

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

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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 *Aethotaxia 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*.

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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 Channichthyidae.

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 nototheniid 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;

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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; Tomaszewicz 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 across 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 (Tomaszewicz 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
<i>Bovichtidae</i>										
	<i>Bovichtus diacanthus</i>	Tristan Da Cunha	ICTI1205	MNHN2005-0102	FKCI042	FKCI042	–	JQ688685	JQ688741	JQ688788
	<i>Cottoyperca gobio</i>	Burdwood Banks	ICTI1233	BB	EATFR003	EATFR003	–	–	JQ688745	JQ688794
<i>Pseudaphritidae</i>										
	<i>Pseudaphritis urvillii</i>	Tasmania, Brown's River, Kingston	ICTI115	1	EATFR004	EATFR004	–	JQ688684	JQ688740	JQ688787
<i>Eleginopsidae</i>										
	<i>Eleginops maclovinus</i>	Falklands	ICTI1234	MNHN2005-0093	FKCI050	FKCI050	JQ688849	JQ688691	JQ688746	JQ688795
<i>Nothotheniidae</i>										
	<i>Aethotaxis mitopteryx</i>	Weddell Sea	ICTI351	W128M	EATFR005	EATFR005	–	JQ688713	JQ688762	JQ688814
	<i>Dissostichus mawsoni</i>	Terre Adélie	ICTI388	MNHN2001-1143	EATFR006	EATFR006	JQ688869	JQ688715	JQ688764	JQ688816
	<i>Dissostichus eleginoides</i>	Burdwood Banks	ICTI1222	BB/65	EATFR007	EATFR007	JQ688847	JQ688688	JQ688743	JQ688791
	<i>Gobionotothen marionensis</i>	South Sandwich Isl.	ICTI1225	MNHN2005-0088	FKCI044	FKCI044	–	JQ688689	JQ688744	JQ688792
	<i>Gobionotothen gibberifrons</i>	Shag Rocks	ICTI1229	MNHN2005-0099	FKCI048	JQ063276	JQ063309	JQ688690	JQ063250	JQ063172
	<i>Gobionotothen acuta</i>	Kerguelen Isl.	Aus36	MNHN2007-1878	EATFR008	EATFR008	JQ688844	JQ688723	JQ688771	JQ688825
	<i>Indonotothenia cyanobranchia</i>	Kerguelen Isl.	ICTIAus30	MNHN2007-1844	EATFR009	EATFR009	JQ688843	JQ688722	JQ688770	JQ688824
	<i>Lepidonotothen mizops</i>	Kerguelen Isl.	ICTI943		FKCI067	FKCI067	JQ688875	JQ688720	JQ688768	JQ688822
	<i>Lepidonotothen nudifrons</i>	Shag Rocks	ICTI1245	SR/LN2	FKCI051	FKCI051	JQ688850	JQ688692	JQ688747	JQ688796
	<i>Lepidonotothen larseni</i>	Bouvet Island	ICTI1247	SAIAB74974	FKCI053	FKCI053	–	JQ688693	JQ688748	JQ688797
	<i>Lepidonotothen squamifrons</i>	Bouvet Island	ICTI1251	BO/2	FKCI057	JQ063278	JQ063311	JQ688694	JQ063252	JQ063174
	<i>Notothenia neglecta</i>	Terre Adélie	ICTI137	TA13	EATFR010	EATFR010	JQ688861	JQ688707	JQ688757	JQ688808
	<i>Notothenia coriiceps</i>	Terre Adélie	ICTI655	MNHN2008-1888	FKCI066	JQ063275	JQ063308	JQ688719	JQ063249	JQ063171
	<i>Notothenia rossii</i>	South Georgia	ICTI1218	SG	FKCI043	FKCI043	JQ688846	JQ688687	JQ688742	JQ688790
	<i>Notothenia angustata</i>		ICTINang1		FKCI069	FKCI069	JQ688876	JQ688724	JQ688772	JQ688826
	<i>Paranotothenia magellanica</i>	Kerguelen Isl.	ICTIAus29	MNHN2007-1843	EATFR011	EATFR011	JQ688842	JQ688721	JQ688769	JQ688823
	<i>Patagonotothen squamiceps</i>	Falklands	ICTI1254	F	–	EATFR012	JQ688852	JQ688695	JQ688749	JQ688799
	<i>Patagonotothen wiltoni</i>	Falklands	ICTI1255	F	FKCI060	FKCI060	JQ688853	JQ688696	JQ688750	JQ688800
	<i>Patagonotothen guntheri</i>	Bouvet Island	ICTI1256	SAIAB75162	FKCI061	JQ063277	JQ063310	JQ688697	JQ063251	JQ063173
	<i>Patagonotothen ramsayi</i>	Falklands	ICTI1262	F	FKCI062	FKCI062	JQ688855	JQ688719	JQ063249	JQ063171
	<i>Patagonotothen tessellata</i>	Falklands	ICTI1268	SAIAB75143	FKCI063	FKCI063	JQ688856	JQ688699	JQ688752	JQ688803
	<i>Pleuragramma antarctica</i>	Weddell Sea	ICTI323	BPN01/W114F	EATFR013	EATFR013	JQ688867	JQ688711	JQ688761	JQ688813
	<i>Trematomus loennbergii</i>	Terre Adélie	ICTI139	MNHN1996-0326	GU997426	GU997314	JQ688864	JQ688708	JQ688758	JQ688810
	<i>Trematomus newnesi</i>	Terre Adélie	TA50TRNE1	MNHN2001-1150	GU997428	GU997349	JQ063361	JQ688712	GU997563	JQ063224
	<i>Trematomus vicarius</i>	South Georgia	ICTI1212		EATF588	GU997388	JQ063385	JQ688686	GU997595	JQ063248
	<i>Trematomus lepidorhinus</i>	Weddell Sea	ICTI1368		GU997424	GU997317	JQ688860	JQ688704	GU997518	JQ688807
	<i>Trematomus eulepidotus</i>	Weddell Sea	ICTI1370		EATF585	GU997298	JQ688862	JQ688705	JQ688756	JQ688809
	<i>Trematomus scotti</i>	Weddell Sea	ICTI1371		GU997448	GU997374	JQ688863	JQ688706	JQ688785	JQ688786
	<i>Trematomus hansonii</i>	Terre Adélie	TA646TrHa1		GU997418	GU997307	JQ688890	JQ688736	GU997509	JQ688840
	<i>Trematomus borchgrevinkii</i>	Terre Adélie	ICTI391	MNHN2002-1711	EATF594	GU997240	JQ688870	JQ688716	JQ688765	JQ688817
	<i>Trematomus bernacchii</i>	Terre Adélie	TA650TrBe1		GU997408	GU997262	JQ688891	JQ688737	JQ688784	JQ688841
	<i>Trematomus nicolai</i>	Terre Adélie	TrNi5		GU997440	GU997358	JQ688877	JQ688738	GU997574	JQ688827
	<i>Trematomus pennellii</i>	Terre Adélie	392TrPe		GU997442	JQ693498	JQ688871	JQ688739	GU997583	JQ688818
	<i>Trematomus tokarevi</i>	Dumont d'Urville Sea	si171n1296	MNHN2009-1250	EATF169	EATF169	JQ688882	JQ688703	GU997591	JQ688832
<i>Channichthyidae</i>										
	<i>Chionodraco hamatus</i>	Terre Adélie	ICTI385	TA41CHHAI	EATFR014	EATFR014	JQ688868	JQ688714	JQ688763	JQ688815
	<i>Neopagetopsis ionah</i>	Terre Adélie	ICTI298	TA59NEOI01	EATFR015	EATFR015	JQ688866	JQ688710	JQ688760	JQ688812
	<i>Dacodraco hunteri</i>	Dumont d'Urville Sea	si136n845	MNHN2009-1126	EATF135	EATF135	JQ688879	JQ688726	JQ688774	JQ688829
	<i>Chionobathyscus dewitti</i>	Dumont d'Urville Sea	si280n2119	MNHN2009-1155	EATF275	EATF275	JQ688887	JQ688733	JQ688781	JQ688837
	<i>Chaenocephalus aceratus</i>	South Sandwich Isl.	ICTI1283	MNHN2005-0092	EATFR016	EATFR016	JQ688858	JQ688701	JQ688754	JQ688805
	<i>Champocephalus gunnari</i>	Shag Rocks	ICTI1271	SR	FKCIR091	FKCIR091	JQ688857	JQ688700	JQ688753	JQ688804
<i>Bathydraconidae</i>										
	<i>Gymnodraco acuticeps</i>	Terre Adélie	ICTI241	TA255GYV11	EATFR017	EATFR017	JQ688865	JQ688709	JQ688759	JQ688811
	<i>Prionodraco evansii</i>	Dumont d'Urville Sea	si291n2190	MNHN2009-1078	EATF286	EATF286	JQ688889	JQ688735	JQ688783	JQ688839
	<i>Gerlachea australis</i>	Dumont d'Urville Sea	si146n893	MNHN2009-1043	EATF145	EATF145	JQ688880	JQ688727	JQ688775	JQ688830
	<i>Cygnodraco mawsoni</i>	Terra Nova Bay	ICTI559	C.maw 01 (2184)	EATFR018	EATFR018	JQ688873	JQ688718	JQ688767	JQ688820
	<i>Vomeridens infuscipinnis</i>	Dumont d'Urville Sea	si248n1964	MNHN2009-1074	EATF243	EATF243	JQ688886	JQ688732	JQ688780	JQ688836
	<i>Parachaenichthys georgianus</i>	South Georgia	ICTI1291	SG	EATFR019	EATFR019	JQ688859	JQ688702	JQ688755	JQ688806
	<i>Acanthodraco dewitti</i>	Dumont d'Urville Sea	si155n719	MNHN2009-1050	EATF154	EATF154	JQ688881	JQ688728	JQ688776	JQ688831
	<i>Racovitzia glacialis</i>	Dumont d'Urville Sea	si225n1698	MNHN2009-1067	EATF220	EATF220	JQ688884	JQ688730	JQ688778	JQ688834
<i>Artedidraconidae</i>										
	<i>Dolloidraco longedorsalis</i>	Dumont d'Urville Sea	si182n1370	MNHN2009-0955	EATF179	EATF179	JQ688883	JQ688729	JQ688777	JQ688833
	<i>Pogonophryne scotti</i>	Dumont d'Urville Sea	si238n1819	MNHN2009-1389	EATF233	EATF233	JQ688885	JQ688731	JQ688779	JQ688835
	<i>Histiodraco velifer</i>	Dumont d'Urville Sea	si283n978	MNHN2009-0967	EATF278	EATF278	JQ688888	JQ688734	JQ688782	JQ688838
	<i>Artedidraco loennbergi</i>	Dumont d'Urville Sea	si11n91	MNHN2009-0938	EATF011	EATF011	JQ688878	JQ688725	JQ688773	JQ688828
<i>Harpagiferidae</i>										
	<i>Harpagifer kerguelensis</i>	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 μ l (5% of DMSO, 5 μ g of bovine serum albumine, 300 μ M of each dNTP, 0.3 μ M of Taq DNA polymerase (Qiagen), 2.5 μ l 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 TelR1	5'-TCGACTAATCAYAAAGAYATYGGCAC-3' 5'-ACTTCTGGGTGNCCAAARAATCARAA-3'	52°C	*	Dettai et al. (2011)
Nuclear	Rh	Rhodopsin retrogene	Partial sequence	GroupXII (809,696-810,650)	≈ 830 bp	RhF193 RhR1039	5'-CNTATGAATAYCCTCAGTACTACC-3' 5'-TGCTTGTTCATGCAGATGTAGA-3'	50°C	*	Chen et al. (2003)
	Pkd1	Polycystic kidney disease 1	5' part of exon 20 (Transcript ENSGACT00000025103)	GroupIX (14,963,056-15,005,610)	≈ 890 bp	Pkd1F62 Pkd1R952 Pkd1F1511 Pkd1F1605 Pkd1R2587	5'-CATGAGYGTCTACAGCATCCT-3' 5'-YCCTCTNCCAAAGTCCCACT-3' 5'-YATGTTTACACNTCCGCTC-3' 5'-GTNCARCCTGAGCTGGAGG-3' 5'-GAGCNGTGAGRTTCACCATGT-3'	50°C	*	Lautredou et al. (2010) This study
	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 HECWF160 HECWF170 HECWR838	5'-CAATGGTSCITGTACTATGRAGA-3' 5'-GCTTGTACTATGNAGAYGACAG-3' 5'-ATGAAGAYGACAGYGTGTGGC-3' 5'-CTCACCTGAATGGGKGAAG-3'	55°C	*	Lautredou et al. (submitted for publication)
	PPM1d	Protein phosphatase, Mg2+/Mn2+ dependent, 1D	Almost complete exon 1	GroupI (18,501,183-18,506,137)	≈ 410 bp	PPMF45 PPMR457	5'-AGGRGNAGGAAATACATGGA-3' 5'-CAGGCTAYRAATCCTTTGCC-3'	50°C	*	This study
	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 SSRPR600	5'-RTTTCCTGAAGCCGAGGTG-3' 5'-GCAAAGCTGAGCTATGGTTGT-3'	55°C	*	Lautredou et al. (submitted for publication)

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*

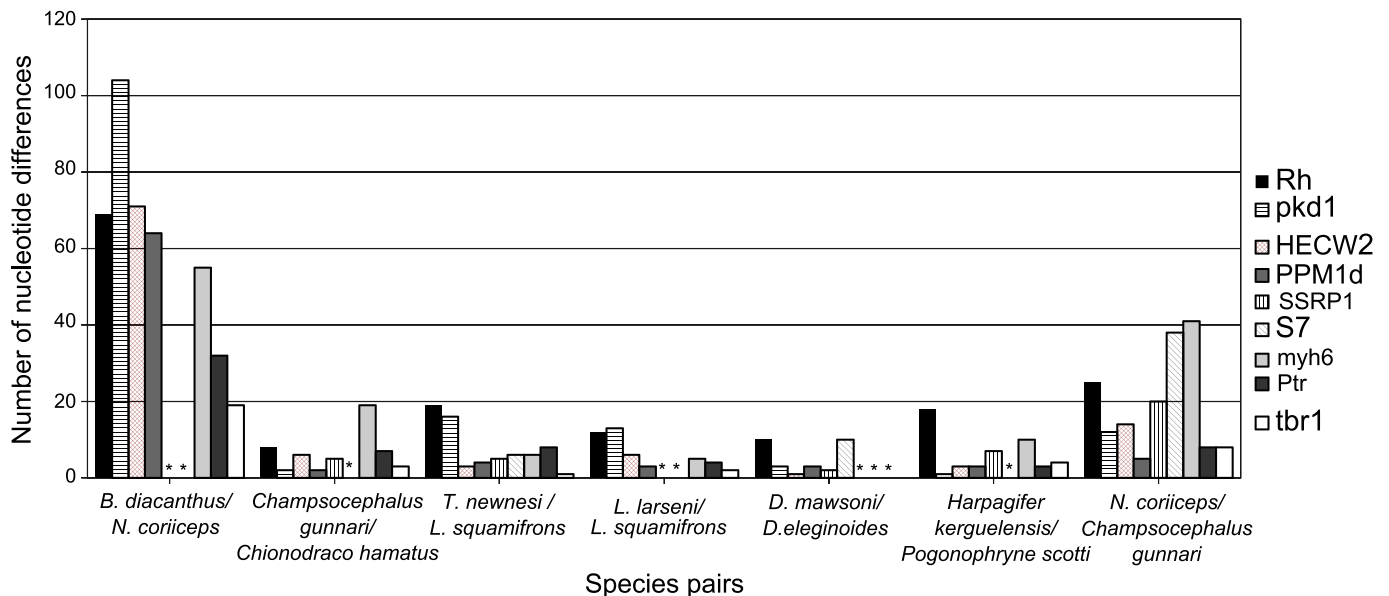


Fig. 1. Divergence of species pairs for the different markers. The pairs were chosen to represent both closely and less closely related species, and to maximize the sampling overlap between the present study, Near and Cheng (2008) and Rutschmann et al. (2011). Markers for which there is no data for a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

Clade content	Clade Abbreviation	Maximum parsimony									
		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
<i>Notothenia</i> and <i>Paranotothenia</i> clade closest to H	NH	?	X	?	X	X	no	X	X	no	G closer
<i>Gobionotothen</i> species with clade NH	GNH	?	X	?	X	X	no	X	X	no	X
<i>Trematomus</i> , <i>Lepidonotothen</i> , <i>Patagonotothen</i> clade	TLP	?	?	X	X	X	X	?	X	X	X
<i>Paranotothenia magellanica</i> included in <i>Notothenia</i>		?	X	SG to <i>N. angustata</i>	?	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
<i>Indonotothenia cyanobranca</i> with or within clade TLP		?	X	X	X	X	X	?	X	–	–
<i>Trematomus</i> monophyly	T	?	no	?	?	X	no	?	no	X	?
<i>Patagonotothen</i> monophyly	Pa	X	?	?	X	X	X	?	X	X	X
<i>Lepidonotothen</i> monophyly	L	?	no	?	?	no	no	?	no	?	?
<i>Lepidonotothen squamifrons</i> with <i>Patagonotothen</i>		?	?	?	?	X	X	?	X	?	?
Position of <i>Pleuragramma antarctica</i>	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 <i>Aethotaxis mytopteryx</i>	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 <i>Dissostichus eleginoides</i> and <i>D. mawsoni</i>	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 cyanobranca* 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 mytopteryx* 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. cyanobranca* 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 Tomaszewicz 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; Tomaszewicz 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

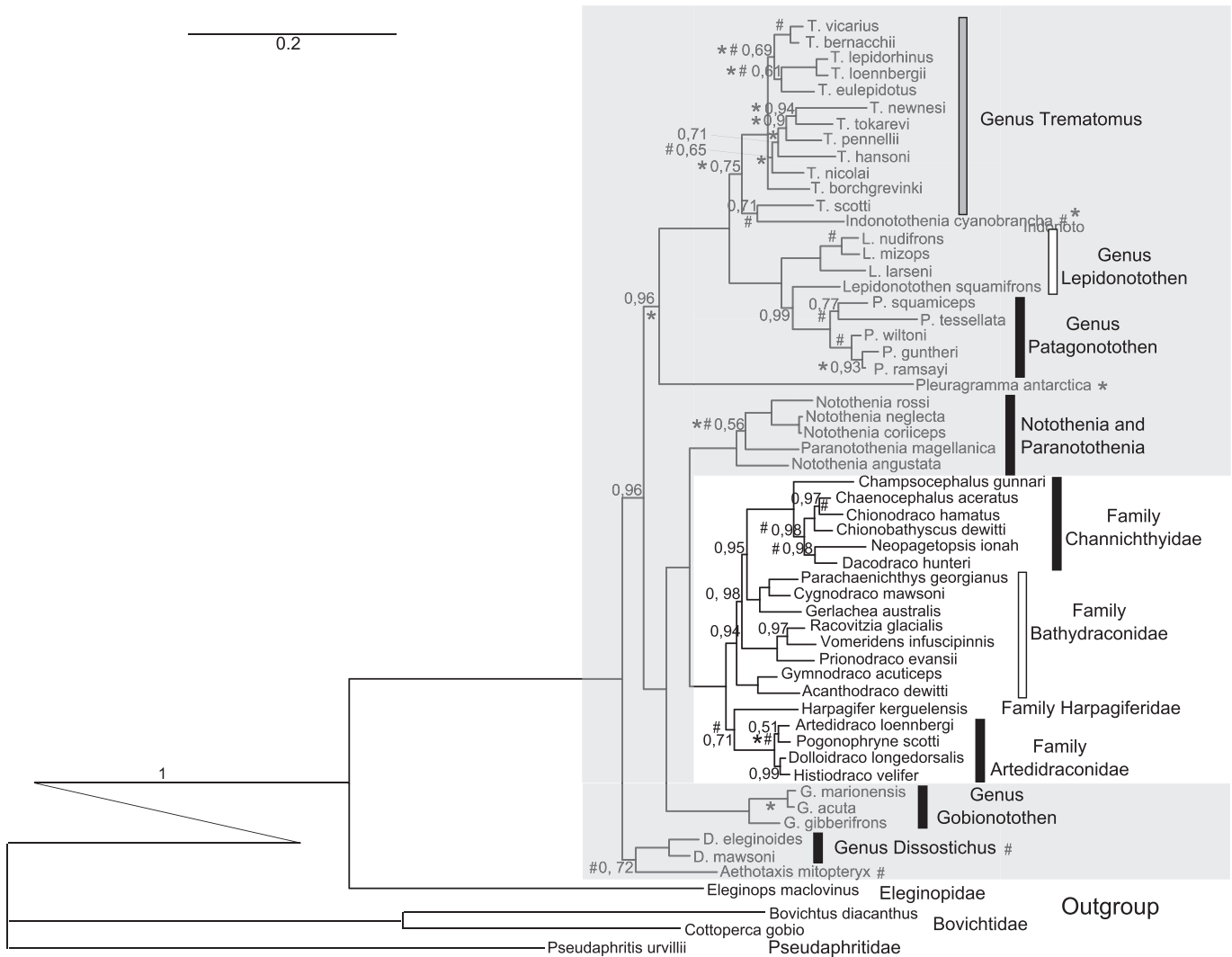


Fig. 2. BI tree for the simultaneous analysis of all the markers (nuclear coding and non-coding, and COI). Monophyletic groups are indicated by black bars, non-monophyletic groups by white bars. Posterior probabilities are indicated below the corresponding branches, branches with no indication have a 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 Tomasiewicz 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 Nototheniidae 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 an incomplete '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.

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