Marine Genomics 8 (2012) 49–58

Contents lists available at SciVerse ScienceDirect

Marine Genomics

journal homepage: www.elsevier.com/locate/margen

# Tracking the elusive monophyly of nototheniid fishes (Teleostei) with multiple mitochondrial and nuclear markers

A. Dettai [a,*], M. Berkani [a], A.-C. Lautredou [a], A. Couloux [b], G. Lecointre [a], C. Ozouf-Costaz [a], C. Gallut [a]

[a] Muséum national d'Histoire naturelle (MNHN), Département Systématique et Évolution, UMR 7138, CP 26, 43, rue Cuvier, 75231 Paris Cedex 05, France
[b] Genoscope, Centre National de Séquençage, 2, rue Gaston Crémieux, CP5706, 91057 Évry cedex, France

ARTICLE INFO

*Article history:*
Received 28 January 2012
Received in revised form 24 February 2012
Accepted 29 February 2012

*Keywords:*
Cytochrome oxidase 1
Phylogeny
Congruence
Simultaneous analysis

ABSTRACT

Since the first molecular study of the suborder Notothenioidei in 1994, many phylogenetic studies have been published. Among these, those with a sufficient number of taxa have all suggested that the Nototheniidae, as currently defined, is monophyletic only with the inclusion of the Channichthyidae, Artedidraconidae, Bathydraconidae and Harpagiferidae. This is corroborated by more recent studies including more taxa, but in these studies either the number of nuclear markers or the number of taxa included remained low. We obtained sequences for a large sampling covering most nototheniid genera for five markers described previously for other samplings (COI, Rhodopsin retrogene, Pkd1, HECW2, and SSRP1) and one nuclear marker never used before in phylogenetic inference (PPM1d). The topology for the combined analysis of the nuclear coding genes, as well as the topology for SSRP1 (non-coding) and the combined analysis for all markers all support the paraphyly of Nototheniidae, the genus *Notothenia* (including *Paranotothenia*) is the sister group of the clade Channichthyidae, Artedidraconidae, Bathydraconidae and Harpagiferidae, and genus *Gobionotothen* is a sister group to both. As in previous studies, *Trematomus*, *Lepidonotothen* and *Patagonotothen* form a clade that also includes *Indonotothenia cyanobrancha*. The position of *Pleuragramma antarctica*, *Dissostichus* species and *Aethotaxis mitopteryx* remains unstable and dependant on markers and analyses.
We therefore propose the inclusion of the four families of the High Antarctic clade in the Nototheniidae, and their transformation into subfamilies. We transfer *Paranotothenia magellanica* to the genus *Notothenia*, as *Notothenia magellanica*.



## 1. Introduction

The teleost fish suborder Notothenioidei is a relatively recent clade (Near, 2004; 2009; Matschiner et al., 2011). The eight families of the suborder include 134 currently recognized species (De Witt, 1970; Gon and Heemstra, 1990; Eakin et al., 2009; Froese and Pauly, 2011). Most Notothenioidei are endemic to the Southern Ocean. They provide a rare marine example of rapid diversification in an extreme and isolated environment. This has been proposed by Eastman and McCune (2000) and investigated as an example of a marine adaptative radiation (Matschiner et al., 2011). Notothenioids represent approximately 76% of the fish species of the Antarctic shelf, and more than 91% for both abundance and biomass (Eastman, 2005). These fishes possess unique physiological attributes and adaptations to their environment (Chen et al., 1997; Cheng et al., 2006; Rutschmann et al., 2011), including antifreeze glycoproteins in five out of eight of the families (except in Bovichthidae, Pseudaphritidae, and Eleginopidae), to the complete loss of hemoglobin in the Channichtyidae.

A good knowledge of their phylogenetic relationships is of utter importance for both optimal taxon selection to represent the group in any functional study and the interpretation of the results. Interrelationships within this crucial component of the Antarctic marine biodiversity must be well characterized for further studies on Antarctic ecosystems and their possible responses to climate change.

There is overall agreement over the position of the three non-Antarctic notothenioid families Bovichthidae, Pseudaphritidae and Eleginopidae. They have diverged first and in this order (Baluskin, 1992; Bargelloni et al., 1994; Lecointre et al., 1997; Ritchie et al., 1997). Four of the remaining families consistently form a clade: Artedidraconidae, Bathydraconidae, Harpagiferidae and Channichthyidae. This group is found in all studies, morphological or molecular (Eakin, 1981; Bargelloni et al., 1994, 2000; Derome et al., 2002; Near et al., 2004; Near and Cheng, 2008; Sanchez et al., 2007). Harpagiferidae and Artedidraconidae are each monophyletic and are sister-groups in all studies. Bathydraconidae and Channichthyidae appear to be closely related. We will hereafter call this group the «High Antarctic Clade» following Near et al. (2004).

The last family, Nototheniidae, is the richest family in terms of both number of species and ecological diversification (Eastman, 1993;

* Corresponding author.
*E-mail addresses:* adettai@mnhn.fr (A. Dettai), mohamed.berkani@hotmail.fr (M. Berkani), ac.lautredou@orange.fr (A.-C. Lautredou), acouloux@genoscope.cns.fr (A. Couloux), lecointr@mnhn.fr (G. Lecointre), ozouf@mnhn.fr (C. Ozouf-Costaz), cyril.gallut@upmc.fr (C. Gallut).

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doi:10.1016/j.margen.2012.02.003

Rutschmann et al., 2011). The composition of the family has varied in the last thirty years. Currently, it contains 12 genera: the three monotypic genera *Aethotaxis*, *Indonotothenia* and *Pleuragramma*, as well as more species diverse genera like *Cryothenia* (2 species), *Dissostichus* (2 species), *Gobionotothen* (5 species), *Gvozdarus* (2 species), *Lepidonotothen* (4 species), *Notothenia* (7 species), *Paranotothenia* (2 species), *Patagonotothen* (14 species), *Trematomus* (15 species when including the two *Cryothenia* and the two *Pagothenia* species, Lautredou et al., submitted for publication).

The two clades *Trematomus-Pagothenia* and *Lepidonotothen-Patagonotothen* (Bargelloni et al., 2000, mitochondrial 12SrDNA and 16SrDNA) were repeated in later studies, and even found to form a clade together (Near et al., 2004; Near and Cheng, 2008; Sanchez et al., 2007; Rutschmann et al., 2011), and *Pagothenia* was integrated into *Trematomus* (Near and Cheng, 2008; Kuhn and Near, 2009; Lautredou et al., submitted for publication). A «pelagic» clade grouping *Aethotaxis*, *Dissostichus*, *Pleuragramma* was found by Near et al. (2004, 16S), Sanchez et al. (2007), based on the recoding of Balushkin (2000) and Near and Cheng (2008) but only for some datasets and analyses. *Gvozdarus* might also be part of this group, but for this last genus, results are only based on morphological characters (Sanchez et al., 2007). The components of this "pelagic" clade are dispersed in the tree in other analyses (Near and Cheng, 2008; Rutschmann et al., 2011). The genera *Gobionotothen, Dissostichus, Patagonotothen* appear monophyletic in all studies. So does the genus *Notothenia,* although *Paranotothenia* clusters within this genus in the studies where it is present (Near et al., 2004; Near and Cheng, 2008; Sanchez et al., 2007).

However the monophyly of Nototheniidae remains elusive. The first molecular study including a sufficient number of taxa (Bargelloni et al., 1994) did not recover the family as monophyletic in its currently defined composition, whether based on partial 12S rDNA, 16SrDNA, or hemoglobin sequences. The three families from the High Antarctic clade (Artedidraconidae, Channichthyidae and Bathydraconidae) present in the study were nested within the Nototheniidae. Their sister group was either the representatives of the genus *Notothenia* (Parsimony Analysis for both 12S, 16S and hemoglobin) or a *Notothenia*/*Gobionotothen*/*Dissostichus* cluster (NJ distance method). The nototheniid genus *Trematomus* appeared in both trees as the first to diverge after the outgroup.

Most subsequent molecular studies have either failed to resolve the Nototheniidae as a group (Bargelloni et al., 2000; Sanchez et al., 2007), or cast doubt on its monophyly (Near and Cheng, 2008; Matschiner et al., 2011; Rutschmann et al., 2011; Tomasziewicz et al., 2011), almost always with the same (((High Antarctic clade) *Notothenia*)*Gobionotothen*) topology. Only a minority of studies recovered the family as a clade (Near et al., 2004; Near and Cheng, 2008), but not consistently accross markers, and depending on whether the analysis was performed with parsimony analysis (PA), Bayesian inference (BI) or maximum likelihood (ML)(Near and Cheng, 2008).

However, most of the studies cited above are based only on mitochondrial data, and one of them pointed out a possible incongruence between nuclear and mitochondrial data (Near and Cheng, 2008). The others include either a single nuclear marker (Bargelloni et al., 1994; Near and Cheng, 2008) or a reduced taxonomic sampling (Bargelloni et al., 1994; Matschiner et al., 2011; Rutschmann et al., 2011).

The present phylogenetic study samples most genera of the High Antarctic Clade and Nototheniidae genera, and most species within the later (Table 1). The study is based on five variable nuclear markers, including one never used before, the partial sequence of the protein phosphatase, Mg2+/Mn2+ dependent, 1D (PPM1d)-coding gene. The other four nuclear markers had previously been used with success on the genus *Trematomus* (Lautredou et al., submitted for publication): the partial rhodopsin retrogene, Pkd1, HECW2 and the intron 9 of SSRP1 and flanking exonic sequences. These markers present a relatively high rate of nucleotide divergence even among closely related species, which is necessary for a better support of the nodes from the nuclear data. One mitochondrial marker, the partial sequence coding for the cytochrome oxidase 1 was also included, as previous studies had shown its value within notothenioids (Tomasziewicz et al., 2011; Lecointre et al., 2011). Comparing inferences drawn from independent markers or combinations of markers allows examination of the reliability of clades. It helps to detect problems due to a single marker imposing its signal (whether phylogenetic or not) on the whole analysis (Dettai and Lecointre, 2004). The results obtained here were thus compared with those of Near and Cheng (2008) for the mitochondrial 16S, ND2 and three tRNAs and nuclear partial S7 protein-coding gene and those of Rutschmann et al. (2011) for the mitochondrial partial cytochrome b, and nuclear partial myh6, Ptr, tbr1 coding genes. Comparing these three studies allows to take into account almost all datasets studied previously for the group with multiple analysis methods, without shared marker across studies, yielding independent corroboration by multiple large datasets.

## 2. Material and methods

### 2.1. New marker selection

The new marker PPM1d was identified using the complete genomes available in the ENSEMBL database following the Lautredou et al. (submitted for publication) protocol modified from Li et al. (2009). A list of shared protein-coding sequences was obtained through genome/genome filtering in the ENSEMBL database release 41 using the Biomart mining tool (Haider et al., 2009). *Tetraodon nigroviridis* was used as a dataset, and only sequences that also had unique best hits in the genomes of *Takifugu rubripes* and *Danio rerio* were retained. In the resulting list, coding sequences longer than 500 base pairs were investigated, beginning with the sequences with high sequence divergence between *T. nigroviridis* and *T. rubripes* to maximize our chances to identify markers that provide information at a smaller scale. These were checked for the presence of exons of sufficient length (>500 bp), first on *T. nigroviridis* and then on all other teleost genomes from ENSEMBL. Sequences of long exons were downloaded from ENSEMBL, and sequences for additional teleost taxa were recovered for some markers in GenBank using the *Gasterosteus aculeatus* sequences as a blast query. All available sequences were aligned using BioEDIT (Hall, 1999). The sequence alignments were used to identify conserved regions by visual inspection, and primers were defined in these areas. They were then checked using OLIGO 4.1. Several primers were ordered and tested for each new marker, and the markers that could be amplified straightforwardly were retained. The location of the markers, of the amplified fragment, as well as the description of the corresponding gene in the release 62 of the genome of *Gasterosteus aculeatus* (the closest relative within the available genomes) are given in Table 2.

### 2.2. Amplification and sequencing

The same specimen was consistently used for all markers, even if it meant having some missing sequences. Members of the three first notothenioid families to diverge (Bovichthidae, Pseudaphritidae, and Eleginopidae) were included as outgroup (Lecointre et al., 1997; Sanchez et al., 2007; Near et al., 2004; Near and Cheng, 2008). Samples are listed in Table 1. Tissue samples were stored in 85% ethanol and extracted following Winnepenninckx et al. (1993). Published primers were used for the markers already used before: the Barcode region of the cytochrome oxidase I (Folmer et al., 1994), the partial rhodopsin retrogene (Chen et al., 2003), Pkd1 (Lautredou et al., 2010) and HECW2 and SSRP1 (Lautredou et al., submitted for publication). The primers for the new marker were

**Table 1**
Taxonomic sampling and sequence accession numbers.
Accession and/or Barcode reference numbers for new sequences are in bold.

| Family | Genus and species | Location | Sample ref | Voucher ref | COI | Rh | SSRP1 | PPM1d | PKD1 | HECW2 |
|---|---|---|---|---|---|---|---|---|---|---|
| *Bovichtidae* | | | | | | | | | | |
| | *Bovichtus diacanthus* | Tristan Da Cunha | **ICTI1205** | MNHN2005-0102 | **FKCI042** | **FKCI042** | **–** | **JQ688685** | **JQ688741** | **JQ688788** |
| | *Cottoperca gobio* | Burdwood Banks | **ICTI1233** | BB | **EATFR003** | **EATFR003** | **–** | **–** | **JQ688745** | **JQ688794** |
| *Pseudaphritidae* | | | | | | | | | | |
| | *Pseudaphritis urvillii* | Tasmania, Brown's River, Kingston | **ICTI115** | 1 | **EATFR004** | **EATFR004** | **–** | **JQ688684** | **JQ688740** | **JQ688787** |
| *Eleginopsidae* | | | | | | | | | | |
| | *Eleginops maclovinus* | Falklands | **ICTI1234** | MNHN2005-0093 | **FKCI050** | **FKCI050** | **JQ688849** | **JQ688691** | **JQ688746** | **JQ688795** |
| *Nothotheniidae* | | | | | | | | | | |
| | *Aethotaxis mitopteryx* | Weddell Sea | **ICTI351** | W128M | **EATFR005** | **EATFR005** | **–** | **JQ688713** | **JQ688762** | **JQ688814** |
| | *Dissostichus mawsoni* | Terre Adélie | **ICTI388** | MNHN2001-1143 | **EATFR006** | **EATFR006** | **JQ688869** | **JQ688715** | **JQ688764** | **JQ688816** |
| | *Dissostichus eleginoides* | Burdwood Banks | **ICTI1222** | BB/65 | **EATFR007** | **EATFR007** | **JQ688847** | **JQ688688** | **JQ688743** | **JQ688791** |
| | *Gobionotothen marionensis* | South Sandwich Isl. | **ICTI1225** | MNHN2005-0088 | **FKCI044** | **FKCI044** | **–** | **JQ688689** | **JQ688744** | **JQ688792** |
| | *Gobionotothen gibberifrons* | Shag Rocks | **ICTI1229** | MNHN2005-0099 | **FKCI048** | **JQ063276** | **JQ063309** | **JQ688690** | **JQ063250** | **JQ063172** |
| | *Gobionotothen acuta* | Kerguelen Isl. | **Aus36** | MNHN2007-1878 | **EATFR008** | **EATFR008** | **JQ688844** | **JQ688723** | **JQ688771** | **JQ688825** |
| | *Indonotothenia cyanobrancha* | Kerguelen Isl. | **ICTIAus30** | MNHN2007-1844 | **EATFR009** | **EATFR009** | **JQ688843** | **JQ688722** | **JQ688770** | **JQ688824** |
| | *Lepidonotothen mizops* | Kerguelen Isl. | **ICTI943** | | **FKCI067** | **FKCI067** | **JQ688875** | **JQ688720** | **JQ688768** | **JQ688822** |
| | *Lepidonotothen nudifrons* | Shag Rocks | **ICTI1245** | SR/LN2 | **FKCI051** | **FKCI051** | **JQ688850** | **JQ688692** | **JQ688747** | **JQ688796** |
| | *Lepidonotothen larseni* | Bouvet Island | **ICTI1247** | SAIAB74974 | **FKCI053** | **FKCI053** | **–** | **JQ688693** | **JQ688748** | **JQ688797** |
| | *Lepidonotothen squamifrons* | Bouvet Island | **ICTI1251** | BO/2 | **FKCI057** | **JQ063278** | **JQ063311** | **JQ688694** | **JQ063252** | **JQ063174** |
| | *Notothenia neglecta* | Terre Adélie | **ICTI137** | TA13 | **EATFR010** | **EATFR010** | **JQ688861** | **JQ688707** | **JQ688757** | **JQ688808** |
| | *Notothenia coriiceps* | Terre Adélie | **ICTI655** | MNHN2008-1888 | **FKCI066** | **JQ063275** | **JQ063308** | **JQ688719** | **JQ063249** | **JQ063171** |
| | *Notothenia rossii* | South Georgia | **ICTI1218** | SG | **FKCI043** | **FKCI043** | **JQ688846** | **JQ688687** | **JQ688742** | **JQ688790** |
| | *Notothenia angustata* | | **ICTINang1** | | **FKCI069** | **FKCI069** | **JQ688876** | **JQ688724** | **JQ688772** | **JQ688826** |
| | *Paranotothenia magellanica* | Kerguelen Isl. | **ICTIAus29** | MNHN2007-1843 | **EATFR011** | **EATFR011** | **JQ688842** | **JQ688721** | **JQ688769** | **JQ688823** |
| | *Patagonotothen squamiceps* | Falklands | **ICTI1254** | F | – | **EATFR012** | **JQ688852** | **JQ688695** | **JQ688749** | **JQ688799** |
| | *Patagonotothen wiltoni* | Falklands | **ICTI1255** | F | **FKCI060** | **FKCI060** | **JQ688853** | **JQ688696** | **JQ688750** | **JQ688800** |
| | *Patagonotothen guntheri* | Bouvet Island | **ICTI1256** | SAIAB75162 | **FKCI061** | **JQ063277** | **JQ063310** | **JQ688697** | **JQ063251** | **JQ063173** |
| | *Patagonotothen ramsayi* | Falklands | **ICTI1262** | F | **FKCI062** | **FKCI062** | **JQ688855** | **JQ688698** | **JQ688751** | **JQ688802** |
| | *Patagonotothen tessellata* | Falklands | **ICTI1268** | SAIAB75143 | **FKCI063** | **FKCI063** | **JQ688856** | **JQ688699** | **JQ688752** | **JQ688803** |
| | *Pleuragramma antarctica* | Weddell Sea | **ICTI323** | BPN01/W114F | **EATFR013** | **EATFR013** | **JQ688867** | **JQ688711** | **JQ688761** | **JQ688813** |
| | *Trematomus loennbergii* | Terre Adélie | **ICTI139** | MNHN1996-0326 | **GU997426** | **GU997314** | **JQ688864** | **JQ688708** | **JQ688758** | **JQ688810** |
| | *Trematomus newnesi* | Terre Adélie | **TA50TRNE1** | MNHN2001-1150 | **GU997428** | **GU997349** | **JQ063361** | **JQ688712** | **GU997563** | **JQ063224** |
| | *Trematomus vicarius* | South Georgia | **ICTI1212** | | **EATF588** | **GU997388** | **JQ063385** | **JQ688686** | **GU997595** | **JQ063248** |
| | *Trematomus lepidorhinus* | Weddell Sea | **ICTI1368** | | **GU997424** | **GU997317** | **JQ688860** | **JQ688704** | **GU997518** | **JQ688807** |
| | *Trematomus eulepidotus* | Weddell Sea | **ICTI1370** | | **EATF585** | **GU997298** | **JQ688862** | **JQ688705** | **JQ688756** | **JQ688809** |
| | *Trematomus scotti* | Weddell Sea | **ICTI1371** | | **GU997448** | **GU997374** | **JQ688863** | **JQ688706** | **JQ688785** | **JQ688786** |
| | *Trematomus hansoni* | Terre Adélie | **TA646TrHa1** | | **GU997418** | **GU997307** | **JQ688890** | **JQ688736** | **GU997509** | **JQ688840** |
| | *Trematomus borchgrevinki* | Terre Adélie | **ICTI391** | MNHN2002-1711 | **EATF594** | **GU997240** | **JQ688870** | **JQ688716** | **JQ688765** | **JQ688817** |
| | *Trematomus bernacchii* | Terre Adélie | **TA650TrBe1** | | **GU997408** | **GU997262** | **JQ688891** | **JQ688737** | **JQ688784** | **JQ688841** |
| | *Trematomus nicolai* | Terre Adélie | **TrNi5** | | **GU997440** | **GU997358** | **JQ688877** | **JQ688738** | **GU997574** | **JQ688827** |
| | *Trematomus pennellii* | Terre Adélie | **392TrPe** | | **GU997442** | **JQ693498** | **JQ688871** | **JQ688739** | **GU997583** | **JQ688818** |
| | *Trematomus tokarevi* | Dumont d'Urville Sea | **si171n1296** | MNHN2009-1250 | **EATF169** | **EATF169** | **JQ688882** | **JQ688703** | **GU997591** | **JQ688832** |
| *Channichthyidae* | | | | | | | | | | |
| | *Chionodraco hamatus* | Terre Adélie | **ICTI385** | TA41CHHAI | **EATFR014** | **EATFR014** | **JQ688868** | **JQ688714** | **JQ688763** | **JQ688815** |
| | *Neopagetopsis ionah* | Terre Adélie | **ICTI298** | TA59NEOI01 | **EATFR015** | **EATFR015** | **JQ688866** | **JQ688710** | **JQ688760** | **JQ688812** |
| | *Dacodraco hunteri* | Dumont d'Urville Sea | **si136n845** | MNHN2009-1126 | **EATF135** | **EATF135** | **JQ688879** | **JQ688726** | **JQ688774** | **JQ688829** |
| | *Chionobathyscus dewitti* | Dumont d'Urville Sea | **si280n2119** | MNHN2009-1155 | **EATF275** | **EATF275** | **JQ688887** | **JQ688733** | **JQ688781** | **JQ688837** |
| | *Chaenocephalus aceratus* | South Sandwich Isl. | **ICTI1283** | MNHN2005-0092 | **EATFR016** | **EATFR016** | **JQ688858** | **JQ688701** | **JQ688754** | **JQ688805** |
| | *Champsocephalus gunnari* | Shag Rocks | **ICTI1271** | SR | **FKCIR091** | **FKCIR091** | **JQ688857** | **JQ688700** | **JQ688753** | **JQ688804** |
| *Bathydraconidae* | | | | | | | | | | |
| | *Gymnodraco acuticeps* | Terre Adélie | **ICTI241** | TA255GYVI1 | **EATFR017** | **EATFR017** | **JQ688865** | **JQ688709** | **JQ688759** | **JQ688811** |
| | *Prionodraco evansii* | Dumont d'Urville Sea | **si291n2190** | MNHN2009-1078 | **EATF286** | **EATF286** | **JQ688889** | **JQ688735** | **JQ688783** | **JQ688839** |
| | *Gerlachea australis* | Dumont d'Urville Sea | **si146n893** | MNHN2009-1043 | **EATF145** | **EATF145** | **JQ688880** | **JQ688727** | **JQ688775** | **JQ688830** |
| | *Cygnodraco mawsoni* | Terra Nova Bay | **ICTI559** | C.maw 01 (2184) | **EATFR018** | **EATFR018** | **JQ688873** | **JQ688718** | **JQ688767** | **JQ688820** |
| | *Vomeridens infuscipinnis* | Dumont d'Urville Sea | **si248n1964** | MNHN2009-1074 | **EATF243** | **EATF243** | **JQ688886** | **JQ688732** | **JQ688780** | **JQ688836** |
| | *Parachaenichthys georgianus* | South Georgia | **ICTI1291** | SG | **EATFR019** | **EATFR019** | **JQ688859** | **JQ688702** | **JQ688755** | **JQ688806** |
| | *Acanthodraco dewitti* | Dumont d'Urville Sea | **si155n719** | MNHN2009-1050 | **EATF154** | **EATF154** | **JQ688881** | **JQ688728** | **JQ688776** | **JQ688831** |
| | *Racovitzia glacialis* | Dumont d'Urville Sea | **si225n1698** | MNHN2009-1067 | **EATF220** | **EATF220** | **JQ688884** | **JQ688730** | **JQ688778** | **JQ688834** |
| *Artedidraconidae* | | | | | | | | | | |
| | *Dolloidraco longedorsalis* | Dumont d'Urville Sea | **si182n1370** | MNHN2009-0955 | **EATF179** | **EATF179** | **JQ688883** | **JQ688729** | **JQ688777** | **JQ688833** |
| | *Pogonophryne scotti* | Dumont d'Urville Sea | **si238n1819** | MNHN2009-1389 | **EATF233** | **EATF233** | **JQ688885** | **JQ688731** | **JQ688779** | **JQ688835** |
| | *Histiodraco velifer* | Dumont d'Urville Sea | **si283n978** | MNHN2009-0967 | **EATF278** | **EATF278** | **JQ688888** | **JQ688734** | **JQ688782** | **JQ688838** |
| | *Artedidraco loennbergi* | Dumont d'Urville Sea | **si11n91** | MNHN2009-0938 | **EATF011** | **EATF011** | **JQ688878** | **JQ688725** | **JQ688773** | **JQ688828** |
| *Harpagiferidae* | | | | | | | | | | |
| | *Harpagifer kerguelensis* | Kerguelen Isl. | **ICTI412** | MNHN2000-0269 | **EATF605** | **EATF605** | **JQ688872** | **JQ688717** | **JQ688766** | **JQ688819** |

defined for this study (see Table 2). Primers were defined and tested for a second segment of Pkd1 (Table 2), but no specimens were sequenced for the present study. The PCRs were run in a final volume of 20 μl (5% of DMSO, 5 μg of bovine serum albumine, 300 μM of each dNTP, 0.3 μM of Taq DNA polymerase (Qiagen), 2.5 μl of the corresponding buffer, and 1.7 pM of each of the two primers). After denaturation for 2 min at 94 °C, the PCR ran for 45 to 55 cycles of (20 s, 94 °C; 25 s, 50 °C; 1 mn 72 °C), with a terminal elongation of 3 min at 72 °C on Biometra thermocyclers. Purification and sequencing of the PCRs were performed at the Genoscope (http://www.genoscope.cns.fr/) using the same primers. All sequences were obtained in both directions and checked manually against their chromatogram using Sequencher 4.8 (Gene Codes Corporation). They were aligned by hand using Bioedit (Hall, 1999), and were controlled for mix-ups and contaminations by pairwise sequence comparison. The new COI and rhodopsin retrogene sequences were deposited in the Barcode of Life database with the specimen and collection data, the sequences for the other markers were deposited in GenBank. The SSRP1 and HECW2 datasets contained long insertions for a few species, which were trimmed to the parts with a reliable alignment. The analysed datasets are available in the additional material.

### 2.3. Sequence analyses

Using MEGA, numbers of differences between sequences for shared pairs of species were calculated for all the nuclear markers currently available (Near and Cheng, 2008; Rutschmann et al., 2011; Matschiner et al., 2011; present study).

All markers were analysed separately using PA and BI. Two concatenated datasets were assembled: one with all datasets, and one with only the four coding nuclear markers (Rhodopsin, HECW2, PPM1d and Pkd1). In this second dataset, SSRP1 was not included because it has more missing data than the other datasets. COI was left out because it has a number of informative characters much higher than any of the other datasets (Table 3), and might therefore have a disproportionate influence on the result when included. Both AIC and BIC approaches as implemented in Modeltest 3.7 (Posada and Buckley, 2004) were used to identify the level of complexity of the model of nucleotide substitution that best fit the combined dataset.

Parsimony analysis: Tree searching was done with PAUP* 4.0b10 (Swofford, 2003), using heuristic searches with 100 random additions, TBR (Tree Bisection and Reconnection) branch swapping algorithm, keeping a maximum of 10,000 multiple equi-parsimonious trees. A strict consensus was calculated over the resulting trees. The same parameters were used for all datasets. A bootstrap analysis was performed for the four concatenated coding nuclear markers, using 1000 replicates of heuristic searches with 100 random additions, TBR branch swapping algorithm and keeping multiple equi-parsimonious trees.

Bayesian inference: For the concatenated datasets, 8 analyses were run with 10,000,000 generations sampled every 500th step, unlinked partitioning by codon positions and a GTR + G + I model, which was the most complex model suggested for a partition by Modeltest (Posada and Buckley, 2004). 4 analyses were run for each separate marker, with 10,000,000 generations sampled every 500th step, unlinked partitioning by codon positions and a GTR + G + I model in order to be as similar as possible to the concatenated analysis using Mr. Bayes (Huelsenbeck and Ronquist, 2001). The first 25% of the sampled trees were eliminated after checking that the burnin was within this region. After checking convergence had been reached, the trees and parameters resulting from the analyses that had reached convergence were pooled and combined in a consensus.

## 3. Results

The description of the datasets is given in Table 3. There is little missing data, except in SSRP1 where only *Eleginops maclovinus* could be obtained among the outgroups. The nuclear markers used in this study are all located on different linkage groups (corresponding to chromosomes) in the stickleback genome (see Table 1) and have no

**Table 2**
Marker list and primer sequences.
Full names of the markers and location in the *Gasterosteus aculeatus* (stickleback) genome are given using the ENSEMBL release 62. The second fragment of Pkd1 was not amplified for the present study as it is not independent in location and function from the first fragment, however the primers work over the whole range of species.

| | Gene | Description (Source: HGNC Symbol) | Location of the amplified fragment in the gene* | Gene location: Group (position)* | Frag. Size | Primer name | Primers | Hyb. temp. | Best pair | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| Mitoc. | COI | Cytochrome oxidase 1 | 3′ end (Folmer region) | Mitochondrial genome | ≈650 bp | TelF1 | 5′-TCGACTAATCAYAAAGAYATYGGCAC-3′ | 52°C | * | Dettai et al. (2011) |
| | | | | | | TelR1 | 5′-ACTTCTGGGTGNCCAAARAATCARAA-3′ | | * | |
| Nuclear | Rh | Rhodopsin retrogene | Partial sequence | GroupXII (809,696-810,650) | ≈830 bp | RhF193 | 5′-CNTATGAATAYCCTCAGTACTACC-3′ | 50°C | * | Chen et al. (2003) |
| | | | | | | RhR1039 | 5′-TGCTTGTTCATGCAGATGTAGA-3′ | | * | |
| | Pkd1 | Polycystic kidney disease 1 | 5′ part of exon 20 (Transcript ENSGACT00000025103) | GroupIX (14,963,056-15,005,610) | ≈890 bp | Pkd1F62 | 5′-CATGAGYGTCTACAGCATCCT-3′ | 50°C | * | Lautredou et al. (2010) |
| | | | | | | Pkd1R952 | 5′-YCCTCTNCCAAAGTCCCACT-3′ | | * | |
| | | | | | Not sequenced | Pkd1F1511 | 5′-YATGTTCTACACNTCCGCTC-3′ | | | This study |
| | | | | | | Pkd1F1605 | 5′-GTNCARCGTGAGCTGGAGG-3′ | | | |
| | | | | | ≈1050 bp | Pkd1R2587 | 5′-GAGCNGTGAGRTTCACCATGT-3′ | | | |
| | HECW2 | HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2 | Partial exon 9 | GroupI (25,889,267-25,906,930) | ≈670 bp | HECWF153 | 5′-CAATGGTSCTTGTTACTATGRAGA-3′ | 55°C | | Lautredou et al. (submitted for publication) |
| | | | | | | HECWF160 | 5′-GCTTGTTACTATGNAGAYGACAG-3′ | | | |
| | | | | | | HECWF170 | 5′-ATGAAGAYGACAGYGTGTGGC-3′ | | * | |
| | | | | | | HECWR838 | 5′-CTCACCTGAATGGGKGAAAG-3′ | | * | |
| | PPM1d | Protein phosphatase, Mg2 + /Mn2 + dependent, 1D | Almost complete exon 1 | GroupI (18,501,183-18,506,137) | ≈410 bp | PPMF45 | 5′-AGGRGGNAGGAAATACATGGA-3′ | 50°C | * | This study |
| | | | | | | PPMR457 | 5′-CAGGCTAYRAATCCTTTGCG-3′ | | * | |
| | SSRP1 | Structure specific recognition protein 1 | Exon 9, intron 9–10, partial exon 10 (Transcript ENSGACT00000023577) | GroupIV (9,245,999-9,256,786) | ≈560 bp | SSRPF34 | 5′-RTTTCCTTGAAGCGCAGGTG-3′ | 55°C | * | Lautredou et al. (submitted for publication) |
| | | | | | | SSRPR600 | 5′-GCAAACTGAGCTATGGTTGT-3′ | | * | |

**Table 3**
Description of the datasets for parsimony analysis.

| Marker | Dataset length | Nb of sequences | Constant characters | PA informative characters | % PA informative characters | Number of parsimonious trees | Tree length | Retention index (PA) |
|---|---|---|---|---|---|---|---|---|
| SSRP1 | 519 | 51 | 373 | 60 | 11.6 | 7976 | 226 | 0.8825 |
| COI | 651 | 56 | 396 | 233 | 35.8 | 6 | 1540 | 0.6431 |
| PPM1d | 495 | 56 | 333 | 72 | 14.5 | 2 | 223 | 0.8935 |
| Rh | 777 | 57 | 582 | 128 | 16.5 | >10,000 | 496 | 0.7400 |
| HECW2 | 687 | 57 | 410 | 132 | 19.2 | >10,000 | 433 | 0.8187 |
| pkd1 | 867 | 57 | 626 | 142 | 16.4 | 7395 | 367 | 0.8477 |
| Concatenation of Rh, HECW2, PPM1d, pkd1 | 2826 | 57 | 1951 | 474 | 16.7 | 599 | 1559 | 0.7779 |
| Concatenation of all markers | 3996 | 57 | 2628 | 767 | 19.2 | 2 | 3385 | 0.70 |

commonality of function. They can therefore be considered independent. The nucleotide divergence for species pairs shared in several studies (Near and Cheng, 2008; Rutschmann et al., 2011) for the different markers is presented in Fig. 1. The present study includes both nuclear markers that are among the most variable available for the group (Rh, pkd1, and SSRP1), as well as less variable ones (PPM1d).

The clades present in the topologies resulting from the separate and simultaneous analyses, as well as those present in Near and Cheng (2008) and Rutschmann et al. (2011) are summarized in Table 4. Only the clades that are repeated, shared by several studies, and necessary for the discussion are scored.

### 3.1. Maximum parsimony topologies

The separate analyses in PA present many polytomies (question marks in Table 4). Most clades found in the two simultaneous analyses (nuclear coding markers only and all markers) are also present in several of the trees inferred from the separate analyses of the different markers (Table 4), so they are not imposed by a single marker over the others (Dettai and Lecointre, 2004). For many clades, there are no contradictors (alternative resolved clade) for any marker. Most of the relationships at small scale (within genera, and within families in the High Antarctic Clade) are either unresolved (most separate analyses) or vary from one analysis to the other depending on markers and analysis method.

The High Antarctic Clade is mostly either recovered (simultaneous analyses, Pkd1, SSRP1, HECW2), or not resolved (Rh, PPM1d). It is not recovered for COI. However, the basis of the ingroup in the COI PA consensus tree is *Prionodraco evansii*, a bathydraconid, followed by the rest of the bathydraconids, and the other three families from the High Antarctic Clade. This is a very unusual position for the root of the tree, as the High Antarctic Clade is consistently monophyletic in the other analyses, and *P. evansii* is also consistently associated with other bathydraconids. Our sequences for COI for all these taxa were checked using blast searches on the Barcode of Life Database, and none appears to be a contamination. The monophyly of Bathydraconidae is only recovered by PPM1d and the mitochondrial dataset in Near and Cheng (2008), while the monophyly of Artedidraconidae and Channichthyidae is recovered by almost all analyses (separate and simultaneous), or unresolved (Artedidraconids with pkd1). The presence of the group uniting Harpagiferidae and Artedidraconidae is marker dependent.

The monophyly of the family Nototheniidae is not recovered with any of the nuclear markers. Nototheniidae are paraphyletic in the topologies obtained with the simultaneous analyses and all separate analyses but Rh (polytomy). The same contradicting topology is repeated in the simultaneous analyses as well as in several separate analyses (pkd1, PPM1d, SSRP1 and S7 of Near and Cheng, 2008): the clade including the three species of *Notothenia* and *Paranotothenia magellanica* is the sister group of the High Antarctic Clade. In a similarly repeated fashion, the *Gobionotothen* genus is the sister group of this clade. This topology is not present in HECW2 and Rh (polytomy), as well as in COI and the PA analysis of the mitochondrial dataset of Near and Cheng (2008). These last analyses are the only PA ones that recover the monophyly of the Nototheniidae. As described previously (Bargelloni et al., 2000; Near et al., 2004; Sanchez et al., 2007; Near and Cheng, 2008), *Trematomus*, *Lepidonotothen*

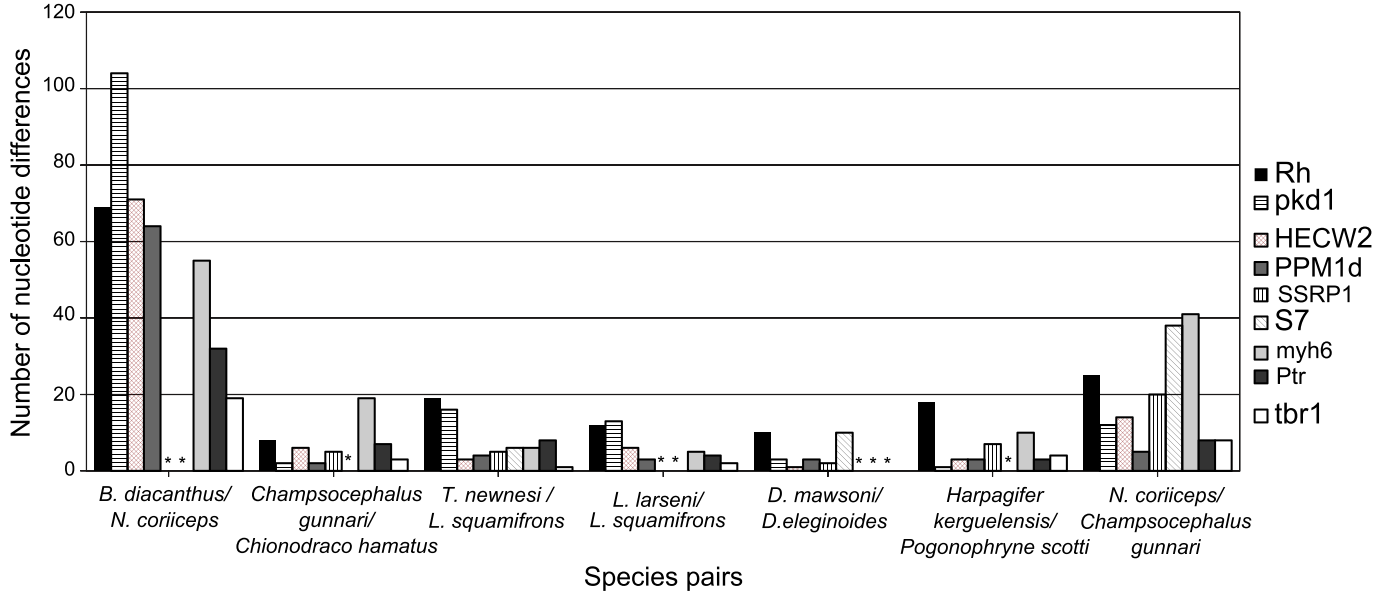


**Fig. 1.** Divergence of species pairs for the different markers. The pairs were chosen to represent both closely and less closely related species, and to maximize the sampling overlap between the present study, Near and Cheng (2008) and Rutschmann et al. (2011). Markers for which there is no data for a pair are indicated by a *.

**Table 4**
Clade repetition in the various analyses.
"–" indicates clades not evaluated because of missing taxa, "X" denotes clade presence, "no" marks the presence of a contradictory clade (some of the elements of the clade are associated with other terminals in an incompatible group), "?" marks a polytomy. "SG" is the abbreviation for sister group. Clades in grey are the ones considered for the evaluation of the markers. For the BI analyses, X in bold have a posterior probability superior or equal to 90.

| | | Maximum parsimony | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Clade content | Clade Abbreviation | Rh | pkd1 | HECW2 | PPM1d | Combined nuc. coding genes | COI | SSRP 1 | Combined all datasets | Mitochondrial Near and Cheng (2008) | S7 Near and Cheng (2008) |
| Nototheniid monophyly | Not | ? | no | no | no | no | no | no | no | X | no |
| High Antarctic clade monophyly | H | ? | X | X | ? | X | no | X | X | X | X |
| *Notothenia* and *Paranotothenia clade* closest to H | NH | ? | X | ? | X | X | no | X | X | no | G closer |
| *Gobionotothen* species with clade NH | GNH | ? | X | ? | X | X | no | X | X | no | X |
| *Trematomus, Lepidonotothen, Patagonothoten* clade | TLP | ? | ? | X | X | X | X | ? | X | X | X |
| *Paranotothenia magellanica* included in *Notothenia* | | ? | X | SG to *N. angustata* | ? | X | X | X | X | X | ? |
| Bathydraconid monophyly | B | ? | ? | ? | X | no | no | no | no | X | ? |
| Artedidraconid monophyly | A | X | ? | X | X | X | X | X | X | X | X |
| Channichthyid monophyly | C | X | X | X | X | X | X | X | X | X | X |
| *Indonotothenia cyanobrancha* with or within clade TLP | | ? | X | X | X | X | X | ? | X | – | - |
| *Trematomus* monophyly | T | ? | no | ? | ? | X | no | ? | no | X | ? |
| *Patagonotothen* monophyly | Pa | X | ? | ? | X | X | X | ? | X | X | X |
| *Lepidonotothen* monophyly | L | ? | no | ? | ? | no | no | ? | no | ? | ? |
| *Lepidonotothen squamifrons* with *Patagonotothen* | | ? | ? | ? | ? | X | X | ? | X | ? | ? |
| Position of *Pleuragramma antarctica* | P | ? | ? | SG to rest of ingroup | SG to rest of ingroup | SG to rest of ingroup | SG of A–D, then SG to TLP | ? | SG to ingroup but A–D | A SG to P | SG of *N* |
| Position of *Aetothaxis mytopterix* | A | ? | SG to all but D | ? | ? | A SG to D, then SG to all but P | | – | A SG to D then to rest of ingroup | | A SG of D, then SG to ingroup |
| Position of *Dissostichus eleginoides* and *D. mawsoni* | D | ? | SG to ingroup | ? | ? | | | ? | | SG to A–P, within Not | |

and *Patagonotothen* species form a clade, in the simultaneous analyses and several separate analyses (HECW2, PPM1d, COI), and are never contradicted. *Indonotothenia cyanobrancha* is always within this clade, although its position changes from one analysis to another. Within this group, the monophyly of the genus *Trematomus* is not recovered in the separate analyses, only in the simultaneous ones (except in the PA analysis of all markers), the mitochondrial dataset of Near and Cheng (2008) and Rutschmann et al. (2011). The monophyly of the genus *Patagonotothen* is, on the contrary, recovered by several separate analyses and contradicted by none. The monophyly of the genus *Lepidonotothen* is either not recovered or contradicted, and the problem is always *L. squamifrons* appearing closer to the *Patagonotothen* clade (COI, combined nuclear coding genes). The position of *P. antarctica*, the two *Dissostichus* species, and *Aethotaxis mitopteryx* varies from one marker to another. They are often at the basis of the ingroup, but in variable configurations (cf. Table 4).

### 3.2. Bayesian inference topologies

While the topologies are slightly different between PA and BI, the position and composition for most clades are similar, although the supporting datasets vary. The rooting of COI within Bathydraconidae is also present for the BI analysis.

The monophylies of Artedidraconidae, Channichthyidae and *Patagonotothen* are always either recovered (with high posterior probabilities in both the simultaneous and the separate analyses) or unresolved. The monophyly of the *Trematomus-Lepidonotothen-Patagonotothen* clade, the inclusion of *I. cyanobrancha* in it, the inclusion of *P. magellanica* in the genus *Notothenia*, and the monophyly of the High Antarctic Clade are supported or not contradicted by all separate and combined analyses but one (either COI, or Rh), with high posterior probabilities in most. The (((High Antarctic clade)*Notothenia*)*Gobionotothen*) topology is supported by Pkd1, SSRP1, our simultaneous analyses (Fig. 2), with high posterior probabilities except for Pkd1, and the simultaneous analyses of Near and Cheng (2008) and Rutschmann et al. (2011). Again, the Bathydraconidae and *Lepidonotothen* are repeatedly contradicted or not recovered.

## 4. Discussion

### 4.1. Marker efficiency

While the separate analyses often lack resolution, they still separately support many of the clades. The new nuclear markers perform better at recovering clades present in the other studies (Near and Cheng, 2008; Rutschmann et al., 2011) and in the combined analysis (see Fig. 2) than the COI and the rhodopsin retrogene. Of the 11 clades scored in Table 4

| Bayesian inference | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Rh | pkd1 | HECW 2 | PPM1d | Combined nuc coding genes | COI | SSRP 1 | Combined all datasets | Mitochondrial Near and Cheng (2008) | S7 Near and Cheng (2008) | Combined Near and Cheng (2008) | Rutschmann et al. (2011) |
| no | **no** | ? | ? | **no** | no | no | no | no | **no** | **no** | no |
| X | X | **X** | ? | **X** | no | **X** | **X** | **X** | **no** | **X** | **X** |
| no | X | no | ? | **X** | no | **X** | **X** | X | X (within H) | **X** | X |
| no | **X** | no | ? | **X** | no | **X** | **X** | no | X | **X** | X (includes P) |
| no | **X** | **X** | X | **X** | **X** | ? | **X** | **X** | **X** | **X** | **X** |
| no | X | **X** | ? | **X** | X | **X** | X | **X** | ? | **X** | – |
| no | ? | ? | ? | no | no | no | no | no | **no** | no | – |
| **X** | ? | **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** | – |
| **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** | **X** |
| no | **X** | **X** | X | **X** | **X** | ? | **X** | – | – | – | – |
| no | no | no | ? | **X** | ? | ? | X (with Indo) | X | ? | X | **X** |
| **X** | ? | X | **X** | **X** | **X** | ? | **X** | X | X | X | **–** |
| ? | no | ? | ? | **no** | no | ? | **no** | no | ? | no | – |
| ? | X | ? | ? | **X** | X | ? | **X** | no | ? | no | – |
| in T | SG to TLP | ? | P, A, D in polytomy with TLP | SG to TLP | P in polytomy with A–D and TLP | ? | SG to TLP | A SG to P | SG of N | P in a polytomy with GNH and TLP | SG to G |
| A SG to D, in polytomy within TLP | SG to ingroup but D | ? | | **SG to ingroup but D** | | – | A SG to D then to rest of ingroup | | A SG to D, then SG to all but P | A SG to D, then SG to ingroup | SG to ingroup |
| | **SG to ingroup** | SG to N-G clade | | SG to ingroup | | ? | | SG to A–P and TLP | | | SG to TLP |

for the combined analyses that are robust to the change of method (present in both PA and BI) (Dettai and Lecointre, 2004; Mueller, 2006) most markers recover a high proportion of the clades and give similar topologies for the two methods: SSRP1 recovers 6 and fails to resolve 7 for both methods, Pkd1 recovers 6 and 8 respectively, but contradicts for both approaches the monophyly of *Trematomus*. Although it has a low nucleotide diversity compared to most of the others, PPM1d recovers 7 and 5, but is the only one in PA to recover the bathydraconids. However, it has very little to no resolution between closely related species (genus *Trematomus*, Artedidraconidae), and would bring little to a study interested in such relationships. HECW2 recovers 5 and 7, but fails to recover the monophyly of genus *Trematomus* and the sister group relationship between *Notothenia*, *Gobionotothen* and the High Antarctic clade. The rhodopsin retrogene is the nuclear marker presenting the most problems, although it is apparently one with the highest divergence between species pairs. It has little resolution for the PA, and a topology in disagreement with most of the others for the BI. This might explain the lack of resolution observed in the study of Sanchez et al. (2007). COI and Rh both produce topologies where the root is located within groups that are always recovered monophyletic otherwise. The *Notothenia-Paranotothenia* clade is basal and paraphyletic in the Rh BI separate analysis, and the tree is rooted within one component of the High Antarctic Clade for the COI analysis. This is contrary to the topology obtained for this last marker and approach by Tomasziewicz et al. (2011) with a reduced sampling (although some sequences are shared between the two studies). The topology for that study was very close to the simultaneous analysis topology obtained here. Over the different studies, the mitochondrial markers tend to give different results depending on the analysis method for the notothenioids (Bargelloni et al., 1994; Near and Cheng, 2008; Tomasziewicz et al., 2011; present study). Neither Rh nor COI imposed the unusual part of their topology on the simultaneous analyses they were included in.

Still, the mitochondrial markers are complementary to the nuclear markers. They give the best resolution for closely related groups, where the nuclear markers present little or no divergence, as could already be observed in previous studies (Near and Cheng, 2008; Sanchez et al., 2007).

### 4.2. Notothenioid phylogeny

#### 4.2.1. Position of Pleuragramma, Dissostichus and Aethotaxis

These three genera have a highly variable position depending on the approach (PA or BI) and the marker. Their position even differs among the simultaneous analyses depending on the approach employed. The *Dissostichus* species form a clade, but their association with *Aethotaxis* suggested in previous studies (Near et al., 2004; Near and Cheng, 2008; Sanchez et al., 2007) is also labile. Their most

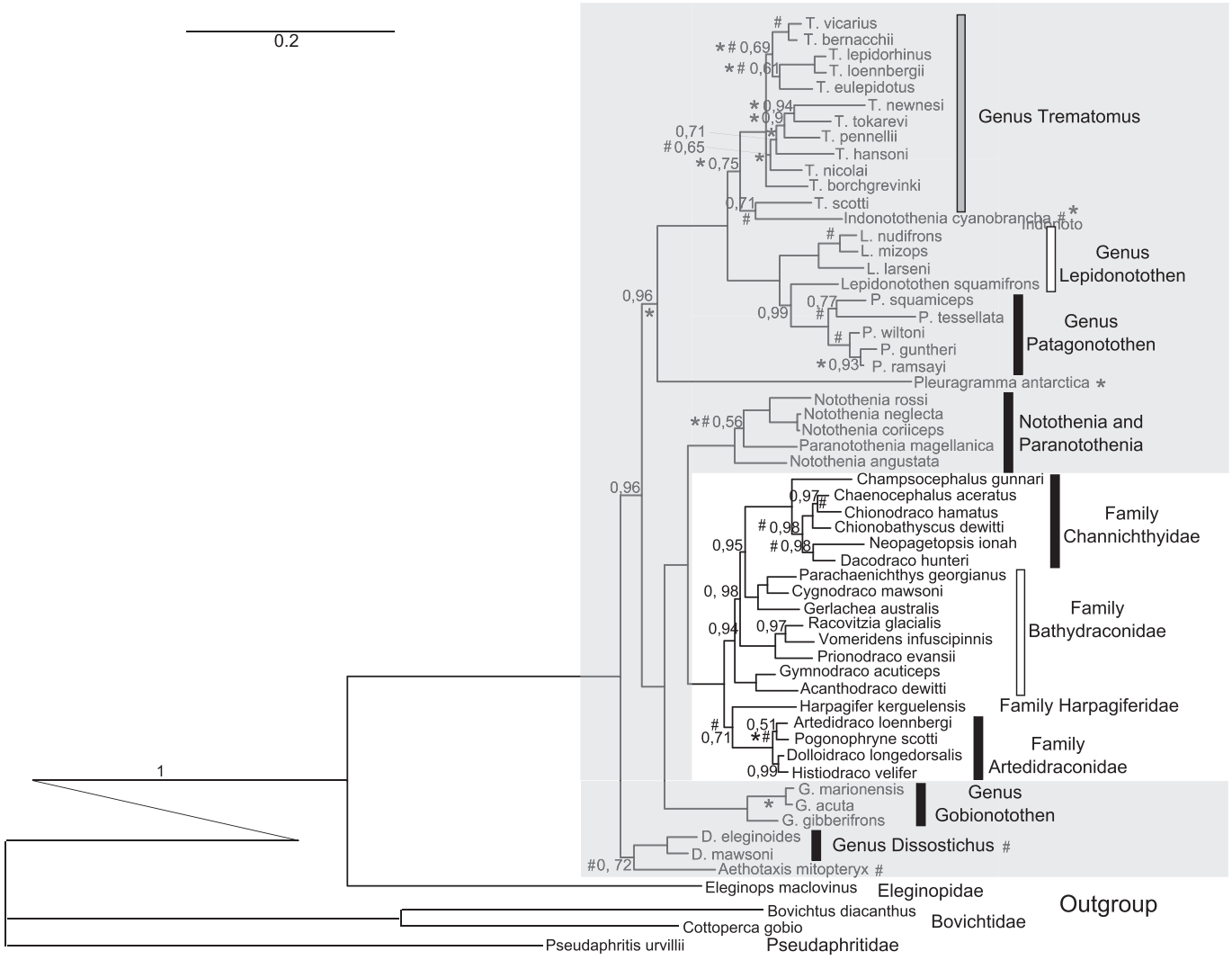


**Fig. 2.** BI tree for the simultaneous analysis of all the markers (nuclear coding and non-coding, and COI).Monophyletic groups are indicated by black bars, non-monophyletic groups by white bars. Posterior probabilities are indicated below the corresponding branches, branches with no indication have a posterior probability of 1. The groups that are placed differently on the PA topology for the same dataset are indicated by a *, the ones that are placed differently for the BI analysis of the four nuclear coding datasets with a #.

frequent position is either as a sister group of the rest of the ingroup, or at the base of one of the other large clades. *Pleuragramma antarctica* is rarely associated with the two other genera: only with mitochondrial datasets (COI in PA, Mt in PA and BI of Near and Cheng, 2008). Its position cannot be considered resolved for now.

*4.2.2. Genera Trematomus, Lepidonotothen and Patagonotothen*

The clade uniting the genera *Trematomus*, *Lepidonotothen* and *Patagonotothen* is again (as in Near and Cheng, 2008; Sanchez et al., 2007; Rutschmann et al., 2011) recovered in almost all analyses here (except Rh in BI). It can be considered as strongly corroborated, as almost all species from these genera are included in the present study. *Indonotothenia cyanobrancha* is included in this clade, as had already been suggested by Bargelloni et al. (2000) using other mitochondrial markers. Its position within the group varies among markers here, and it is not present in published studies with larger datasets, so its exact location remains to be determined.

The representatives of the genus *Patagonotothen* always form a clade (or are included in an irresolution). The relationships of fish of the genus *Trematomus* have been under intense enquiry recently (Kuhn and Near, 2009; Janko et al., 2011; Lautredou et al., submitted for publication). The monophyly of the genus *Trematomus* poses more problems, as it is recovered only with the simultaneous analyses (with sometimes *I. cyanobrancha* inserted in it) and mitochondrial data (Near and Cheng, 2008; Lautredou et al., submitted for publication).

*Lepidonotothen squamifrons* repeatedly groups with the *Patagonotothen* clade rather than with the other *Lepidonotothen* representatives, as it does in Tomasziewicz et al. (2011) for COI. In Near and Cheng (2008), it is either unresolved (S7) or a sister group to all other *Patagonotothen* and *Lepidonotothen* (Mt and simultaneous analyses), and it was sister group to the others in the *Patagonotothen-Lepidonotothen* clade in Bargelloni et al. (2000). The relationships within the clade would benefit from a study including all these groups with very variable markers and more samples per species.

*4.2.3. High Antarctic clade*

The clade formed by the four families Harpagiferidae, Channichthyidae, Bathydraconidae and Artedidraconidae is corroborated by all simultaneous analyses (Near and Cheng, 2008; Rutschmann et al., 2011; present study) and many of the markers separately. The relationships within this group are difficult to evaluate for the time being, and a denser sampling would be needed in this area. However, a few conclusions emerge. The monophyly of Artedidraconidae and Channichthyidae is steadily corroborated for now by almost all analyses whether separate or simultaneous (Near and Cheng, 2008; Rutschmann et al., 2011; present

study). On the contrary, the monophyly of the Bathydraconidae is assessed only with PPM1d in PA, while it is contradicted by SSRP1 and all simultaneous analyses except Rutschmann et al. (2011), but this study includes only two samples for the group.

*4.2.4. Nototheniid paraphyly*

The sister group of the High Antarctic clade, as it now emerges from multiple studies and markers, is the *Notothenia*/*Paranotothenia* clade, and the sister group to this clade is the genus *Gobionotothen* (Near and Cheng, 2008; Rutschmann et al., 2011; present study). Therefore, the family Nototheniidae is not monophyletic except when the four families from the High Antarctic clade are included in it.

The non-monophyly of the family Nototheniidae as it was previously defined is now corroborated with multiple nuclear markers and multiple studies, and is not approach dependent. Our study of the evolutionary history of the Nototheniidae has to take into account these relationships, and the closer relationships of some nototheniids with the Channichthyidae, Harpagiferidae, Artedidraconidae and Bathydraconidae. A wide number of studies explore the ecology, physiology and adaptations of the five families, and include discussions about the evolution of various characters. It is important to update the state of the classification of the group to reflect the current knowledge about its systematics, so that non-systematicists would not discuss their data on incorrect classifications and topologies. The classification can either be corrected by splitting Notothenidae in a number of new families (most of which would only be including a single genus), or by integrating the four families of the High Antarctic clade into it, and creating subfamilies to conserve the clades: channichthyinae, harpagiferinae, artedidraconinae and bathydraconinae. This last solution avoids creating multiple small families. Moreover, choosing the first solution would base taxonomic decisions on a uncomplete 'state of the art' in the phylogenetic knowledge, as the position of several nototheniid genera is not resolved yet (*Dissostichus*, *Aethotaxis*, and *Pleuragramma*). The second solution allows flexibility while being scientifically correct. At a smaller scale, *Paranotothenia magellanica* is clearly included within genus *Notothenia*, and is therefore *Notothenia magellanica*.

## 5. Conclusions

The use of multiple nuclear markers, as predicted (Near and Cheng, 2008; Sanchez et al., 2007), has brought clarification on the systematics of the nototheniid group, yielding positive evidence for the paraphyly of the family. We now have to take into account the fact that *Notothenia* is more closely related to the High Antarctic clade than to the rest of nototheniids, and that *Gobionotothen* is more closely related to these two than to the rest. Defining new markers for teleost phylogeny remains useful, as the number of available markers remains low. Moreover, for the resolution of smaller scale relationships, and especially in the case of rapid diversifications like Nototheniidae (Matschiner et al., 2011; Rutschmann et al., 2011), it is important to have not only a high number of markers, but also to have some that are variable enough for the problem at hand. The new markers developed in Lautredou et al. (submitted for publication) and present study fulfil these criteria. Comparing the separate analyses with each other, and with the combined analyses brings valuable information on the individual support of the groups, but also on what each marker brings to the analysis. While some clades might be lost with some of the markers that are individually not informative enough, they are recovered by other markers provided that the number of available markers is high enough. When analysing the markers simultaneously, the topologies (Near and Cheng, 2008; Rutschmann et al., 2011; present study) have a better resolution. They are also highly congruent, despite the fact that they share neither samples nor markers. The reliability of the results, in this case, ultimately comes from the corroboration by multiple studies from diverse teams, with different samplings and markers.

## Acknowledgements

We thank the crew and participants of the cruises involved in the capture of the samples, and especially the chief scientists of the CAML-CEAMARC cruises (IPY project no. 53) were supported by the Australian Antarctic Division, the Japanese Science Foundation, the French Polar Institute IPEV, the CNRS, the MNHN and the ANR (White Project ANTFLOCKS USAR no. 07-BLAN-0213-01 directed by Guillaume Lecointre). G. Lancelot is thanked for his help for language corrections and many constructive comments. The present work was also partly based on samples collected during the ICEFISH 2004 cruise (supported by National Science Foundation grant OPP 01-32032 to H. William Detrich (Northestern University)) and the EPOS cruise (1989) for which we are grateful to the Alfred Wegener Institute and the European Science Foundation. We also thank C. Cheng for additional samples. This project is a contribution to the EBA-SCAR program.

This work was supported by a BQR from the Muséum national d'Histoire naturelle "Isolation de marqueurs moléculaires pour la phylogénie inter-ordinale et la cytogénétique intra-familiale des acanthomorphes (teleostéens)"; by the 'Service de Systématique Moléculaire' of the MNHN (UMS 2700 CNRS and the 'Consortium National de Recherche en Génomique': it is part of the agreement number 2005/67 between the Genoscope and the Muséum national d'Histoire naturelle on the project 'Macrophylogeny of life' directed by G. Lecointre.

We also thank the editor and two anonymous reviewers for their helpful comments.

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