrusible jaw, the I-shaped junction between the epihyal and the ceratohyal (Iwami, 1985). Bathydraconids are defined by the absence of the spinous dorsal fin (Iwami, 1985). However, Hastings (1993) concluded that the bathydraconids were paraphyletic, the Bathydraconinae being the sister-group of the Channichthyidae and the Gymnodraconinae the sister-group of both. The characters excluding gymnodraconines were the ascending processes of the premaxillar and the spine on the dorsal margin of the cleithrum both reduced or absent in bathydraconines and channichthyids. These characters remain doubtful because these two structures are only reduced in bathydraconines and absent in channichthyids. Even if researchers do perceive a tendency towards reduction of such structures among the highest notothenioids, their reduction is of doubtful homology with their complete absence. So, it is clear that improvement of our knowledge of the phylogeny of bathydraconids requires supplementary characters, including molecular ones. Hence, the present study increases the taxonomic sampling on the same portions of cytochrome *b* and control region as in Chen et al. (1998), by sequencing these two mitochondrial genes in bathydraconids, artedidraconids, harpagiferids, and two nototheniids as outgroups. Moreover, a matrix of morphological characters based on the studies of Eakin (1981), Iwami and Abe (1984), Iwami (1985), Hastings (1993), and Voskoboynikova (1993) was produced to evaluate the degree of congruence or conflict with the present molecular data.

2. Materials and methods

2.1. Taxon sampling and DNA extraction

Taxa are listed in Table 1. Bathydraconids contain 11 genera and 16 species. The recently described *Acanthodraco dewitti* (Skóra, 1995; Voskoboynikova and Skóra, 1996) was included in the study. Harpagiferids contain one genus and six species, artedidraconids four genera and 24 species, and channichthyids 11 genera and 15 species. All the genera of each of the four families were sampled, except the two very rare bathydraconids *Akarotaxis* and *Vomeridens*. Small piece of muscle tissues was stored at −80 °C or fixed in 70% ethanol. DNA extraction followed the standard phenol/chloroform method described in Winnepenninckx et al. (1993). Part of the present data was retrieved from a previous work (Chen et al., 1998).

2.2. DNA amplification and sequencing

DNA amplification was performed via Polymerase Chain Reaction (PCR) (Mullis and Faloona, 1987; Saiki et al., 1988) in 50 μl volume usually containing (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 U) Hi-Taq polymerase (Bioprobe), 50 pmol each of the two primers, and 0.3–1.2 μg template DNA. Primers used for amplifying the different genes are listed in Table 2. PCR was carried out using a Biometra trioblock cycler. The thermocycles were: 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 AT was between 50 and 60 °C depending on the species and the regions amplified. PCR products were visualized then purified by agarose gel extraction using Qiaex II Kit (Quiagen). Thermo Sequenase Cycle Sequencing Kit (Amersham) was used for direct sequencing followed with numbers of thermocycles: 95 °C/3 min, 72 °C/2 min then 95 °C/30 s, 53 °C/60 s, 72 °C/60 s for 30 cycles, and 72 °C/10 min. The reacted samples were loaded after denaturation in each lane of an acrylamide-urea electrophoresis gel. Radiolabels were previously incorporated into the primers used for sequencing by end-labelling the 5 end of the primers with T4 polynucleotide kinase and [^{33}P]ATP. The primers used for sequencing the different domains or genes were the same as those for PCR. However, internal primers were also necessary for completing the sequencing when PCR products were longer than 500 bp (Table 2). After electrophoresis, the gel was dried and then exposed to an X-ray film for at least one night.

2.3. Quality of molecular data

The possibility of sequence errors was checked by comparing our sequences to the sequence obtained from a second exemplar or to the sequence made from a new DNA extraction. Sequences were obtained and checked several times, from two to four times. Sequences were read and entered twice using the MUST package (Philippe, 1993). Alignments were performed using ED of MUST. Hypervariable regions were kept for phylogenetic analysis because the high levels of variability in these regions usually came from only a few divergent taxa. Only two marginal segments were excluded from the analysis because of incomplete taxonomic sampling (alignments available upon request). Alignments of the cytochrome *b* sequences of 37 taxa provided 744 positions among which 212 were informative for parsimony, i.e., having at least two types of nucleotides each present at least twice. Control region sequences of 38 taxa provided 298 positions among which 178 were informative for parsimony. Mutational saturation was explored for each gene by plotting the pairwise number of observed nucleotide differences against the pairwise number of inferred substitutions (Hassanin et al., 1998; Lavoué et al., 2000; Philippe et al., 1994). For cytochrome *b* sequences, this was performed at each codon

Table 1
Taxonomic sampling, accession numbers, geographic area of origin, and collectors

		Cytochrome *b*	d Loop	Location	Collector
Outgroups	*Notothenia neglecta*	AF490648	AF490671	Terre Adélie	Ozouf-Costaz
	Dissostichus eleginoides	AF490649	AF490672	Kerguelen Islands	Duhamel
Harpagiferidae	*Harpagifer antarcticus*	AF490647	AF490669	Signy Island	White
	Harpagifer georgianus	AF490646	AF490668	King George Island	Bargelloni
	Harpagifer kerguelensis	AF490645	AF490670	Kerguelen Islands	Duhamel
Artedidraconidae	*Artedidraco skottsbergi*	AF490642	AF490665	Weddell Sea	Ozouf-Costaz
	Artedidraco mirus	AF490640	AF490661	Weddell Sea	Ozouf-Costaz
	Dolloidraco longedorsalis	AF490641	AF490664	Weddell Sea	Ozouf-Costaz
	Histiodraco velifer	AF490644	AF490660	Weddell Sea	Ozouf-Costaz
	Pogonophryne barsukovi	AF490637	AF490663	Weddell Sea	Ozouf-Costaz
	Pogonophryne marmorata	AF490639	AF490666	Weddell Sea	Ozouf-Costaz
	Pogonophryne mentella	AF490638	AF490667	Weddell Sea	Ozouf-Costaz
	Pogonophryne scotti	AF490643	AF490662	Weddell Sea	Ozouf-Costaz
Bathydraconidae	*Acanthodraco dewitti*	AF490636	AF490650	Terra Nova Bay	Vacchi
	Bathydraco macrolepis	AF490630	AF490659	Weddell Sea	Ozouf-Costaz
	Bathydraco marri	AF490632	AF490654	Weddell Sea	Ozouf-Costaz
	Cygnodraco mawsoni	AF490633	AF490652	Weddell Sea	Ozouf-Costaz
	Gerlachea australis	AF490631	AF490655	Weddell Sea	Ozouf-Costaz
	Gymnodraco acuticeps	AF037109	AF037133	Weddell Sea	Ozouf-Costaz
	Parachaenichthys charcoti	No	AF490653	King George Island	Bargelloni
	Parachaenichthys georgianus	AF490635	AF490658	Weddell Sea	Ozouf-Costaz
	Prionodraco evansii	AF490628	AF490657	Weddell Sea	Ozouf-Costaz
	Psilodraco breviceps	AF490634		South Georgia Island	Bargelloni
			AF490651	Terre Adélie	Ozouf-Costaz
	Racovitzia glacialis	AF490629	AF490656	Weddell Sea	Ozouf-Costaz
Channichthyidae	*Chaenocephalus aceratus*	AF037121	AF037136	Shetland Islands	Ozouf-Costaz
	Chaenodraco wilsoni	AF037116	AF037128	Weddell Sea	Ozouf-Costaz
	Champsocephalus gunnari	AF037110	AF037132	Heard Island	Ozouf-Costaz
	Channichthys rhinoceratus	AF037115	AF037126	Heard Island	Ozouf-Costaz
	Chionobathyscus dewitti	AF037120	AF037137	Weddell Sea	Ozouf-Costaz
	Chionodraco hamatus	AF037122	AF037135	Terre Adélie	Ozouf-Costaz
	Chionodraco myersi	AF037117	AF037130	Weddell Sea	Ozouf-Costaz
	Chionodraco rastropinosus	AF037118	AF037135	Weddell Sea	Ozouf-Costaz
	Cryodraco antarcticus	AF037114	AF037131	Weddell Sea	Ozouf-Costaz
	Dacodraco hunteri	AF037123	AF037129	Lazarev Sea	Zimmermann
	Neopagetopsis ionah	AF037111	AF037124	Weddell Sea	Ozouf-Costaz
	Pagetopsis maculatus	AF037113	AF037127	Weddell Sea	Ozouf-Costaz
	Pagetopsis macropterus	AF037112	AF037125	Prydz Bay	Ozouf-Costaz
	Pseudochaenichthys georgianus	AF037119	AF037138	Weddell Sea	Ozouf-Costaz

Sequences of *G. acuticeps* and all channichthyid sequences were taken from Chen et al. (1998).

Table 2
Primers used

Gene	Name	Sequence ($5' \rightarrow 3'$)
Control Region	LPR-02	AAC-TCC-CAC-CAC-TAA-CTC-CCA-AGG-C
	HDL2	AGG-TAG-GAA-CCA-GAT-GCC-AGN-AAT
	L. 15926	TCA-AAG-CTT-ACA-CCA-GTC-TTG-TTA-ACC
	H. 16498	CTT-GAA-GTA-GGA-ACC-AGA-TG
Cytochrome *b*	L. 14724	TGA-CTT-GAA-GAA-CCA-CCG-TTG
	L. 15026	CCG-AGG-VCT-DTA-CGG-CTC
	L. 15047	TAC-CTA-TAC-AAA-GAA-ACN-TGA-AA
	L. 15053	CCA-AAA-GAA-ACC-TGA-AAY-ATY-GG
	L. 125	TTC-TTY-GCC-TTC-CAC-TTC-TC
	H. 506	CGG-AAT-GTT-AGG-CCT-CGT-TGT-T
	H. 15930	CCT-CGA-TCT-TCG-RTT-TAC-AAG

position for transitions and transversions separately. The COMP-MAT program of MUST was used, the pairwise number of observed differences being computed by MUST and the pairwise number of inferred substitutions being computed using PAUP 3.1.1. (Swofford, 1993) as the number of steps met in the path joining the two species in the most parsimonious tree. The patristic distance matrix was obtained by saving the MP tree with its branch lengths from PAUP and transferring it to the AF_PAUP3 and TREEPLOT programs of MUST.

2.4. Morphological data

In a majority of morpho-anatomical studies of the Bathydraconidae, characters are described but hypotheses of homology are not formally translated into a matrix. We have reanalysed the morphological and anatomical characters discussed by Eakin (1981), Iwami and Abe (1984), Iwami (1985), Hastings (1993), and Voskoboynikova (1993), at the genus level, leading to a matrix containing 15 genera and 26 characters (Table 3). The morphological taxonomic sampling was not detailed within channichthyids because of anatomical homogeneity within this family concerning those characters that offer suitable variation between the families. *Akarotaxis* and *Vomeridens* were not included in the morphological matrix because they were not included in molecular data sets. To allow a real comparison between morphological and molecular data, a molecular matrix containing the taxonomic sample of the morphological matrix was created to allow tests for character incongruence and tree comparisons using PAUP and Mac Clade (Maddison and Maddison, 1992).

2.5. Analysis of character congruence

Data sets were delineated with respect to their putative independence, in terms of functional constraints and selective pressures (Slowinski and Page, 1999): control region, cytochrome *b*, and morphological data. It can be objected that the control region and the cytochrome *b* gene are not really independent because both are mitochondrial. However, the way each of them accumulated signal and homoplasy is different in the sense that the second encodes a transmembraneous protein. With the purpose to explore the properties of the data, the significance of character incongruence between molecular partitions (cytochrome *b* versus control region), and between the combined molecular data and the morphological partition, was tested using the Incongruence Length Difference test (Farris et al., 1995; Mickevich and Farris, 1981). Both the ARNIE program from the Random Cladistics package of Mark Siddall (Siddall, 1997; commands cc-.; mh; bb-; with 1000 iterations; the package is available at http://www.vims.edu/~mes/mes/rchelp.html#arnie) and the partition homogeneity test of PAUP4 (Swofford, 1998) were used. When two DNA data sets were found to be incongruent to each other (*P* value $<5\%$), visual inspection of the MP trees obtained separately rapidly provided the scope of incongruence. Removal of each species separately followed by a new ILD analysis sometimes allows the identification of the species responsible for incongruence (e.g., in Lecointre et al., 1998). If not, blocks of species can be removed the same way to detect the source of incongruence. This was performed from the present molecular data. The ILD test is not used here as a procedure of "conditional combination" (Bull et al., 1993), but is used to under-

Table 3
Matrix of morpho-anatomical characters

	00000000011111111112222222
	12345678901234567890123456
Notothenia	00000000000000000000001000
Harpagifer	01111111100100010000001000
Artedidraco	11111111110000011010001000
Dolloidraco	11111111110000011010001000
Histiodraco	21111111110000012010002000
Pogonophryne	21111111110000012010002000
Bathydraco	11111111101211100000101000
Cygnodaco	11111111101211110000101000
Gerlachea	11111111101211100000101000
Gymnodraco	11111111101200110101111000
Parachaenichthys	11111111101211110000101000
Psilodraco	11111111101200110101111000
Prionodraco	11111111101211110000101000
Racovitzia	11111111101211100000101000
Channichthys	21111111101222012000102111

1. Anterior pleural ribs (Eakin, 1981): present: 0, reduced: 1, absent: 2; 2. Postcleithrum (Baluskin, 1992): present: 0, absent: 1; 3. Last ray of the second dorsal and anal fins (Eakin, 1981): divided to the base: 0, not divided: 1; 4. Basisphenoid (Eakin, 1981): present: 0, absent: 1; 5. Prootics (Iwami, 1985): in contact ventrally: 0, separated: 1; 6. dorsal spine of the penultimate vertebra (Eakin, 1981): shorter than the spine of the antepenultimate vertebra: 0, same length: 1; 7. Second basibranchial (Iwami and Abe, 1984): cartilaginous: 1, ossified: 0; 8. Third and fourth hypurals: separated: 0, fused: 1; 9. Baudelot's ligament (Eakin, 1981): attached to the basioccipital: 0, attached to the first vertebra: 1; 10. Upper margin of the opercle (Eakin, 1981): straight: 0, hooked-shaped: 1; 11. Epihyal–ceratohyal junction (Iwami, 1985): L-shaped: 0, I-shaped: 1; 12. Uroneurals (Eakin, 1981): autogenous: 0, partly fused to the urostylar vertebra: 1, non-autogenous: 2; 13. Ascending process of the maxillae (Iwami, 1985): normal: 0, reduced: 1, absent: 2; 14. Spine on the cleithrum (Iwami, 1985): normal: 0, reduced: 1, absent: 2; 15. Spinous dorsal fin (Eakin, 1981): present: 0, absent: 1; 16. Scales (Eakin, 1981): present: 0, absent: 1; 17. Epipleural ribs on first vertebra (Eakin, 1981): present: 0, reduced: 1, absent: 2; 18. fang-like teeth (Voskoboynikova, 1993): present: 0, absent: 1; 19. Mental barbel (Eakin, 1981): present: 1, absent: 0; 20. Metapterigoid upper lobe (Voskoboynikova, 1993): present: 0, absent: 1; 21. Jaw protrusibility (Eakin, 1981): yes: 0, no: 1; 22. Pseudochoanae (Voskoboynikova, 1993): present: 1, absent: 0; 23. Pleural posterior ribs (Eakin, 1981): normal: 0, reduced: 1, absent: 2; 24. Third hypobranchial (Iwami and Abe, 1984): triangular: 0, rod-shaped: 1; 25.Third and fourth pharyngobranchials (Iwami and Abe, 1984): autogenous: 0, fused: 1; 26. First basibranchial (Iwami and Abe, 1984): ossified: 0, cartilaginous: 1.

stand the structure of our data. Whatever the issue, after separate analyses of each partition, data will be combined for a simultaneous analysis.

2.6. Phylogenetic analysis sensu stricto

Separate and simultaneous phylogenetic analyses (Lecointre and Deleporte, 2000; Nixon and Carpenter, 1996) were performed using parsimony with PAUP4 (Swofford, 1998). Separate analyses are useful to record repeated clades and detect tree reconstruction artefacts, while the tree based on simultaneous analysis is the one on which natural history of characters must be discussed, because it is the tree maximizing the congruence of all available characters. In that tree, reliable clades are those repeated across previous separate analyses (Lecointre and Deleporte, 2000). The MP trees were obtained either through Branch-and-Bound search or heuristic search with 100 random stepwise addition sequences (MULPARS on), followed by TBR swapping trees without steepest descent. Gaps were treated as fifth state of characters. For separate and simultaneous analyses, MP trees were obtained without weighting strategies, and regardless of saturation detected in transitions, because it has been shown from various sequence data that homoplasy is not homogeneously spread across the tree (Philippe et al., 1996), probably an effect of unequal rates through times and among lineages. This partly explains why underweighting transitions and/or third codon positions more often leads to signal loss and less phylogenetic accuracy than extracting phylogenetic signal (Hassanin et al., 1998; Källersjö et al., 1999; Sennblad and Bremer, 2000; Wenzel and Siddall, 1999). Moreover, some types of transitions accumulate saturation while others do not (Hassanin et al., 1998). The taxonomic congruence was determined through comparing separate phylogenetic trees without consensus. By principle, consensus techniques were not used for comparing trees from different sources of data because special attention was paid to (1) repeated clades and (2) branch lengths to retain the possibility of detecting artifactual branchings (e.g., when a taxon escapes from its clade only in one of the three trees because of a rate acceleration in the evolution of the gene in this taxon). This information would have been lost in a strict consensus tree. To allow a better exploration of taxonomic incongruence between molecular trees and morphological trees, the strength of the conflict between topologies was explored by assessing the significance of tree length differences using Wilcoxon's signed-rank test (Templeton, 1983) as performed by PAUP4. For robustness analyses, Bremer supports were calculated (Bremer, 1994, 1988) and bootstrap proportions (Felsenstein, 1985) were obtained from 1000 iterations using PAUP.

3. Results

3.1. Separate analyses

Absolute saturation plots exhibited a certain degree of dispersion due to homoplasy; however, no plateau was detected. The 744 positions of the cytochrome *b* sequences (among which 212 were informative for parsimony) provided through the heuristic search of PAUP4 with 1000 random addition sequences two trees of 891 steps (C.I. = 0.464, R.I. = 0.692), while the 298 positions of the control region (among which 178 were informative) provided eight equiparsimonious trees of 776 steps (C.I. = 0.539, R.I. = 0.745). These molecular trees (not shown) agreed upon their most robust parts. Both molecular data sets showed the monophylies of the Channichthyidae, the Artedidraconidae, and the Harpagiferidae. Both molecular data sets neither recovered monophyletic Bathydraconidae nor provided statistical support (in terms of bootstrap proportions) for the paraphyly of the family. The interesting point is that both separately recovered the same bathydraconid subgroups. These subgroups are shown as in Fig. 2 on the tree based on the combined molecular data: the clade GY corresponding to the Gymnodraconinae (*Gymnodraco*, *Psilodraco*, *Acanthodraco*), the clade CY which could be called the Cygnodraconinae (*Cygnodraco*, *Gerlachea*, *Parachaenichthys*), and the clade BA which could be called the Bathydraconinae (*Bathydraco*, *Racovitzia*, *Prionodraco*), a definition that is not the same as the wider Bathydraconinae of Hastings (1993) but which is practical in the present context. There was neither consensus nor significant bootstrap support for any hypothesis about how these three clades are related to each other and to channichthyids.

The two most parsimonious trees obtained from morpho-anatomy (Fig. 3) contradicted the molecular trees (as well as the tree Fig. 2) on several points: (1) harpagiferids were the sister-group of all the rest; (2) within artedidraconids *Pogonophryne* was the sister-group of *Histiodraco* (as in Eakin, 1981); (3) bathydraconids were monophyletic (though this is not really a contradiction, as molecular data simply failed to show monophyly as well as paraphyly of that family); and (4) within bathydraconids, *Bathydraco*, *Gerlachea*, and *Racovitzia*, were grouped on the basis of the presence of scales. Each contradicting clade was based on a single synapomorphy: (1) The exclusion of harpagiferids was based on anterior pleural ribs that are normal in nototheniids and harpagiferids and reduced in others, even absent in *Histiodraco*, *Pogonophryne*, and channichthyids (character 1); (2) *Histiodraco* and *Pogonophryne* were sister-groups on the basis of several losses: the loss of anterior pleural ribs (character 1), epipleural ribs on first vertebra (character 17), and pleural posterior ribs

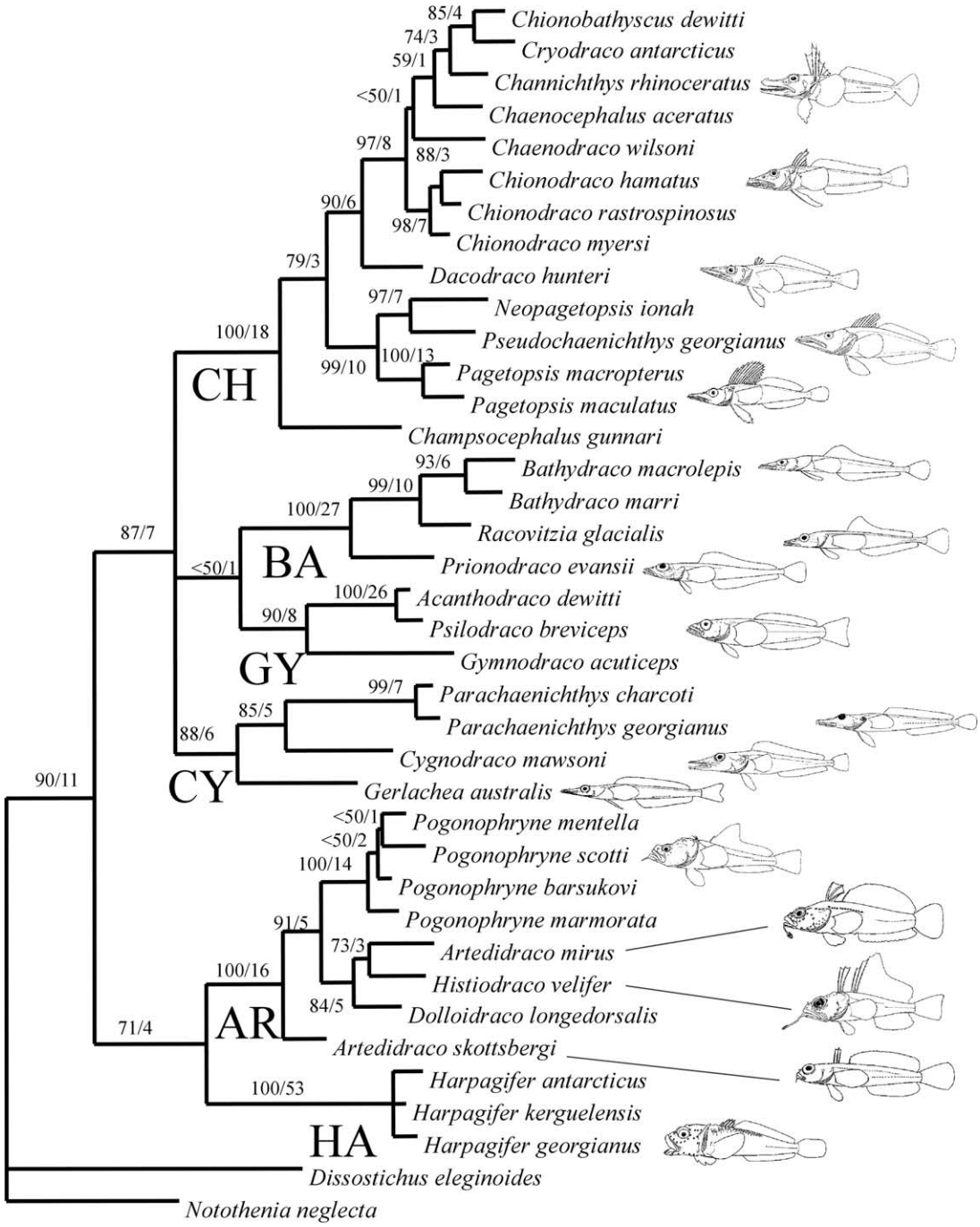

Fig. 2. Strict consensus of the four equiparsimonious trees obtained from the combined molecular matrix (cytochrome *b* and control region sequences providing 1042 positions, among which 390 are informative for parsimony), through the heuristic search of PAUP4 with 1000 random addition sequences. The length of the four trees is 1686, C.I. = 0.532, R.I. = 0.727. Branch lengths are given under ACCTRAN. The first number above nodes is the bootstrap proportion obtained from 1000 iterations. The second number is the Bremer support. AR: Artedidraconidae, BA: Bathydraconinae, CH: Channichthyidae, CY: Cygnodraconinae, GY: Gymnodraconinae, HA: Harpagiferidae (BA, CY, and GY are components of the Bathydraconidae).

(character 23); (3) bathydraconids were grouped on the basis of the loss of spinous dorsal fin (character 15, no homoplasy); (4) Three bathydraconids, *Bathydraco*, *Gerlachea*, and *Racovitzia*, were grouped on the presence of scales (character 16, no homoplasy).

3.2. Character incongruence between genes

The null hypothesis of character congruence between the two molecular data sets was rejected (Table 4: line 1). Removing taxa one by one, followed by new ILD

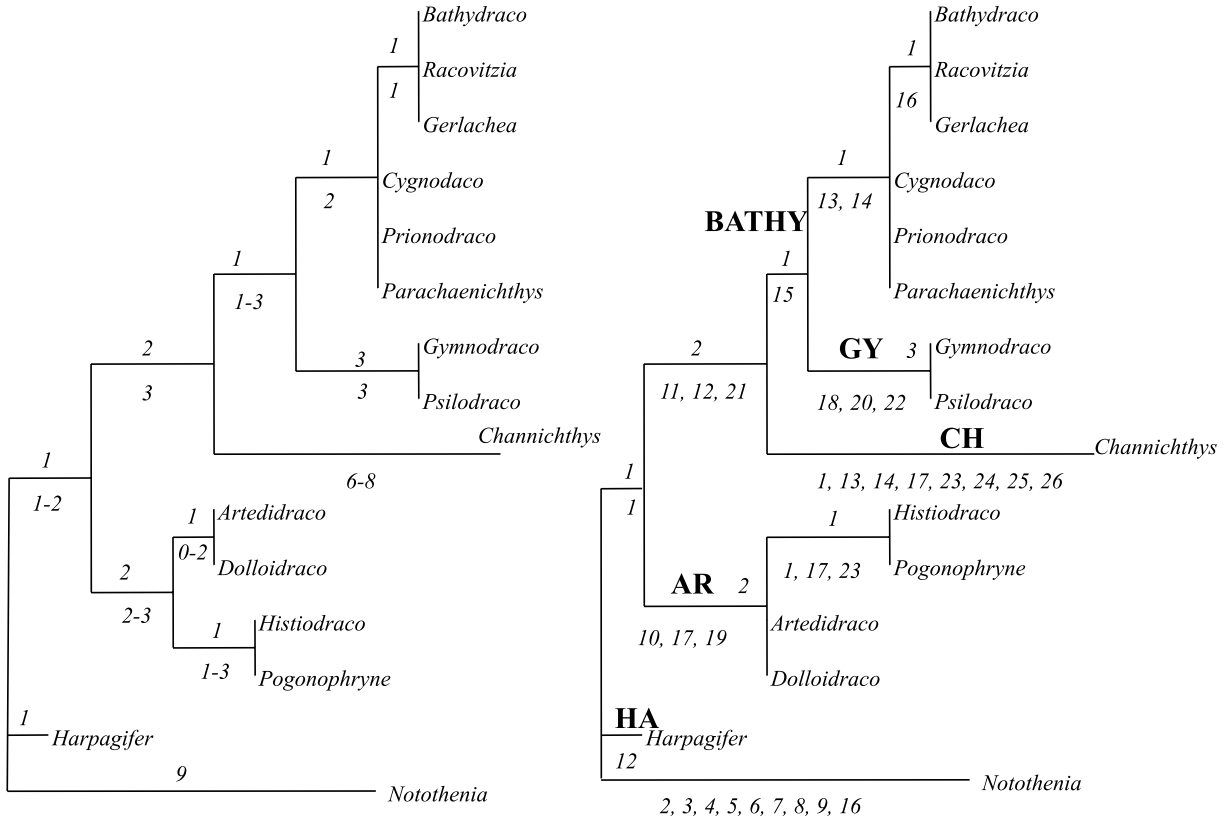

Fig. 3. The two equiparsimonious trees obtained from a Branch-and-Bound search of PAUP4 conducted on the morphological matrix. The length is 35, C.I. = 0.886, R.I. = 0.920. Branch lengths are given under ACCTRAN. Numbers above nodes are Bremer supports (Bremer, 1988, 1994). In the left tree, branch length depends on the optimization of homoplasies and the range of branch lengths is given under each node. In the right tree, as the branch length does not depend on the optimization, numbers under nodes are the list of characters changing at this node. BATHY: Bathydraconidae (the family), other abbreviations: see the legend of Fig. 2.

Table 4
P values of the ILD test performed between the two molecular data sets, with removal of clades, showing that statistically significant character incongruence is localized within the Cygnodraconinae (CY)

Present	Removed	*P* value (ARNIE) (%)	*P* value (PAUP) (%)
All: OUT, HA, AR, BATHY, CH	None	0.5	2
OUT, HA, AR	BATHY, CH	30	37
OUT, CH	HA, AR, BATHY	75	75
OUT, HA, AR, CH	BATHY	17.8	24
OUT, HA, AR, CH, GY	BA, CY	7.3	7
OUT, HA, AR, CH, CY	BA, GY	0.8	2
OUT, HA, AR, CY, BA	CY, GY	13	11
All but Cygnodraco	Cygnodraco	1	1
All but Gerlachea	Gerlachea	1	1
All but *Parachaenichthys charcoti*	*Parachaenichthys charcoti*	1	1
All but *Parachaenichthys georgianus*	*Parachaenichthys georgianus*	4	1

First column: clades present in the ILD test (abbreviations as in Fig. 2). Second column: clades removed in the ILD test. Third column: the resulting *P* value, as calculated by the software ARNIE. Fourth column: the same calculated by PAUP.

Note. No removal of a single species increased the *P* value above the treshold of significance of 5%. AR: Artedidraconidae, BA: Bathydraconinae (a bathydraconid subfamily), BATHY: Bathydraconidae, CH: Channichthyidae, CY: Cygnodraconinae (a bathydraconid subfamily), GY: Gymnodraconinae (a bathydraconid subfamily), HA: Harpagiferidae.

tests as in Lecointre et al. (1998), did not remove incongruence. So, it was not possible to identify a single sequence responsible for character incongruence. Taxa were then removed clade by clade (Table 4), to localize the area of trees where the incongruence plays a role. This incongruence was localized neither at the basal part of the trees (harpagiferids, artedidraconids, Table 4: line 2) nor at the crown (channichthyids, line 3). This anal-

ysis indicated that the significant incongruence was therefore localized within bathydraconid sequences, as the removal of all bathydraconid sequences increased the *P* values above the 5% threshold (compare lines 1–4). Then, it was interesting to detect more precisely in what bathydraconid lineage the incongruence was localized. Lines 5–7 of Table 4 show that it was localized in the Cygnodraconinae (clade CY). In the trees from separate analyses (not shown), a topological difference within cygnodraconines was detected in the relative positions of *Cygnodraco* and *Gerlachea*. There were no means to determine objectively which taxon in which gene was wrong (Table 4, lines 8–11). It was then not possible to remove precisely the stretch of DNA sequence of the gene responsible for incongruence as in Gilles et al. (2001) or in Lecointre and Deleporte (2000).

3.3. Character congruence between molecules and morphology

Molecular characters were not significantly incongruent with morphological data, as shown by the *P* value of the ILD test performed pairwise between the morphological matrix and each of the molecular data sets: the *P* value obtained between morphological matrix and cytochrome *b* sequences was 0.70 and it was 0.05 between the morphological matrix and the control region data. When the ILD test was performed on the three data sets together (i.e., declaring three partitions), the null hypothesis of congruence was not rejected: the *P* value was 0.08, a value close to the treshold of significance of 5%. Comparing the later *P* value to the one obtained by testing morphology against cytochrome *b*, we record a strong decrease which was probably due to the control region data set, as suggested by the incongruence detected above between the two molecular data sets, and also suggested by the extreme *P* value (5%, the threshold value) obtained when morphology was tested against control region.

3.4. Simultaneous analysis of molecular data

The ILD test was used as an exploratory step to understand the structure of the data. The statistically significant incongruence is located within the cygnodraconines without more precise localization. However, in such a situation, it is useful to perform the simultaneous analysis for other parts of the tree, i.e., to see whether this approach can give higher robustness to the position of harpagiferids and the interrelationships of bathydraconid subfamilies. The simultaneous analysis of the two molecular data sets yielded four equiparsimonious trees of 1686 steps. The strict consensus exhibited two collapsed nodes (Fig. 2). These four trees only differed in combinations of the alternative topologies ((BA, GY)(CY, CH)) and (((BA, GY)CY)CH), mixed with two alternative topologies within the genus *Harpagifer*. It is interesting to notice that two of the four equiparsimonious trees recovered monophyletic bathydraconids ((BA, GY)CY). The tree from the simultaneous analysis of all molecular data (Fig. 2) exhibited a general increase of robustness, compared to trees from separate analyses of each gene (not shown), with relationships closer to those given by the cytochrome *b* sequences. Each family was monophyletic, except the bathydraconids, for which neither paraphyly nor monophyly was obtained; harpagiferids were the sister-group of artedidraconids and the sister-group of the channichthyids remained unknown. Interrelationships within the Channichtyidae were well resolved (they were the same in each separate molecular analysis) and strikingly congruent with those found by Iwami (1985) from morphological and anatomical data. These relationships have already been discussed elsewhere (Chen et al., 1998). The interrelationships of artedidraconid genera showed the paraphyly of *Artedidraco*.

3.5. Taxonomic congruence between molecules and morphology

Molecular data failed to recover monophyletic bathydraconids, found three bathydraconid lineages, and found harpagiferids as the sister-group of artedidraconids. Morphological data recovered monophyletic bathydraconids, broke the three bathydraconid lineages as established by molecules, and found harpagiferids as the most basal family. The strength of these topological conflicts was explored by measuring the significance of tree length differences using Wilcoxon's signed-rank test. Using molecular data, monophyletic bathydraconids were constrained with an internal topology respecting the three bathydraconid lineages, as (Cygnodraconinae (Bathydraconinae, Gymnodraconinae)). Using a 5% threshold, this test did not reject the null hypothesis that the length of the optimal molecular tree is not significantly different than that of the constrained tree ($P = 0.62$). The morphological topology within bathydraconids was then constrained. Using a 5% threshold, the test rejected the null hypothesis ($P < 0.001$). Independently, harpagiferids were also constrained to be basal, as in the morphological tree. Using a 5% threshold, the test rejected the null hypothesis ($P = 0.0011$). Difference between molecular tree lengths is therefore not significant when constraining bathydraconid monophyly, but is significant when constraining either the morphological bathydraconid tree or basal harpagiferids. Using morphological data, harpagiferids were constrained to be the sister-group of artedidraconids, which correspond to the molecular hypothesis. The test did not reject the null hypothesis ($P = 0.32$). The topology depicting interrelationships between bathydraconid genera was then constrained to be exactly the one

found by the simultaneous analysis of the two molecules (Fig. 2). The test rejected the null hypothesis ($P = 0.046$). Difference between morphological tree lengths is therefore not significant when constraining harpagiferids to be the sister-group of artedidraconids, but is significant when constraining the three bathydraconid lineages as found by the molecules. The conflict is therefore not strong concerning two points: bathydraconid monophyly can be accepted because it is not significantly rejected by molecular data and the harpagiferids can be considered as the sister-group of artedidraconids, a hypothesis supported by morphology and not significantly rejected by morphological data. The conflict remains for interrelationships of bathydraconid genera and needs to be further explored through the simultaneous analysis of all available data.

3.6. Simultaneous analysis of all available data

Finally, the tree based on the combination of all available data (molecular and morphological) was constructed (15 taxa, Fig. 4). Bathydraconids were found monophyletic and composed of the same three main groups as established by molecular data. Harpagiferids were interestingly not the sister-group of artedidraconids, but were the sister-group of all the rest, the hypothesis supported by morphological data (Fig. 3).

4. Discussion

4.1. Reliability of clades

Reliability in phylogenetics is not statistical robustness, because non-random homoplasy (Hassanin et al., 1998; Naylor et al., 1995), poor taxonomic sampling (Philippe and Douzery, 1994), and processes of discord (Doyle, 1992, 1997; Maddison, 1997) can lead to positively misleading signals. Reliability is closer to the function of confirmation of Carnap (1950), i.e., based on knowledge acquired from different sources. Reliability of clades is based on their repeatability through different investigations (Grande, 1994; Miyamoto and Fitch, 1995; Nelson, 1979). Here, we consider as reliable the monophyly of the Channichthyidae and the interrelationships of genera within this family, corroborated by

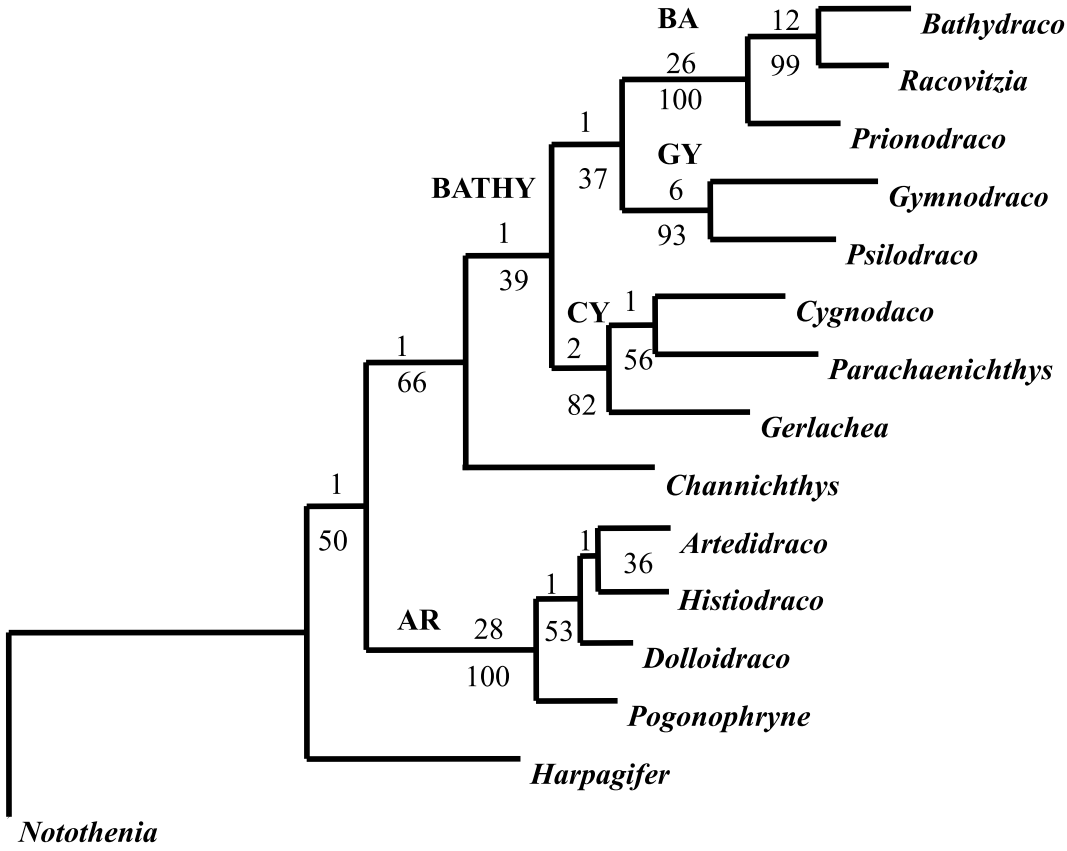

Fig. 4. The most parsimonious tree obtained from the simultaneous analysis of all of the available data (molecular and morphological), through a Branch-and-Bound search of PAUP4. The taxonomic sample is reduced because of the morphological matrix (1068 characters). Tree length is 1070, C.I. = 0.64, R.I. = 0.58. Branch lengths are given under ACCTRAN. The first number is the bootstrap proportion calculated from 1000 iterations, the second number is the Bremer support. Note that the small morphological matrix (26 characters) imposes harpagiferids as the sister-group of all other families, as well as the monophyly of the bathydraconids, while the molecular characters impose bathydraconid intra-relationships.

the study of anatomy of these fishes by Iwami (1985). Also reliable are the monophylies of the Artedidraconidae and the Harpagiferidae. With that definition of reliability, more ambiguous is the status of the three bathydraconid clades (Fig. 2: GY, CY, BA) because if they are independently strongly supported by the present two genes, they are not supported by the present morphological data. Conversely, a lack of reliability affects the position of the harpagiferids and the sister-group of the channichthyids. In the tree based on all the molecular data (Fig. 2), the position of harpagiferids is the same as that in molecular trees based on other molecular data (Bargelloni et al., 2000: 12S and 16S mitochondrial ribosomal sequences) and morphological data (Baluskin, 1992; Eakin, 1981). That position of harpagiferids was found by Hastings (1993) from morphology as equally parsimonious as another solution showing harpagiferids closer to bathydraconids and channichthyids than are artedidraconids, a position never obtained from our data. It is only from our own morphological data that harpagiferids appear as the most basal. Therefore, facing this ambiguous situation, we provisionally consider that harpagiferids may be the sister-group of artedidraconids, because it is, at the moment, the most corroborated scheme across different studies, i.e. moderately supported by our molecular data (Fig. 2: bootstrap proportion of 71% and Bremer support of 4), found from other molecular studies (Bargelloni et al., 2000) and morphological studies (Baluskin, 1992; Eakin, 1981), without being significantly rejected by our morphological data (as shown by Wilcoxon's signed-rank tests) or seriously challenged by any other study. Basal harpagiferids obtained from Fig. 4 can be interpreted as a result of the decrease of the taxonomic sampling, possibly weakening or destroying the molecular signal favouring the clade grouping harpagiferids and artedidraconids.

The three bathydraconid lineages recovered by both molecules (CY, GY, and BA) could become three distinct subfamilies (as is the Gymnodraconinae, Voskoboynikova, 1991; Voskoboynikova and Skóra, 1996); each being monophyletic. However, interrelationships among the three are unclear from our molecular data: topologies are different from one molecule to another and the corresponding nodes all have very low statistical supports. From our simultaneous analysis of all molecular data, two equiparsimonious trees showed monophyletic bathydraconids with the scheme (CH((BA, GY)CY)) and the two others paraphyletic bathydraconids as ((BA, GY)(CY, CH)). In other molecular studies, the number of bathydraconid representatives is not sufficient to allow a precise comparison with the present study. Morphological studies consider bathydraconids as monophyletic, except Hastings (1993) who places the Bathydraconinae (here CY plus BA) closer to the Channichthyidae than to the Gymnodraconinae (GY). Our morphological trees found monophyletic bathydraconids on the basis of the loss of the anterior spinous dorsal fin (character 15 without homoplasy). Morphology and the tree based on all the available data (Fig. 4) lead us to provisionally consider that bathydraconids are monophyletic, a solution that is included in 50% of the equiparsimonious trees involved in the strict consensus of Fig. 2. From Fig. 4, it would be justified to take that result as unreliable, because not robust (based on a bootstrap of 39%) and based on a single morphological trait (Fig. 4). However, the presence/absence of the dorsal spinous fin does not seem to be very labile in perciforms (Nelson, 1994).

4.2. Strength of conflicts

Statistically significant incongruence usually comes from strongly supported conflicting signals. Present conflicts detected through taxonomic incongruence between molecules and morphology did not provoke such character incongruence because within one of the two matrices the signal was rather weak. It is true for the position of harpagiferids, which was not the same as that shown in Figs. 2 and 3, nevertheless, which did not lead to a statistically significant incongruence between molecular and morphological characters (P value of the ILD test is 70% for the comparison cytb/morphology and 5% for the comparison control region/morphology). Harpagiferids were found not to be basal from our molecular data (Fig. 2) and from another gene (Bargelloni et al., 2000). In the morphological matrix, this taxonomic conflict was based on a single morphological character (character 1, Fig. 3). Nevertheless, in the tree based on all the available data (Fig. 4), the small morphological matrix (26 characters) imposed the position of harpagiferids on the molecular data (390 informative positions). It is striking to notice that the morphological matrix did not significantly reject the molecular position of harpagiferids, while the molecular matrix did reject the morphological position of harpagiferids, and that the morphological topology won in the tree based on all data. One could see a contradiction there. However, this contradiction is not real: this may be due to the fact that the taxonomic sampling of the matrix combining all data is reduced compared to the molecular taxonomic sampling, possibly weakening the molecular signal for the clade grouping harpagiferids and artedidraconids, a clade already moderately supported by the present molecular data (Fig. 2: bootstrap proportion of 71% and Bremer support of 4). The same scenario is met within the monophyly of the Bathydraconidae, which is based on a single character without homoplasy (Fig. 3), the loss of the anterior spinous dorsal fin. Once again, the tree based on the whole available data (Fig. 4) does favour the morphological hypothesis, probably because of the lack of molecular

signal either for the paraphyly or for the monophyly of that family. These two examples show that morphological characters, when combined with molecular data, are not automatically "swamped" (Barret et al., 1991; Swofford, 1991). The internal structure of each matrix (for instance high R.I.) or, to put it in another way, the level of internal conflict within each matrix is more important than the number of characters in determining the phylogenetic outcome (Farris et al., 1995).

For the same reasons, molecules can impose some groupings on morphology in the tree based on all the available data, not because they provide more characters, but because they contain a stronger signal for a particular grouping. Within the Bathydraconidae, the morphological tree groups *Bathydraco*, *Racovitzia*, and *Gerlachea* on the basis of a single character state: the presence of scales. This clade is in conflict with the bathydraconinae "BA" and cygnodraconinae "CY" highly supported (in terms of bootstrap proportions) by the two molecular markers independently or jointly. In the tree based on the overall data, this single morphological character does not impose groupings because this time, molecular data strongly support the previous subfamilies. Beyond these methodological outcomes, it is likely that losing and reacquiring scales must be labile event(s) in teleosts. For example, in the Blennioidei, the loss of scales occurred in two families that are not sister-groups (Stepien et al., 1993). Sometimes scales disappear even within the same species (e.g., in carps), though under artificial selection. Interestingly, in Bovichtidae (the most basal notothenioid family), this character is not constant. So the presence of scales in three bathydraconid genera *Bathydraco*, *Racovitzia*, and *Gerlachea* could also be a symplesiomorphy rather than a reversion, depending on the outgroup chosen.

4.3. Bathydraconid monophyly or paraphyly?

In the morphological literature, there is no consensus on the bathydraconid relationships with other families and interrelationships of bathydraconid genera are not known from a morphological cladogram. Bathydraconid monophyly was recovered from our morphological data (on the basis of the synapomorphic loss of the spinous dorsal fin), in two of the four equiparsimonious trees from the simultaneous analysis of the two genes (Fig. 2) and in the tree based on all the available data (Fig. 4). In the molecular strict consensus tree (Fig. 2), there was no signal for or against bathydraconid monophyly, otherwise in other molecular studies, bathydraconid paraphyly is shown through different very poorly supported topologies (e.g., Bargelloni et al., 2000; Ritchie et al., 1997). The global interpretation taking into account morphology and molecules is that the family is monophyletic and composed of three lineages (those highly supported by our two molecular markers). A rapid diversification in a short time span would prevent the recovery of monophyly from molecular data. The way collapsed nodes and well supported nodes are distributed within the tree based on molecular data (Fig. 2) suggests such a rapid diversification. The central collapsed molecular nodes (Fig. 2) are surrounded by upstream robust nodes and downstream robust nodes, suggesting that the collapse is not due to an inappropriate rate of change in the molecules, but rather due to a real short time span between divergence times of bathydraconid subfamilies and the maximum divergence time of the channichthyids (icefishes). Antarctic fishes have been described as one of the very few marine species flocks, which by definition arise quickly and form bush-like phylogenies. If this is true, Antarctic dragonfishes would represent a second burst of diversification, after the sudden nototheniid diversification (Bargelloni et al., 2000).

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