

Barcoding Antarctic Fishes: Species Discrimination and Contribution to Elucidate Ontogenetic Changes in Nototheniidae

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Abstract Fish species richness in the Southern Ocean accounts for approximately 2 % of the world's ocean species, with more than 370 species registered and several awaiting for formal description. Here we explore on the use of DNA barcoding to discriminate fishes from Antarctic Peninsula by compiling our results and placing them into a comparative framework with other previous studies to provide a comprehensive review of available barcodes for Antarctic fishes. A total of 275 specimens, belonging to 36 different putative species were barcoded. Nearly all species exhibit unique barcodes or clusters of closely related haplotypes, and only four species lacked genetic resolution using Barcode Index Numbers (BINs). Thus, ~90 % of the species barcoded in this study could be identified at species level with accuracy using BINs. However the use of nucleotic diagnostic character allowed us to discriminate the remaining species. Compiling our results with previous studies, about 80 species inhabiting the Antarctic Peninsula were already barcoded, representing approximately 60 % of the species occurring in the area. Finally, we highlighted ontogenetic morphological traits observed in some Nototheniidae, which may lead to misidentification of juveniles. DNA Barcoding was a cornerstone element for obtaining a reliable identification of these specimens. These results are crucial for management and conservation purposes since an accurate species-level resolution of juveniles is necessary to determine nursery areas and to clarify species distributions.

Keywords DNA barcodes · Fishes · Antarctic Peninsula · Barcode Index Number · Species discrimination · Ontogenetic variation

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1 Introduction

Fish species richness in the Southern Ocean accounts for approximately 2 % of the world's ocean species, with more than 370 species registered and several awaiting for formal description (Duhamel et al. 2014). The Notothenioidei (with representatives from six families), liparids and zoarcids are the dominant components of the Antarctic fish fauna (Eastman 2005), being snail fishes (Liparidae) the most speciose Antarctic fish family.

Eastman (2005) suggests that after a century of ichthyofaunal research in Antarctic waters, the fauna is fairly well known. However, not all groups are completely understood as revealed by the number of species recently described for notothenioids and other highly diversified fish families such as, Liparidae and Zoarcidae (Duhamel et al. 2010, 2014; Stein 2012). Moreover, although several taxonomic studies have been carried out on Antarctic fishes, the number of species is probably underestimated as some taxa and regions have not been deeply explored. Indeed, the asymptotic level in species richness has not yet been reached (Duhamel et al. 2014). Several families (Rajidae, Muraenolepididae, Harpagiferidae, among others) still require thorough taxonomic revision due to the lack of detailed species diagnoses or because of misidentifications in the scientific record (Duhamel et al. 2014). On the other hand, most taxonomic studies do not usually cover different ontogenetic stages, lacking information about the morphological identification of juveniles.

The Antarctic fish fauna has a remarkably high level of endemism (c.a 90 % of the species recorded are found only in Antarctic waters, Eastman 2005; Smith et al. 2012). This feature, not restricted to fishes, increases the concern about the potential influence of global warming, habitat loss, UV exposure and ocean acidification in the southern ocean ecosystem (Clarke et al. 2005; Thatje 2005; Aronson et al. 2009; Turner et al. 2009; Cook et al. 2005, 2010; Constable et al. 2014), specially because some changes are already visible in the Antarctic Peninsula (Steig et al. 2009; Naish et al. 2009). Therefore, a more comprehensive knowledge of their biodiversity is required.

Over the last few decades several molecular studies have been conducted on Antarctic fishes in order to support morphological research. Some of these works includes the use of DNA Barcoding as a standardized molecular taxonomic approach (Lautrédou et al. 2010; Smith et al. 2008, 2011, 2012; Rock et al. 2008; Rey et al. 2011; Dettai et al. 2011; Duhamel et al. 2010). The Fish Barcode of Life initiative (FISH-BOL; Ward et al. 2009) seeks to establish a mitochondrial 5' cytochrome *c* oxidase subunit I (COI) reference sequence library for the molecular identification of fishes worldwide, following a common protocol that includes links to voucher specimens (Steinke and Hanner 2010). The use of DNA barcoding in fishes can facilitate subsequent species identification by non-specialists, help highlight specimens that represent a range expansion of known species, flag previously unrecognized (e.g. cryptic) species, and enable identifications where traditional methods are not applicable (e.g. fillets, eggs and larvae). As of July 2010, nearly 7800 fish species had been barcoded, including at least one species for ~90 % of all families (Becker et al. 2011). Five years later, this number has risen to nearly 11,000 species (www.fishbol.org).

Many studies have demonstrated the usefulness of DNA barcoding to discriminate marine and freshwater fishes around the world (Pegg et al. 2006; Steinke et al. 2009; Ward and Holmes 2007; Ward et al. 2008; Huber et al. 2008; Valdez-Moreno et al. 2009; Mabragna et al. 2011; Rosso et al. 2012; Knebelberger et al. 2014, among others). Rock et al. (2008) were the first to provide barcodes from Antarctic fishes, analyzing DNA barcoding of 34 putative species representing seven different families, collected in the Scotia Sea. Duhamel et al. (2010) conducted a survey in the eastern sector of the Southern Ocean and provide the first molecular data (COI) for 13 species of liparids, allowing the identification of most species. Lautrédou et al. (2010) analysed the boundaries between 12 species of *Trematomus* collected in different sectors of the southern ocean. Rey et al. (2011) analysed the difference between two species of *Gymnodraco* off Terre Adélie based on morphology and DNA barcoding. Dettai et al. (2011), provided barcodes from 57 species in the Eastern part of the Antarctic continental shelf. Finally, Smith et al. (2012) provided an overview of barcode records for the Ross Sea fishes and a comparison of genetic divergence within the Ross Sea and between this and other regions of the Southern Ocean. They found that DNA barcoding could discriminate 87.5 % of Antarctic species. Nevertheless, these molecular studies showed incomplete species discrimination within some representatives of Notothenidae, Artedidraconidae and Liparidae.

As part of a global project conducted by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) on the systematics and biology of Antarctic organisms, DNA barcodes of fish species around Antarctic Peninsula and adjacent Islands were obtained and their concordance with traditional morphological identification was explored. The overarching objective of this study was to further extend the use of DNA barcoding to discriminate Antarctic fishes by compiling the results of our survey and placing them into a comparative framework with other previous studies to provide a comprehensive review of available barcodes for Antarctic fishes. We also highlighted ontogenetic morphological traits observed in some species of Notothenidae, which may lead to misidentification of juveniles through the use of taxonomical keys.

2 Materials and Methods

2.1 Study Area

The sampling region encompasses part of the Argentine Antarctic Sector including the South Shetland Islands, the South Orkney Islands and the north of the Antarctic Peninsula (Fig. 1). Around the South Shetland Islands the shelf break lies at depths between 225 and 380 m in the north-east (Elephant Island) and between 250 and 450 m in the rest of the archipelago. The Islands are located along 481 km of shelf in a NE–SW direction. Around the South Orkney Islands the shelf is very narrow to the north and a broad plain to the south, breaking mainly below the 500 m isobath (Acosta et al. 1989; Jones 2000). The Antarctic Peninsula is separated from the

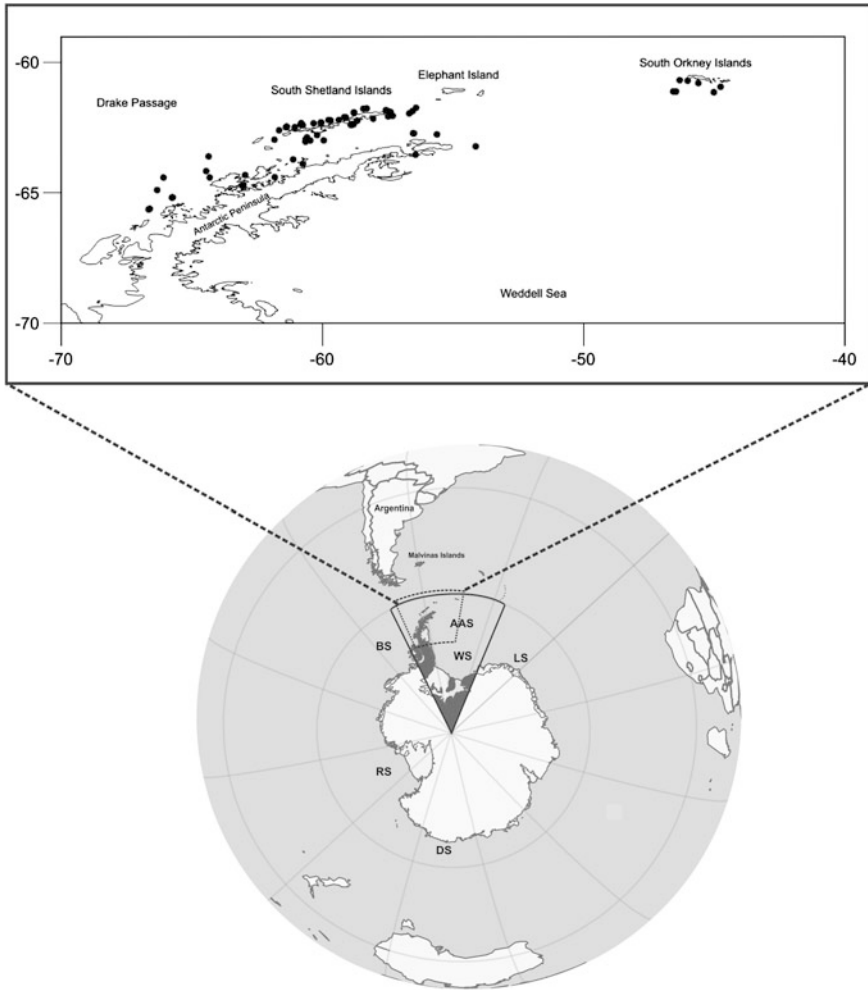


Fig. 1 Collection sites (*black circles*) for specimens examined in this study. *AAS* Argentine Antarctic Sector, *BS* Bellingshausen Sea, *DS* Dumont d’Urville Sea, *LS* Lazarev Sea, *RS* Ross Sea, *WS* Weddell Sea

Shetland Islands by the deep waters of Bransfield Strait (Acosta et al. 1989; Kock et al. 2000). The bottom topography in the west and north of the Antarctic Peninsula might be described as a shelf surrounded by islands, communicating with the open sea by troughs of varying depths (Barrera-Oro 2002). The circulation in the region is controlled by an eastward component of the Antarctic Circumpolar Current which balances the waters coming from the Antarctic Peninsula, the Weddell Sea and waters from the Bellingshausen Sea (Gordon 1988; Barrera-Oro 2002; Turner et al. 2009). In the study area 131 species have been reported (Gon and Heemstra 1990; Andriashev 1998; Matallanas and Pequeño 2000; La Mesa

et al. 2002; Kock 2005; Chernova 2006; Matallanas 2009, 2011; Balushkin and Prirodina 2010; Balushkin 2012).

2.2 *Sample Collection*

Overall 5297 specimens belonging to 40 species and 9 families (Arteidraconidae, Bathydraconidae, Channichthyidae, Harpagiferidae, Myctophidae, Nototheniidae, Liparidae, Rajidae and Zoarcidae) were collected on board the Oceanographic Vessel “Puerto Deseado” of CONICET during summers of 2011, 2012 and 2013. This number represents almost the 30 % of the species reported for the surveyed area. A total of 70 stations were performed (Fig. 1). Fish were collected using two bottom trawls, including a shrimp net (50 mm mesh in the wings, and 20 mm in the cod end; vertical height 1 m, horizontal opening 4 m), and a bottom trawl net (135 mm mesh in the wings, and 60 mm in the cod end; vertical height 3.7 m, horizontal opening 10 m). We also used “long liner gears” in shallow areas. Specimens were identified on board using diagnostic keys (and reexamined in laboratory when necessary, after results of molecular analysis) and measured (total and standard length). Vouchers were morphologically identified following the identification reliability level 2 according to the Fish-BOL collaborator’s protocol (Steinke and Hanner 2010): “specimen identified by a trained identifier who had prior knowledge of the group in the region or used available literature to identify the specimen”. We followed Eschmeyer (2015) for species names and its higher classification.

2.3 *DNA Analysis*

A portion of tissue was taken from representatives of each species and preserved in 96 % ethanol for subsequent molecular analysis. The voucher specimens were labelled, photographed, formalin fixed (with further alcohol long-term preservation) and deposited as vouchers in the fish collection of “Instituto de Investigaciones Marinas y Costeras (IIMyC)- CONICET- Universidad Nacional de Mar del Plata”, Argentina.

DNA extraction, polymerase chain reaction (PCR), and sequencing of the 5’ region of the COI gene were performed following standard DNA barcoding protocols (Ivanova et al. 2006) coupled with primers and primer cocktails developed for fishes (Ward et al. 2005; Ivanova et al. 2007). Extraction and amplification were performed in two International Barcode of Life reference Laboratories of CONICET in Argentina, one located at the Museo Argentino de Ciencias Naturales and the other one at the IIMyC. Sequencing was performed in Advanced Analysis Center’s Genomics Facility (College of Biological Sciences, University of Guelph, Ontario Canadá) and in the Canadian Centre for DNA Barcoding (CCDB) at the Biodiversity Institute of Ontario, (University of Guelph, Ontario, Canada).

Amplification of the 5’ region of COI, corresponding to base positions 6474 to 7126 of the *Danio rerio* mitochondrial genome (Broughton et al. 2001), was first attempted

using FF2d_t1/FR1d_t1 primer combination and C_FishF1t1/C_FishR1t1 primer cocktails (Ivanova et al. 2006). The primer combinations C_FishF1t1 and C_FishR1t1 both contained two primers (FishF2_t1/VF2_t1 and FishR2_t1/FR1d_t1, respectively). PCR reactions were performed in 96-well plates. The reaction master mix consisted of 825 μ l water, 125 μ l 106 buffer, 62.5 μ l MgCl₂ (25 mM), 6.25 μ l dNTP (10 mM), 6.25 μ l each primer (0.01 mM) and 6.25 μ l Taq DNA polymerase (5 U/ μ l). This mixture was prepared for each plate, and each well contained 10.5 μ l of solution and 2 μ l of genomic DNA. The PCR reaction profile was comprised of an initial step of 2 min at 95 °C, and 35 cycles of 30 s at 94 °C, 40 s at 52 °C, and 1 min at 72 °C, with a final extension at 72 °C for 10 min. For specimens that failed to amplify using the primer combinations above, the primer combinations C_VF1LFt1/C_VR1LRt1 (Ivanova et al. 2007) consisting of VF1_t1/VF1d_t1/LepF1_t1/VFli_t1 and VR1_t1/VR1d_t1/LepR1_t1/VRli_t1 primer sets respectively were tried. All primers were appended with M13 tails to facilitate sequencing.

Amplicons were visualized on a 2 % agarose E-GelH 96-well system (Invitrogen). Sequencing reactions applied M13 forward and reverse primers using the BigDyeH Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems Inc.), and the reaction profile was comprised of an initial step of 2 min at 96 °C and 35 cycles of 30 s at 96 °C, 15 s at 55 °C, and 4 min at 60 °C. Products were directly sequenced using an ABI 3730 capillary sequencer, according to manufacturer's instructions.

2.4 Data Analysis

DNA sequences were aligned with SeqScape v.2.1.1 software (Applied Biosystems, Inc.) and further double-checked visually. Barcode sequences were subjected to distance-based, diagnostic character (Maximum Likelihood) and spectral clustering (BIN) analyses.

Sequence divergences were calculated using the Kimura two parameter (K2P) distance model (Kimura 1980), and Neighbor-joining (NJ) trees of K2P distances were created to provide graphic representations of divergence between species, using the software MEGA v5.0 (Tamura et al. 2011). The *p* distance model was also tested. Differences in distance estimates and tree topology between *p* distance and K2P models were minimal. Consequently, K2P model was chosen for comparison purposes, as it is commonly used for describing differences among species in DNA barcoding studies. Nevertheless, K2P has been described as a poorly fitting model at the species level (Collins et al. 2012). Moreover, distance-based models erase all character-based information (DeSalle 2006). As a result, K2P/NJ clusters of taxonomical units with either high intra-specific or low interspecific divergences were more closely inspected by a subsequent character-based analysis. For this purpose, the best nucleotide substitution model was selected to perform a maximum likelihood (ML) analysis using MEGA v5.0 (Tamura et al. 2011).

The Barcode Index Number (BIN) was used to estimate the number of species directly from the barcode records and congruence of these estimates with the

distance based and character based approaches were evaluated. BINs is “an online framework that clusters barcode sequences algorithmically, generating a web page for each cluster. Since clusters show high concordance with species, BINs can be used to verify species identifications as well as document diversity when taxonomic information is lacking” (see boldsystems.org and Ratnasingham and Hebert 2013 for further details on BINs). The public library of BINs in BOLD, was also used to scrutinize whether the literature about DNA Barcoding of Antarctic fishes had incorporated different BINs under this unique nominal taxa (i.e. to ascertain taxonomic conflicts among barcode studies conducted by different teams of researchers). For those species sharing the same BIN, we additionally explored their COI sequences for diagnostic characters with the tool available in BOLD.

The nearest-neighbour distance (NND) distribution analysis, that is, the minimum genetic distance between a species and its closest neighbour-species, was also performed. BOLD was also used to explore the genetic divergence between barcode records of given species of the Argentine Antarctic Peninsula with other available barcode sequences for the corresponding species from other sector of Antarctica.

All sequence assemblies, electropherogram (trace) files, primer sequences and specimen provenance data were deposited in the “Argentinean Antarctic Fishes phase I” (Code AAFI) on the Barcode of Life Database (BOLD, Ratnasingham and Hebert 2007). This included digital images of morphological voucher specimens, sex and ontogenetic stage (juvenile or adult), total and standard length as well as GPS coordinates for all specimen collection localities. Sequence data are also available on GenBank (Accession numbers pending).

3 Results and Discussion

A total of 275 specimens, belonging to 36 different putative species from 8 families, including Artedidraconidae, Bathydraconidae, Channichthyidae, Harpagiferidae, Myctophidae, Nototheniidae, Liparidae and Zoarcidae were successfully barcoded (Table 1). Representatives of family Arhynchobatidae (*Bathyraja maccaini* and *B. murrayi*), as well as some species within families Bathydraconidae (*Bathydraco marri*), Liparidae (*Paraliparis trilobodon*) and Zoarcidae (*Pachycara brachycephalum*) did not amplify following the same protocols. No stop codons, insertions or deletions were found in any of the amplified sequences, showing that all of them constitute functional mitochondrial COI sequences. Four species were represented by only one sequence. The analysis of COI sequences, with variable levels of divergence, revealed that interspecific divergence (D) was relatively high except in some species within Artedidraconidae, Liparidae, Channichthyidae and Zoarcidae ($D < 3\%$) (Fig. 2 and Table 2). The K2P genetic distances averaged 0.27 % within species, 8.96 % within genera, and 14.1 % within families (Table 3). The full K2P/NJ tree also showed that nearly all species exhibit unique barcodes or clusters of closely related haplotypes. The spectral cluster (BIN) did not agree with current taxonomic classification of our specimens in 100 % of cases. BIN analysis

Table 1 Species barcoded during the surveys 2011–2013 on Antarctic Peninsula

Family	Species	Nº	Range TL (mm)
Artedidraconidae	<i>Artedidraco skottsbergi</i> (Lönnberg 1905)	14	45–95
	<i>Pogonophryne scotti</i> (Regan 1914)	5	350
	<i>Pogonophryne permitini</i> (Andriashev 1967)	1	166
	<i>Dolloidraco longedorsalis</i> (Roule 1913)	1	155
Bathydraconidae	<i>Gymnodraco acuticeps</i> (Boulenger 1902)	4	285
	<i>Parachaenichthys charcoti</i> (Vaillant 1906)	12	118–496
	<i>Prionodraco evansii</i> (Regan 1914)	6	109–189
Channichthyidae	<i>Chaenocephalus aceratus</i> (Lönnberg 1906)	7	232
	<i>Chionodraco rastrospinosus</i> (De Witt and Hureau 1979)	5	255–387
	<i>Chaenodraco wilsoni</i> (Regan 1914)	6	163–320
	<i>Champocephalus gunnari</i> (Lönnberg 1905)	2	321–445
	<i>Cryodraco antarcticus</i> (Dollo 1900)	2	140–343
	<i>Pagetopsis macropterus</i> (Boulenger 1907)	4	188–237
	<i>Pseudochaenichthys georgianus</i> (Norman 1937)	3	177–369
Harpagiferidae	<i>Harpagifer antarcticus</i> (Nybelin 1947)	9	48–117
Myctophidae	<i>Electrona antarctica</i> (Günther 1878)	1	81
Nototheniidae	<i>Gobionotothen gibberifrons</i> (Lönnberg 1905)	13	70–395
	<i>Notothenia coriiceps</i> (Richardson 1844)	6	360–411
	<i>Notothenia rossii</i> (Richardson 1844)	5	277–359
	<i>Lepidonotothen squamifrons</i> (Günther 1880)	21	223–295
	<i>Lindbergichthys nudifrons</i> (Lönnberg 1905)	36	58–182
	<i>Nototheniops larseni</i> (Lönnberg 1905)	20	53–203
	<i>Pleuragramma antarctica</i> (Boulenger 1902)	11	167–188
	<i>Trematomus newnesi</i> (Boulenger 1902)	4	133–196
	<i>Pseudotrematomus scotti</i> (Boulenger 1907)	13	60–180
	<i>Pseudotrematomus hansonii</i> (Boulenger 1902)	1	222–371
	<i>Pseudotrematomus eulepidotus</i> (Regan 1914)	9	130–237
	<i>Pseudotrematomus bernacchii</i> (Boulenger 1902)	4	112–136
	Liparidae	<i>Paraliparis antarcticus</i> (Regan 1914)	4
<i>Paraliparis</i> sp.		4	61–104
<i>Careproctus</i> sp.		7	36–93
Zoarcidae	<i>Ophthalmolycus</i> cf. <i>amberensis</i> (Tomo, Marschoff and Torno 1977)	7	269
	<i>Lycenchelys nigripalatium</i> (DeWitt and Hureau 1979)	7	135–196
	<i>Lycenchelys tristichodon</i> (DeWitt and Hureau 1980)	1	269
	<i>Lycenchelys</i> sp.	1	146–181

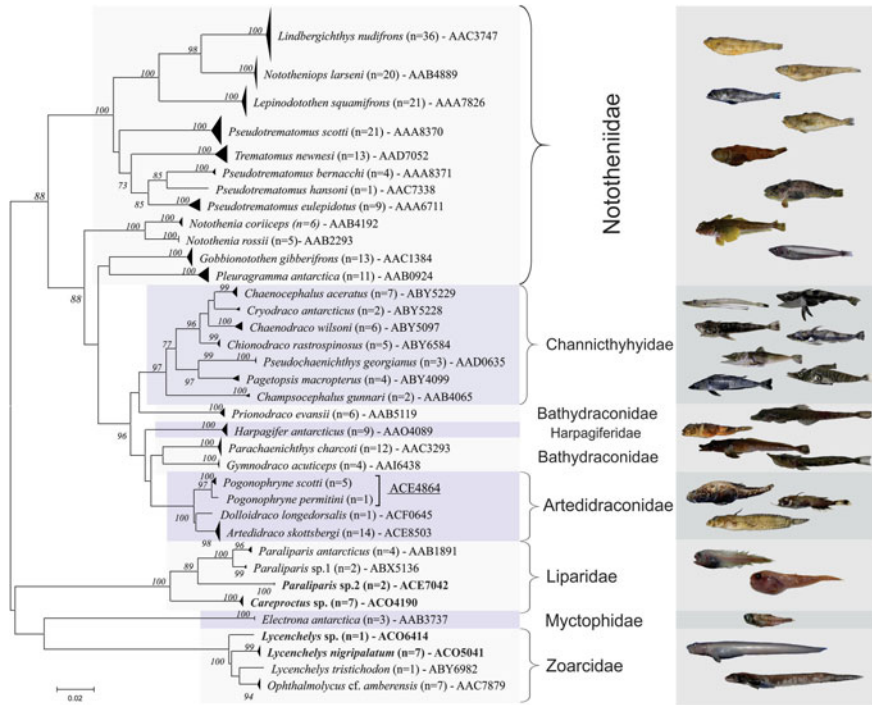


Fig. 2 Neighbour-Joining tree based on K2P distances. *Numbers after taxa* indicate the corresponding BIN. *Solid triangles* represent clusters of multiple specimens, with the vertical dimension proportional to the number of specimens, and the horizontal depth proportional to the genetic variation within that cluster. *Number at nodes* represent bootstrap values, (only values greater than 70 are given). *Underlined BIN* include more than one species. In *bold* those species that represent new BINs for BOLD. Figures contain representatives of each genera

recognized 35 taxonomic units from the 36 putative species (Fig. 2). Indeed albeit forming distinctive clusters in the K2P/NJ tree, *Pogonophryne scotti* and *P. permitini* showed low (0.6 %) genetic divergence (Fig. 2) and were included in the same BIN. Our work yielded barcodes for 13 species of the Antarctic Peninsula region for the first time. Sequence divergence between these specimens and those (public or published) from other regions were compared (Table 4). Most widely distributed species showed little or no sequence divergences among regions, as was observed by Smith et al. 2012.

3.1 Nototheniidae

This family is represented in the Antarctic waters by c.a 38 species, from which 21 were reported for the northern Antarctic Peninsula and adjacent waters (AP).

Table 2 Barcode gap analysis showing the distribution of distances within each species and the distance to the nearest neighbour of each species

Order	Family	Species	Mean intra-Sp	Max intra-Sp	Nearest neighbour	Nearest species	Distance to NN
Mycetophiformes	Mycetophidae	<i>Electrona antarctica</i>	0	0	FARGB1134-12	<i>Pleuragramma antarctica</i>	20.35
Perciformes	Artedidraconidae	<i>Artedidraco skottsbergi</i>	0.26	0.62	FARANI83-14	<i>Dolloidraco longedorsalis</i>	1.92
Perciformes	Artedidraconidae	<i>Dolloidraco longedorsalis</i>	N/A	N/A	FARGB1059-12	<i>Artedidraco skottsbergi</i>	1.92
Perciformes	Artedidraconidae	<i>Pogonophryne permitini</i>	N/A	N/A	FAMSD064-11	<i>Pogonophryne scotti</i>	0.46
Perciformes	Artedidraconidae	<i>Pogonophryne scotti</i>	0.25	0.46	FARGB1054-12	<i>Pogonophryne permitini</i>	0.46
Perciformes	Bathydraconidae	<i>Gymnodraco acuticeps</i>	0.05	0.15	FAMSD018-11	<i>Parachaenichthys charcoti</i>	5.95
Perciformes	Bathydraconidae	<i>Parachaenichthys charcoti</i>	0.13	0.46	FARANI27-14	<i>Gymnodraco acuticeps</i>	5.95
Perciformes	Bathydraconidae	<i>Pronodraco evansii</i>	0.2	0.46	FARGB1031-12	<i>Gymnodraco acuticeps</i>	7.48
Perciformes	Channichthyidae	<i>Chaenoecephalus aceratus</i>	0.19	0.46	FARANI85-14	<i>Cryodraco antarcticus</i>	2.49
Perciformes	Channichthyidae	<i>Chaenodraco wilsoni</i>	0.21	0.64	FARANI76-14	<i>Cryodraco antarcticus</i>	2.81
Perciformes	Channichthyidae	<i>Champsoccephalus gunnari</i>	0.31	0.31	FARGB1050-12	<i>Chaenoecephalus aceratus</i>	7.33
Perciformes	Channichthyidae	<i>Chionodraco rastrospinosus</i>	0.15	0.31	FARGB1050-12	<i>Chaenoecephalus aceratus</i>	2.51
Perciformes	Channichthyidae	<i>Cryodraco antarcticus</i>	0.33	0.33	FAMSD042-11	<i>Chaenoecephalus aceratus</i>	2.49

(continued)

Table 2 (continued)

Order	Family	Species	Mean intra-Sp	Max intra-Sp	Nearest neighbour	Nearest species	Distance to NN
Perciformes	Channichthyidae	<i>Pagetopsis macropterus</i>	0.31	0.62	FARAN154-14	<i>Pseudochaenichthys georgianus</i>	5.11
Perciformes	Channichthyidae	<i>Pseudochaenichthys georgianus</i>	0.11	0.16	FARAN054-12	<i>Pagetopsis macropterus</i>	5.11
Perciformes	Harpagiferidae	<i>Harpagifer antarcticus</i>	0.3	0.62	FARGB1095-12	<i>Artedidraco skottsbergi</i>	7.35
Perciformes	Nototheniidae	<i>Gobionotothen gibberifrons</i>	0.28	0.77	FARGB1136-12	<i>Pleuragramma antarctica</i>	9.38
Perciformes	Nototheniidae	<i>Lepidonotothen squamifrons</i>	0.09	0.46	FARGB627-12	<i>Nototheniops larseni</i>	8.98
Perciformes	Nototheniidae	<i>Lindbergichthys nudifrons</i>	0.22	0.62	FARGB627-12	<i>Nototheniops larseni</i>	5.91
Perciformes	Nototheniidae	<i>Notothenia coriiceps</i>	0.2	0.46	FARGB980-12	<i>Notothenia rossii</i>	3.74
Perciformes	Nototheniidae	<i>Notothenia rossii</i>	0	0	FARGB839-12	<i>Notothenia coriiceps</i>	3.74
Perciformes	Nototheniidae	<i>Nototheniops larseni</i>	0.24	0.77	FARGB1020-12	<i>Lindbergichthys nudifrons</i>	5.91
Perciformes	Nototheniidae	<i>Pleuragramma antarctica</i>	0.39	0.94	FARGB838-12	<i>Gobionotothen gibberifrons</i>	9.38
Perciformes	Nototheniidae	<i>Pseudotrematomus bernacchii</i>	0.08	0.15	FARGB1071-12	<i>Pseudotrematomus hansonii</i>	4.81
Perciformes	Nototheniidae	<i>Pseudotrematomus eulepidotus</i>	0.54	1.24	FARGB1051-12	<i>Pseudotrematomus bernacchii</i>	5.63
Perciformes	Nototheniidae	<i>Pseudotrematomus hansonii</i>	N/A	N/A	FARGB1051-12	<i>Pseudotrematomus bernacchii</i>	4.81
Perciformes	Nototheniidae	<i>Pseudotrematomus scotti</i>	0.43	1.09	FARGB1071-12	<i>Pseudotrematomus hansonii</i>	9.43

(continued)

Table 2 (continued)

Order	Family	Species	Mean intra-Sp	Max intra-Sp	Nearest neighbour	Nearest species	Distance to NN
Perciformes	Nototheniidae	<i>Trematomus newnesi</i>	0.69	1.55	FAMSD036-11	<i>Pseudotrematomus bernacchii</i>	7.31
Perciformes	Zoarcidae	<i>Lycenchelys nigripalatium</i>	0.13	0.46	FARGB963-12	<i>Lycenchelys tristichodon</i>	2.66
Perciformes	Zoarcidae	<i>Lycenchelys sp.</i>	N/A	N/A	FARGB963-12	<i>Lycenchelys tristichodon</i>	2.7
Perciformes	Zoarcidae	<i>Lycenchelys tristichodon</i>	N/A	N/A	FAMSD028-11	<i>Ophthalmolycus sp.</i>	2.35
Perciformes	Zoarcidae	<i>Ophthalmolycus sp.</i>	0.17	0.46	FARGB963-12	<i>Lycenchelys tristichodon</i>	2.35
Scorpaeniformes	Liparidae	<i>Careproctus georgianus</i>	0.05	0.2	FARGB971-12	<i>Paraliparis sp.</i>	8.27
Scorpaeniformes	Liparidae	<i>Paraliparis antarcticus</i>	0.15	0.31	FARAN129-14	<i>Paraliparis sp.</i>	1.55
Scorpaeniformes	Liparidae	<i>Paraliparis sp.</i>	4.51	6.81	FARGB1046-12	<i>Paraliparis antarcticus</i>	1.55

Where the species is a singleton, N/A is displayed for intra-specific values. Distances are highlighted if the nearest neighbour is less than 2 % divergent, or when the distance to the nearest neighbour is less than the max intra-specific distance

Table 3 Summary of distribution of sequence divergence at each taxonomic level

	n	Taxa	Comparisons	Min Dist (%)	Mean Dist (%)	Max Dist (%)	SE Dist (%)
Within species	270	30	1877	0	0.27	6.81	0
Within Genus	69	5	409	0.46	8.96	11.6	0.01
Within Family	263	6	11545	1.92	14.1	23.7	0

Table 4 Intraspecific K2P divergences within specimens from Antarctic Peninsula and adjacent Islands (AP) and among representative of AP and other regions of the Southern Ocean (OR)

Species	D (%) AP	D (%) OR
<i>Electrona antarctica</i>	0	0–0.78
<i>Pogonophryne permittini</i>	–	No specimens
<i>Dolloidraco longedorsalis</i>	–	0.16–0.48
<i>Careproctus</i> sp.	0–0.2	No specimens
<i>Paraliparis antarcticus</i>	0–0.31	0–0.64
<i>Paraliparis</i> sp1.*	–	0.93–1.1 %
<i>Gymnodraco acuticeps</i>	0–0.15	0–0.33 %
<i>Chaenodraco wilsoni</i>	0–0.64 %	0–0.77 %
<i>Cryodraco antarcticus</i>	–	0.16–0.33 %
<i>Pagetopsis macropterus</i>	0–0.62 %	0.31–0.46 %
<i>Lycenchelis tristichodon</i>	–	0.62–0.8 %
<i>Ophthalmolycus</i> cf. <i>amberensis</i>	0–0.46 %	0–0.48 % Ross Sea/1.63–2.18 % AAT
<i>Pleuragramma antarctica</i>	0–0.94 %	0–1.1 %

Data included only species that were not previously barcoded in AP. AAT Australian Antarctic Territory

Recently Duhamel et al. (2014) proposed the inclusion of several families (Artedidraconidae, Bathydraconidae, Channichthyidae, Harpagiferidae) as sub-families within Nototheniidae, based on previous molecular analysis. Although this seems to be a reasonably well-supported hypothesis concerning their classification, we followed the accepted classification presented by Eschmeyer (2015) and treated each separately. Several barcoding studies were made on Antarctic representatives of this family (Rock et al. 2008; Lautrédou et al. 2010; Dettai et al. 2011; Smith et al. 2012). These studies included 22 species of genera *Cryothenia*, *Dissostichus*, *Gobionotothen*, *Lepidonotothen*, *Lindbergichthys*, *Notothenia*, *Nototheniops*, *Pleuragramma*, *Pseudotrematomus* and *Trematomus*, showing that COI provided effective species-level discrimination for nearly all species. The exception appeared within the genus *Trematomus* in which lack of COI divergence was reported for

T. loennbergi and *T. lepidorhinus* (Lautrédou et al. 2010; Dettai et al. 2011; Smith et al. 2012). Public data, available on BOLD, reveal that BINs are congruent with all previous results, placing all species, except *T. loennbergi* and *T. lepidorhinus* into different and exclusive BINs. In the present study specimens belonging to 12 species of Nototheniidae were barcoded (Table 1). All these species were previously barcoded. However, no barcode sequences of *Pleuragramma antarctica* from AP were published yet (Rock et al. 2008; Lautrédou et al. 2010; Dettai et al. 2011; Smith et al. 2012). *Pleuragramma antarctica* showed little or no sequence divergences among AP and other regions, sharing haplotypes (similarity ranged 98.9 to 100 %), as was observed by Smith et al. (2012) for other Notothenids.

3.1.1 The Application of Barcode to Elucidate Ontogenetic Changes in Diagnostic Characters

The diagnostic features used for species identification within the family Nototheniidae rely on a specimen range in size from about 10 cm to over 2 m (DeWitt et al. 1990). In that key, the size range of fish used for the diagnosis of some species is not specified. Ontogenetic changes are discussed only for some structures such as otoliths (DeWitt et al. 1990) or in body morphometrics for punctual species (Piacentino and Barrera-Oro 2009). In this respect, we observed that juveniles of several species of Nototheniidae lacked some diagnostic characteristics (e.g. pattern of head squamation) commonly employed in available diagnostic keys as well as species descriptions. Therefore, these juveniles could be erroneously assigned to a different species or even different genus. In this respect, DNA barcodes were useful to clarify the inconsistency and allowed us to properly match each juvenile with the corresponding species.

Notably, we found that juveniles a priori identified as *Gobionotothen* sp., were assigned to *Pseudotrematomus scotti* by BIN analysis after the barcode sequences were obtained. According to the key to genera of Nototheniidae, presented in Dewitt et al. (1990), the presence of scales in the preorbital separates (among others) *Pseudotrematomus* (at the time of publication valid as *Trematomus*) from *Gobionotothen*, being naked in the last genus. The morphological description of *P. scotti* further characterized this species by a fully scaled occipital and interorbital regions, as well as cheeks and opercles. We noted that in juveniles (51–56 mm TL) of *Pseudotrematomus scotti* the head is mostly naked with the sole presence of a few scales in the occipital region (Fig. 3). On the other hand we have note that juveniles (61–74 mm TL) of *Lepidonotothen squamifrons*, lacked scales on dorsal part of eyes while they are present in adults (Fig. 4). Finally, some juveniles identified as *Trematomus* sp. (63–68 mm TL) corresponded to *Nototheniops larseni* (at the time of publication valid as *Lepidonotothen larseni*). The incongruence here was that these specimens presented the pre-orbital region naked whereas this part of the body is scaled in adults (Fig. 5). Diagnostic meristic counts (dorsal-fin, anal-fin and pectoral-fin rays and tubular scales along the upper lateral line) have overlapping ranges between some genera, therefore the presence/absence of scales in

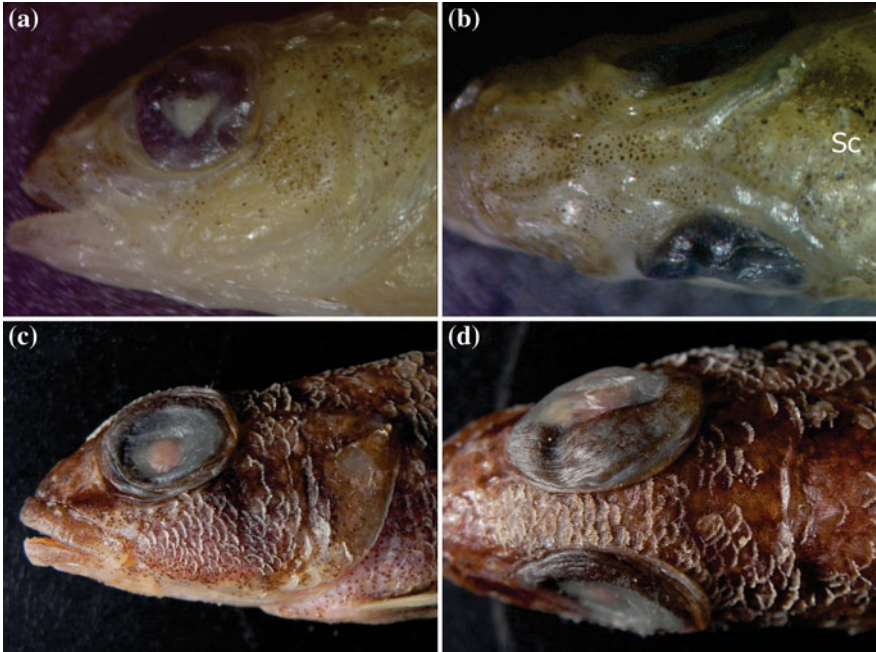


Fig. 3 Ontogenetic changes observed in specimens of *Pseudotrematomus scotti*. Lateral and dorsal view of juveniles **a** and **b** and adults **c** and **d**

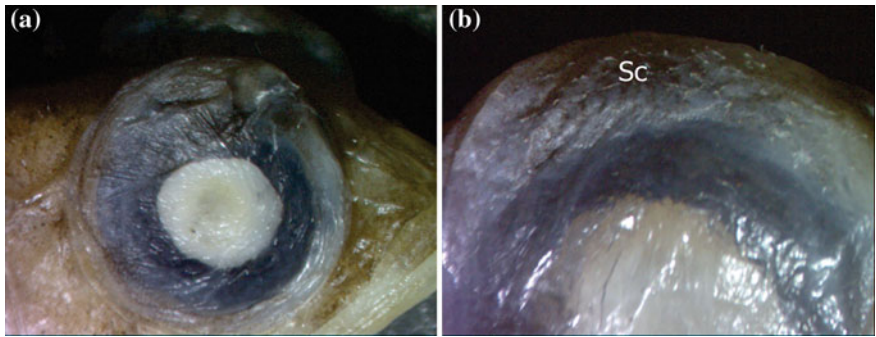


Fig. 4 Ontogenetic changes observed in dorsal squamation of eyes in specimens of *Lepidonotothen squamifrons*. **a** Juveniles and **b** adults. Sc Scales

preorbital and occipital regions are the diagnostic features that primarily distinguish them (Dewitt et al. 1990) and as was mentioned this characteristic was variable among juveniles and adults.

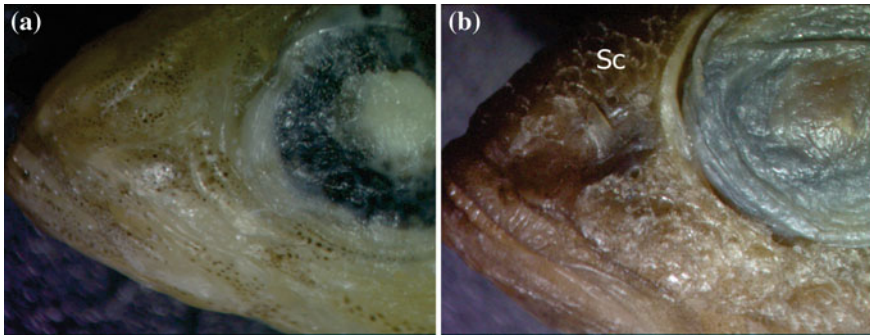


Fig. 5 Ontogenetic changes observed in specimens of *Nototheniops larseni*. **(a)** juveniles **b** Adults. *Sc* Scales

3.2 *Myctophidae*

Myctophids constitutes the dominant fish family of the mesopelagic and bathypelagic Antarctic waters in terms of their species richness, abundance and biomass (Donnelly et al. 1990; Donnelly and Torres 2008; Koubbi et al. 2011). It is represented in Antarctic waters by approximately 36 species, 12 of them are registered in AP (Gon and Heemstra 1990). At least, 9 species of lanternfishes were previously barcoded (Rock et al. 2008; Dettai et al. 2011; Smith et al. 2012). These studies showed that COI provides effective species-level discrimination and also highlighted possible new species (Smith et al. 2012). In turn, BINs mostly supported these findings with each species possessing an exclusive BIN, but *Gymnoscopelus bolini* which harbored different BINs, suggesting the existence of potentially cryptic species (Smith et al. 2012). During our surveys only *Electrona antarctica* was collected and barcoded. *Electrona antarctica* is typically found south of the Antarctic Polar Front (APF). This species has a wide distribution in the southern ocean. Biogeographic data indicate that *Electrona antarctica* has a circumpolar distribution mainly between the Antarctic Slope Front (as delimited by the continental 500 m isobath) and the APF, although small specimens can be taken in the Sub-Antarctic Zone. The southernmost record for the species is at 74.67 °S (Duhamel et al. 2014). There are published barcode records from all Antarctic regions (Rock et al. 2008; Dettai et al. 2011; Smith et al. 2012). Those from the west Antarctic correspond to the Georgias Islands (Rock et al. 2008). Our results showed that specimens from AP presented shallow intra-specific divergences with those from other regions, sharing haplotypes (COI Similarity 99.21–100 %), as was observed by Smith et al. 2012.

3.3 *Artedidraconidae*

Artedidracons, known as plunderfishes, are the less well-known notothenioid fishes (Eakin et al. 2009). This family is composed of approximately 26 species, 10 of which have been recorded in the AP (Eakin 1990). The genus *Pogonophryne* has a circum-Antarctic distribution with some species extending as far north as the South Orkney Islands, and in depths ranging from 100 m to more than 2500 m (Duhamel et al. 2014). The genus comprises nearly 70 % of the diversity within the family. Currently, five species groups are recognized within this genus based on differences in spotting patterns and meristics: the *P. mentella* group, the *P. scotti* group, the *P. barsukovi* group, the *P. marmorata* group and the *P. albipinna* group (Eakin et al. 2009). A molecular phylogenetic analysis of this family was provided by Eakin et al. (2009), showing low genetic divergences among species and limited phylogenetic resolution among the five species groups. Species of the genus *Pogonophryne* are especially difficult to identify because there is scarce meristic separation (Eakin et al. 2009). The mental barbel in *Pogonophryne*, which has been used to distinguish species appears to be highly variable in shape within the various species (Eakin et al. 2001) and makes species identification difficult (Duhamel et al. 2014).

Twenty species of plunderfishes were previously barcoded showing a lack of resolution in many of them (Rock et al. 2008; Dettai et al. 2011; Smith et al. 2012). Based on relatively few specimens, Rock et al. (2008) found that barcodes failed to discriminate *Artedidraco loennbergi* from *A. skottsbergi*. In contrast, Dettai et al. (2011), using more than 100 specimens from 7 species, found that species formed unique molecular clusters excepting some *Pogonophryne* species. However, almost all interspecific differences were smaller than 2 % and several less than 0.8 %. Finally, Smith et al. (2012), analysed barcode data from 4 species of Artedidraconidae and found low divergences among species and highlighted a lack of region-specific haplotypes. Moreover, other molecular markers showed low genetic divergences suggesting recent radiation (Lecointre et al. 2011). In concordance with all these results, when exploring public barcode data only 5 different BINs were obtained within this family: *Artedidraco loennbergi*, *Artedidraco skottsbergi*, *Artedidraco mirus*, *Artedidraco orianae*, and two BINs with several species each, one containing *Dolloidraco longedorsalis*, *Artedidraco shackletoni* and *Histiodraco velifer* and another one with all the species of *Pogonophryne*.

Four species were barcoded in the present study including *Artedidraco skottsbergi*, *Pogonophryne scotti*, *P. permittini* and *Dolloidraco longedorsalis*. All these species but *P. permittini* were previously barcoded but there are no published barcode records for *D. longedorsalis* from the AP. Different BINs were detected for *Artedidraco skottsbergi*, and *Dolloidraco longedorsalis* whereas *Pogonophryne scotti* and *P. permittini*, are included in the same BIN (Fig. 2 and Table 5). Nevertheless, discrimination between these two species was supported by both the

Table 5 Summary of species collected and main information containing each BIN (Barcode Index Number)

Species	BIN	Average/max distance p-dist (%)	Distance to the nearest p-dist (%)	Nearest member
<i>Electrona antártica</i>	AAB3737	0.08/0.81	2.39	<i>Symbolophorus veranyi</i>
<i>Artedidraco skottsbergi</i>	ACE8503	0.27/0.8	1.28	<i>Artedidraco loennbergi</i>
<i>Dolloidraco longedorsalis</i>	ACF0645	0.84/2.25	1.28	<i>Artedidraco orianae</i>
<i>Pogonophryne scotti</i>	ACE4864	0.54/1.29	1.44	<i>Artedidraco loennbergi</i>
<i>Pogonophryne permitini</i>	ACE4864	0.54/1.29	1.44	<i>Artedidraco loennbergi</i>
<i>Gymnodraco acuticeps</i>	AAI6438	0.06/0.47	4.46	<i>Cygnodraco mawsoni</i>
<i>Parachaenichthys charcoti</i>	AAC3293	0.17/1.08	2.25	<i>Parachaenichthys georgianus</i>
<i>Prionodraco evansii</i>	AAB5119	0.19/0.77	4.65	<i>Racovitzia glacialis</i>
<i>Chaenocephalus aceratus</i>	ABY5229	0.2/0.49	2.09	<i>Cryodraco antarcticus</i>
<i>Chaenodraco wilsoni</i>	ABY5097	0.32/0.96	2.09	<i>Chionodraco myersi</i>
<i>Champocephalus gunnari</i>	AAB4065	0.55/1.62	6.74	<i>Chionodraco myersi</i>
<i>Chionodraco rastrispinosus</i>	ABY6584	0.11/0.32	1.77	<i>Chionodraco myersi</i>
<i>Cryodraco antarcticus</i>	ABY5228	0.14/0.49	1.46	<i>Chionobathyscus dewitti</i>
<i>Pagetopsis macropterus</i>	ABY4099	0.26/0.62	2.09	<i>Pagetopsis maculatus</i>
<i>Pseudochaenichthys georgianus</i>	AAD0635	0.06/0.17	4.38	<i>Neopagetopsis ionah</i>
<i>Harpagifer antarcticus</i>	AAO4089	0.47/1.44	6.74	<i>Artedidraco skottsbergi</i>
<i>Gobionotothen gibberifrons</i>	AAC1384	0.22/1.12	4.34	<i>Gobionotothen acuta</i>
<i>Lepidonotothen squamifrons</i>	AAA7826	0.27/1.61	8.07	<i>Patagonotothen tessellata</i>
<i>Lindbergichthys nudifrons</i>	AAC3747	0.37/1.63	1.52	<i>Lindbergichthys mizops</i>
<i>Notothenia coriiceps</i>	AAB4192	0.22/0.85	3.57	<i>Notothenia rossii</i>
<i>Notothenia rossii</i>	AAB2293	0.03/0.32	3.57	<i>Notothenia coriiceps</i>

(continued)

Table 5 (continued)

Species	BIN	Average/max distance p-dist (%)	Distance to the nearest p-dist (%)	Nearest member
<i>Nototheniops larseni</i>	AAB4889	0.26/0.96	6.16	<i>Lindbergichthys mizops</i>
<i>Pleuragramma antarctica</i>	AAB0924	0.26/1.15	8.63	<i>Cryothenia peninsulae</i>
<i>Pseudotrematomus bernacchii</i>	AAA8371	0.18/0.53	4.29	<i>Cryothenia amphitreta</i>
<i>Pseudotrematomus eulepidotus</i>	AAA6711	0.8/1.98	4.31	<i>Trematomus bernacchii</i>
<i>Pseudotrematomus hansonii</i>	AAC7338	0.52/1.32	4.54	<i>Trematomus bernacchii</i>
<i>Pseudotrematomus scotti</i>	AAA8370	0.31/1.12	8.18	<i>Trematomus</i> sp.
<i>Trematomus newnesi</i>	AAD7052	0.58/1.87	7.06	<i>Pagothenia borchgrevinki</i>
<i>Lycenchelys nigripalatium</i>	ACO5041	0.21/0.55	1.14	<i>Lycenchelys aratrirostris</i>
<i>Lycenchelys</i> sp.	ACO6414	–	2.27	<i>Lycenchelys aratrirostris</i>
<i>Lycenchelys tristichodon</i>	ABY6982	0.24/0.92	1.94	<i>Lycenchelys aratrirostris</i>
<i>Ophthalmolycus</i> cf. <i>amberensis</i>	AAC7879	0.14/0.48	1.61	<i>Ophthalmolycus amberensis</i>
<i>Careproctus</i> sp.	ACO4190	0.05/0.2	2.53	<i>Careproctus longipectoralis</i>
<i>Paraliparis antarcticus</i>	AAB1891	0.27/0.66	1.08	<i>Paraliparis</i> aff. <i>longipectoralis</i>
<i>Paraliparis</i> sp.1	ABX5136	0.58/1.14	2.25	<i>Paraliparis</i> sp.
<i>Paraliparis</i> sp.2	ACE7042	–	1.13	<i>Paraliparis</i> aff. <i>longipectoralis</i>

NJ (Fig. 2) and ML (Fig. 4) analyses and also by NDC. Interestingly, a single BIN includes all species of *Pogonophryne* already barcoded (12 species). Compared with public data on BOLD our specimen identified as *P. permittini* clustered together with specimens of *Pogonophryne barsukovi* (from Smith et al. 2012) and *Pogonophryne* sp1 (from Dettai et al. 2011). The NDC did not discriminate these species but, as a group, two NDC, #97 (A) and #603 (A), allowed to differentiate them from the remaining *Pogonophryne*.

There are no previous barcode records for *Dolloidraco longedorsalis* from the AP. COI similarity with conspecific from other regions (Dettai et al. 2011; Smith et al. 2012) ranged 99.52–99.84 %. As noted above, exploring public BOLD data we found that, along with *Dolloidraco longedorsalis*, two more species are included in the same BIN: *Histiodraco velifer* and *Artedidraco shackletoni*. Interspecific

Table 6 Nucleotide position for each diagnostic character in some of the species analysed in this study

Species/nucleotic position	#39	#321	#336	#351	#468	#492	#495	#531	#621
<i>Dolloidraco longedorsalis</i>	T	C	C	T	T	C	T	T	C
<i>Artedidraco shackletoni</i>	T	C	C	C	C	G	C	T	C
<i>Histiodraco velifer</i>	C	T	T	T	T	C	C	G	T
Species/nucleotic position	#252	#555	#579						
<i>Harpagifer antarcticus</i>	T	T	A						
<i>H. bispinnis/H. paliolatus</i>	C	C	G						

divergence among them is low (<2 %), but specimens from each species yielded a cohesive cluster. In addition, the three species could be discriminating through NDC (Table 6).

3.4 *Bathydraconidae*

Antarctic dragonfishes are slender-bodied species endemic to the Southern Ocean and live mostly on the shelf and upper slope. They are represented by 15 species in Antarctic waters, 7 of them were recorded in AP (Gon 1990). All species of dragonfishes were previously barcoded. Rock et al. (2008) stated that certain species (they barcoded 3 species of *Bathydraco*) were not resolved by COI, representing one single MOTU. In the same way Smith et al. (2012) reported lack of species resolution within *Bathydraco* species with shared haplotypes among two pair of species. Exploring public BOLD data, 11 different BINs were obtained within this family. Each species, except those of the genus *Bathydraco* formed an exclusive BIN.

Three dragonfishes were barcoded in this study, *Gymnodraco acuticeps*, *Prionodraco evansii* and *Parachaenichthys charcoti*. Each species formed unique BINs. No barcode records of *G. acuticeps* from the AP were published yet. Similarity percentage between ours specimens and those from other regions ranged from 99.67 to 100 % showing shallow intra-specific divergences among them.

3.5 *Channichthyidae*

Crocodile icefishes are unique among vertebrates in lacking haemoglobin. The family is represented by 18 species in Antarctic waters, 15 of them were registered for AP (Iwami and Kock 1990; Kock 2005; La Mesa et al. 2002). DNA barcoding was applied to 15 species of Channichthyidae (Rock et al. 2008; Dettai et al. 2011;

Smith et al. 2012) and with the exception of *Cryodraco antarcticus*, no problems of resolution with COI were detected (Rock et al. 2008; Dettai et al. 2011; Smith et al. 2012). Smith et al. 2012 found that *C. antarcticus* and *C. atkinsoni* shared haplotypes. In concordance with these studies 14 different public BINs are available on BOLD, all species except *C. antarcticus* and *C. atkinsoni* formed different BINs.

Seven species of crocodile icefishes were successfully barcoded in this study. All these species were previously barcoded. Each species formed unique BINs (Fig. 2 and Table 5). No published barcode records are available for *Chaenodraco wilsoni*, *Cryodraco antarcticus* and *Pagetopsis macropterus* in west Antarctic waters. The three species are distributed around the Antarctic continental shelf but also occur in small numbers as far north as the southern Scotia Arc (Kock 1992). Our results show shallow intra-specific divergences among AP and other regions (Table 3).

3.6 *Harpagiferidae*

Spiny plunderfishes are small benthic/epibenthic species, confined to coastal waters from 0 m (under rocks in pools at low tide) to 200 m depth. They are ecologically and morphologically similar species (Eastman 2005). Currently, 10 species of the Genus *Harpagifer* have been described of which 8 are restricted to the sub-Antarctic islands and southern Scotia Arc islands. *Harpagifer antarcticus* is present along the western side of the Antarctic Peninsula. Littoral and sublittoral zones seem inhabited by different species (Neyelov and Prirodina 2006). *H. permitini* seems restricted to South Georgia; *H. crozetensis* and *H. spinosus* to the Crozet Islands; *H. kerguelensis* and *H. nybelini* to Kerguelen Islands and Heard Islands; *H. marionensis* to the Prince Edward Islands; *H. macquarensis* and *H. andriashevi* to Macquarie Island and *H. georgianus* in South Georgia, Prince Edward and Macquarie islands (Duhamel et al. 2014).

No published barcode records are available for any species. In the present study we barcoded specimens of *Harpagifer antarcticus*, which formed a unique BIN (Table 3). The same BIN also contains *H. bispinnis*, *H. palliolatus*, *H. georgianus* and *H. spinosus*. *H. bispinnis* and *H. palliolatus* are restricted to south Patagonian waters and yielded a unique cluster whereas specimens of *H. antarcticus* were not all clustered together. Specimens of *H. antarcticus* showed 0–0.62 % intraspecific divergence and 98.6–99.53 % similitude with those of *H. bispinnis*/*H. palliolatus*. However, NDC allowed discrimination between *H. bispinnis*/*H. palliolatus* from *H. antarcticus* (Table 6). The lack of resolution between valid species of *Harpagifer* using COI is not surprising. Hüne et al. (2014) found low level of genetic divergence between *Harpagifer antarcticus* (from Antarctica) and *H. bispinnis* (from Patagonia) using the mitochondrial control region D-loop (a rapid mitochondrial marker) suggesting a recent (Quaternary) colonization of Patagonia from the Antarctic Peninsula.

3.7 Zoarcidae

Eelpouts are an important component of benthic ecosystems in Antarctic waters with approximately 32 reported species, 22 of them have been recorded in AP (Matallanas 2009, 2010, 2011; Matallanas et al. 2012). At least 9 species of eelpouts were previously barcoded. COI showed a high species-level resolution and highlighted possible new species (Rock et al. 2008; Dettai et al. 2011; Smith et al. 2012). Four different species were collected in this study: *Ophthalmolycus* cf. *amberensis*, and three species of *Lycenchelys*, *L. nigripalatum*, *L. tristichodon* and an unidentified species *Lycenchelys* sp. Each species formed unique BINs (Fig. 2 and Table 5). Discrimination among these species was also supported by both NJ (Fig. 2) and ML (Fig. 5) trees. *Ophthalmolycus amberensis* is widely distributed along the Antarctic continent to the Antarctic Peninsula (Duhamel et al. 2014).

The seven specimens of *Ophthalmolycus* cf. *amberensis* were included in the same BIN. There is no previous barcode data of this species from the AP. The BIN also contains specimens of *O. amberensis* from the Ross Sea. Similarity between specimens from both regions ranged 99.84–100 %. The nearest neighbor of this BIN contained *O. amberensis* from the Durmont d'Urville Sea. The analysis of public barcode sequences yielded two different clusters, one containing *O. amberensis* from the Ross Sea and those collected in AP, and the other containing specimens from Australian Antarctic Territory (AAT). Smith et al. (2012), already highlighted the high divergence between specimens from Ross Sea and AAT and suggested that this high level of divergence would be indicative of species-level divergence. The authors stressed the need for analyze additional specimens from the type locality (Ambers Islands, Antarctic Peninsula) to resolve the taxonomic status of specimens from the Ross Sea and AAT. As mentioned above, our specimens collected in the Antarctic Peninsula, matched specimens of *O. amberensis* from the Ross Sea and are included in the same BIN. If the type locality is Antarctic Peninsula, it is probable that specimens from this BIN represent the real *O. amberensis* and those from the AAT be a new species. Further morphological analysis, comparing vouchers from the different regions are needed to corroborate this hypothesis (Fig. 6).

The seven specimens of *Lycenchelys nigripalatum* were clustered together and formed a unique BIN (Fig. 2 and Table 5) which is new for BOLD (ACO5041). There is no previous barcode data of this species from any region of Antarctica. The nearest neighbor is *Lycenchelys aratrirostris* with a percentage similarity of 98.54–98.72 %. Regarding *Lycenchelys tristichodon* the specimens formed a unique BIN. This species was already barcoded (Dettai et al. 2011) but there is no public barcode data of specimens from AP. The similarity percentage with specimens from other regions range 99.22–99.38 %. There were no sharing haplotypes between regions and 3 different NDC were also found: Site#172 (G vs A) Site#181 (C vs T); Site#541 (T vs C). The nearest neighbor (97.98 % similarity) is *L. aratrirostris*. Finally *Lycenchelys* sp. constitute a singleton forming a unique and new BIN for BOLD. The distance to the nearest neighbor (*Lycenchelys aratrirostris*) is 2.27 %

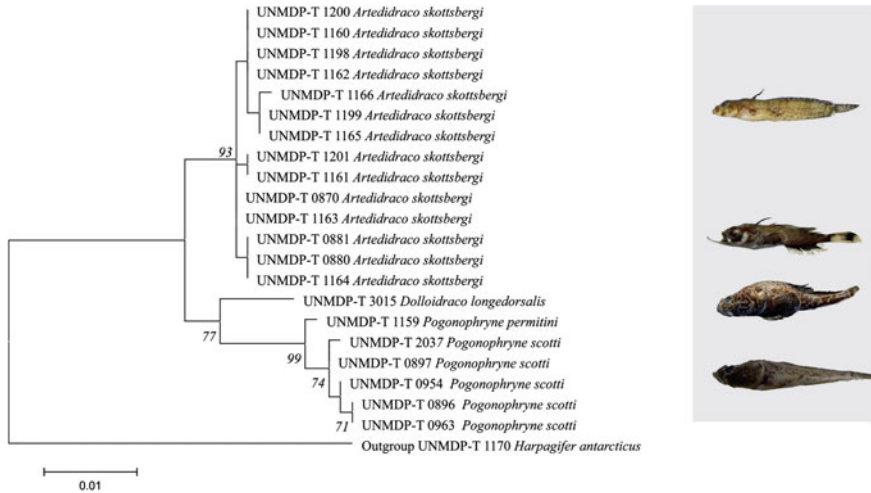


Fig. 6 COI relationships among specimens of Artedidraconidae collected in the Antarctic Peninsula. ML tree rooted with *Harpagifer antarcticus*; number at nodes are bootstrap percentage (>70 %) after 500 replicates based on ML. Scale bar is a K2+G distance reference. Code numbers represent BOLD sample IDs. Photos on the right correspond to each species

(p-dist). The record of zoarcid species and their geographical distribution in the Southern Ocean need to be completed. Species identification is still a problem in some species. For example, very few species have been recorded from East Antarctica (Duhamel et al. 2014).

3.8 Liparidae

Snailfishes are the most speciose family of Antarctic fishes with approximately 66 species described, 17 of which were recorded for the AP and adjacent waters (Heemstra and Duhamel 1990; Andriashev 1998; Matallanas and Pequeño 2000; Chernova 2006; Balushkin and Prirodina 2010; Balushkin 2012; Stein 2012). Identification requires a high level of expertise and a thorough study of osteological characters. Given the fact that several species were barcoded (see Duhamel et al. 2010) including species within genera *Careproctus* and *Paraliparis*, and the identification of specimens were made by specialists, assignation of specific names for our specimens were based on results from BINs. At least 13 species of Liparidae were previously barcoded (Rock et al. 2008; Duhamel et al. 2010; Dettai et al. 2011; Smith et al. 2012), showing different degrees of resolution. Duhamel et al. (2010) found that all individuals from a single species are grouped together in the molecular trees. The distance among species is mostly over 2 %, except for a few pairs of *Paraliparis* species (*P. charcoti*-*P. leobergi*, *P. rosaceus*-*P. neelovi*). Smith

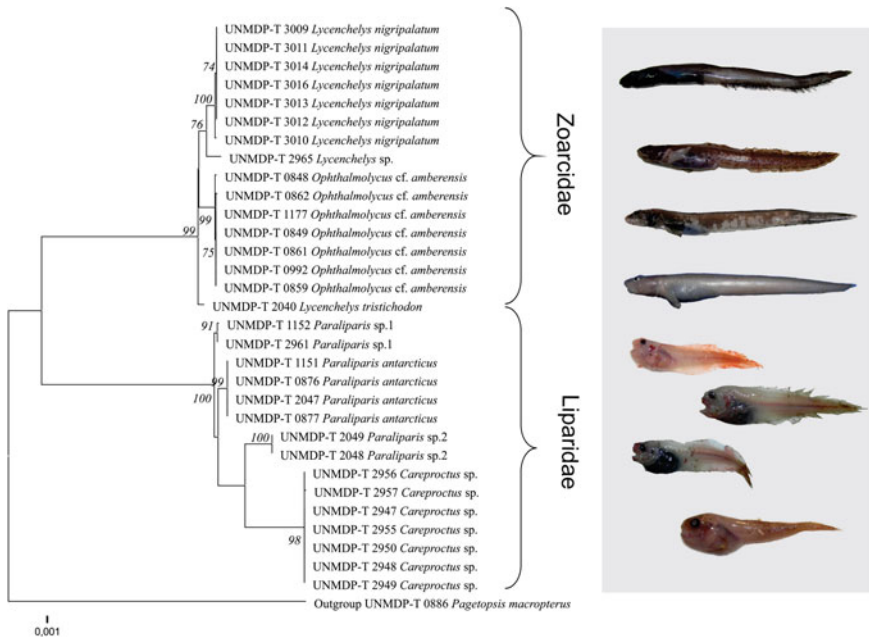


Fig. 7 COI relationships among specimens of Zoarcidae and Liparidae collected in the Antarctic Peninsula. ML tree rooted with *Pagetopsis macropterus*; number at nodes are bootstrap percentage (>70 %) after 500 replicates based on ML. Scale bar is a K2+G distance reference. Code numbers represent BOLD sample IDs. Photos on the right correspond to each species

et al. (2012) noticed lack of resolution among Ross Sea liparids with shallow or zero divergence among recently described species (Stein 2012) of the genus *Paraliparis* (Fig. 7).

In the present study, four different BINs were recorded within this family, corresponding to a species initially identified as *Careproctus georgianus*, *Paraliparis antarcticus* and another two species that could not be identified to species level and were named *Paraliparis* sp. One of them, corresponding to the BIN ACE7042, showed low ($D < 2\%$) interspecific divergence (Fig. 2) related to *Paraliparis antarcticus*, but both the NJ (Fig. 2) and ML (Fig. 5) trees, and BIN approaches supported it is a different species. The other cluster of *Paraliparis* sp. (BIN ABX5136) showed a high distance (>8 %).

Seven specimens of *Careproctus* sp. were barcoded. All of them were clustered together and constituted the same BIN (Fig. 2 and Table 5). No previous barcode data were available on this species representing a new BIN for BOLD. All specimens came from AP. The distance (p-dist) to Nearest Neighbor, *Careproctus longipectoralis* (BIN AAI6622), is 2.53 %. The species were initially identified as *C. georgianus*, but the similarity with public records of *C. georgianus* in BOLD, one from Antarctic waters (Rock et al. 2008) and two from the northern Pacific (Steinke et al. 2009) is 93.91–93.93 %, indicating represent another species. For the

West Antarctic Ocean at least 12 species of *Careproctus* have been described (Andriashev and Prirodina 1990; Duhamel 1992; Andriashev and Stein 1998): *C. acifer*, *C. eltianae*, *C. federovi*, *C. georgianus*, *C. improvisus*, *C. lacmi*, *C. leptorhinus*, *C. parviparratus*, *C. polarstein*, *C. rimiventris*, *C. scoplopterus* and *C. steini*. There are only barcode data of *C. georgianus*. There are barcodes from species of other Antarctic regions (*C. crozotensis*, *C. continentalis*, *C. discoveryae*, *C. longipectoralis*). Our specimens are most similar to *C. longipectoralis*, although correspond to a different BIN and has a genetic divergence (K2P) >3 %.

Regarding *Paraliparis antarcticus* there is no previous barcode record of this species from the West Antarctic. In our samples, all specimens formed a unique BIN in which conspecifics from other regions were also present (Fig. 2 and Table 5). The similarity percentage with specimens from other regions ranged 99.36–100 %, showing shallow intra-specific divergences among AP and other regions, sharing haplotypes. Two specimens of *Paraliparis* sp1. were barcoded in this study. These specimens formed a unique BIN (Fig. 2 and Table 5) which also contained several *P. mawsoni* from other Antarctic region (Dettai et al. 2011). In the NJ tree these two species clustered separately with a percentage similarity ranging 98.9–99.07 %. Two specimens of *Paraliparis* sp2. were also barcoded in this study. These specimens formed a unique BIN (Fig. 2 and Table 5) which also contained another *Paraliparis* sp. from the same area (Rock et al. 2008). The nearest neighbor was *P. aff longipectoralis* that formed another BIN.

3.8.1 Extending the Distribution of Some Antarctic Fish Species

Most species collected during our survey were previously cited for the study area. However some of them constitute new records or an expansion in the distribution range of the species. Within liparids, *P. antarcticus* was previously cited for the east Antarctic shelf, and southernmost Weddell Sea, thus these records extended its distribution to the northern part of the Antarctic Peninsula. Besides, if *Paraliparis* sp1 correspond to *P. mawsoni* (according to BIN), the presence of this species is new for the area because it had been reported only for the south eastern Lazarev Sea (Heemstra and Duhamel 1990), east Antarctic (Terre Adélie and George V Land) (Duhamel et al. 2010), and Weddell Sea (Matallanas, 1999). Although *Paraliparis antarcticus* and *P. mawsoni* have been mentioned to occur in a wider circum-antarctic distribution, knowledge of the distribution of Southern Ocean liparids is still limited due to poor coverage of the deep-sea (Duhamel et al. 2014). *Lycenchelis nigripalatum* and *L. tristichodon* were previously registered off Adelaide Island and Durmont d'Urville Sea (Dettai et al. 2011), thus our records for these species in the study area spread its distribution to the northern part of Antarctic Peninsula.

4 Conclusions

Fishes from Antarctic Peninsula and adjacent waters represent 35 % of fish richness in Antarctic waters. In this study, four new BINs were generated and incorporated in BOLD. They corresponded to *Lycenchelys nigripalatum*, *Lycenchelys* sp., *Careproctus* sp., and a *Paraliparis* sp. Even though barcode records from all the other species barcoded in this study are available at BOLD, this work contributed to the Barcode reference library with samples from an area not fully barcoded (see Rock et al. 2008; Lautrédou et al. 2010; Duhamel et al. 2010; Dettai et al. 2011; Smith et al. 2012).

In this study, 35 different BINs from 36 putative species were obtained, with only *Pogonophryne scotti* and *P. permittini* sharing the same BIN. However, when exploring public barcode data, we found that two more BINs corresponding to *Dolloidraco longedorsalis* and *Harpagifer antarcticus* also included other valid species. Thus, using only a conservative approach (BINs), 88.6 % of the species barcoded in this study could be identified at species level with accuracy. A similar level of species discrimination using BINs was reported for the fish fauna of the Northeast Atlantic (Knebelberger et al. 2014). However the use of nucleotic diagnostic character allowed us to discriminate the remaining species.

Compiling our results with previous studies, about 80 species inhabiting the Antarctic Peninsula were already barcoded, representing approximately 60 % of the species occurring in the area. Over 75 % of the barcoded species could be identified at species level with accuracy using BINs. Our results suggest that the use of nucleotic diagnostic character may help to improve the level of species discrimination.

In the present work, juveniles of some species of Nototheniidae were not able to be identified to the species level using external morphology since they did not share with the corresponding adults the same diagnostic features. These results evidenced an important component of ontogenetic variation in the character state of diagnostic features commonly used to construct taxonomic keys for this family. DNA Barcoding was, therefore, a cornerstone element for obtaining a reliable identification of these specimens. These results are very relevant for management and conservation purposes since an accurate species-level resolution of juveniles is necessary to determine nursery areas and to clarify species distributions.

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