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Fish diversity and molecular taxonomy in the Prydz Bay during the 29th CHINARE

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Abstract
In 2013, the 29th Chinese National Antarctic Research Expedition (CHINARE) prospected the Prydz Bay on the Antarctic continental shelf, and the Chinese R/V *Xuelong* icebreaker sampled all of the examined locations. The nature of Antarctic fish diversity in the high-latitude Prydz Bay is virtually unknown, and the accuracy of relevant estimates has not been established. Thus, it is necessary to evaluate this diversity and propose protective measures. In total, ninety-nine specimens were collected from various locations. To overcome uncertainties associated with identifying species based on morphology, DNA barcoding (COI gene) was employed to reconstruct phylogenetic relationships with delimited references from NCBI. Twenty-two species representing six families were unambiguously identified from a neighbor-joining (NJ) tree and barcoding gaps. With the morphological identification, thirteen species were identified correctly, five species were identified correctly at the genus level, and four species were identified at the close sister species level. Notothenioid dominance was not evident in the Prydz Bay, in contrast to other published studies. The low species diversity and catch biomass during this CHINARE were severely constrained by limited fishing methods and localized sites, which led to biased underestimation. Our analyses indicate that DNA barcoding is an effective tool for the identification of fish species in the Prydz Bay. The identification and distribution of Antarctic fish should be an integral component of understanding Antarctic fish biodiversity and biogeography, and large-scale studies are necessary for the further taxonomic identification of Antarctic fish.

Key words: DNA barcoding, Prydz Bay, Antarctic fish, phylogenetic relationship, barcoding gap

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1 Introduction

Species identification is the most significant task in many fields of biological research and for conservation efforts. Traditional morphological identification is not fully effective for eggs, larvae, juveniles and adults lacking distinctive morphological characteristics. Congeneric or confamilial species sometimes resemble each other to a high degree, which can lead to unreliable identification. Moreover, commonly used morphological features can change with developmental age and thus, may not provide definitive identification. Moreover, identification must be based on a sound knowledge of taxonomy, as a faulty delineation of species limits often precludes identification altogether (Dettai et al., 2011b).

Molecular species identification based on mitochondrial DNA (mtDNA) has been utilized for several decades but has recently acquired a new dimension through larger-scale projects using a standardized approach with high quality control. One proposed method is DNA barcoding, which uses the mtDNA gene cytochrome c oxidase subunit I (COI) for molecular taxonomy (Hebert et al., 2003a, b). Sequences from the same species are generally considered to be correctly identified when they form a monophyletic cluster on a neighbor-joining (NJ) tree with intraspecific distances that are below a given threshold (Srivathsan and Meier, 2012). At present, this approach has proven to be highly efficient and reliable in many fish groups (Ward et al., 2005; Keskin et al., 2013; Loh et al., 2014; Murphy et al., 2017) and is regularly used for a variety of applications, such as fishery management, biodiversity assessment and conservation (Dettai et al., 2011a, b; Keskin et al., 2013; Loh et al., 2014; Murphy et al., 2017; Shen et al., 2016). Both the evaluation of the approach and the development of ameliorations are still underway; however, these approaches seem promising for numerous taxa and developmental stages.

Antarctic waters are home to a largely benthic and highly endemic ichthyofauna, dominated by actinopterygian members that arose through nested adaptive radiations within the isolated Southern Ocean (Eastman, 2005; Lecointre et al., 2013; Fallon et al., 2016). Five families—Artedidraconidae, Bathydraconidae, Channichthyidae, Harpagiferidae, and Nototheniidae—are traditionally recognized, encompassing predominantly endemic Antarctic species (Gon, 1990; Eastman and Eakin, 2014). The majority of Antarctic notothenioid species have benthic lifestyles and limited home ranges as adults (Miyamoto and Tanimura, 1999). The Prydz Bay is the third largest bay in Antarctica. The world's largest glacier, the Lambert Glacier, enters this bay, forming the vast Amery Ice Shelf, which is a key area for scientific investiga-

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tion in the Southern Ocean. The Prydz Bay is an important source of bottom water in Antarctica and is a focus of research on physical oceanography, marine geology, marine geophysics, and marine ecology (Van de Putte et al., 2010; Gao et al., 2013; Yin et al., 2014). Unfortunately, no dedicated studies of fish diversity and molecular taxonomy in the Prydz Bay have been performed. In contrast, several studies of high-latitude fish barcoding and molecular taxonomy have been conducted further east in the Dumont d'Urville Sea and McMurdo Sound (Dettai et al., 2011a, b ; Murphy et al., 2017).

The ecological community is greatly impacted by global warming. With changes already visible in the Antarctic, a biodiversity baseline inventory is necessary to monitor these changes. A large amount of taxonomic work, especially for fish, is still needed in the Antarctic (Dettai et al., 2011b). We employed a DNA barcoding approach as a molecular tool for the identification of fish species collected from the Prydz Bay using available data from NCBI as a reference. We reconstructed phylogenetic relationships to assign species identity and evaluated the identification success rates based on the Kimura's two-parameter (K2P) model. Furthermore, the present study of fish diversity and molecular taxonomy can provide important information regarding biodiversity, biogeography and conservation in Antarctic coastal waters.

2 Materials and methods

2.1 *Specimen collection and morphological identification*

All specimens from 15 stations were collected from the Prydz Bay based on a triangular bottom trawl net (2.2 m wide, 0.65 m high, and 6.5 m long; 20 mm mesh size) during the 29th Chinese National Antarctic Research Expedition (CHINARE) in 2013 (Table 1), which was surveyed on the R/V *Xuelong* icebreaker. Every net was operated for 10–60 min, with variations due to differences among the seabeds at a speed of 3–4 kn. A few specimens were obtained from each station, and all specimens from the same station were packaged together and sorted again in the laboratory. Morphological identification was performed by visual inspection, and the fish were taxonomically classified by taxonomic specialists (Fischer and Hureau, 1985). Photographs of as many fresh specimens as possible were taken on board. Muscle samples were obtained and preserved in 95% ethanol or frozen for DNA extraction after specimen identification and morphological characterization. Subsequently, the whole fish were fixed in a 10% formaldehyde solution and stored as voucher samples in the Third Institute of Oceanography, State Oceanic Administration.

2.2 *DNA extraction, amplification and sequencing*

Genomic DNA was isolated from muscle tissue by proteinase

Table 1. Information on Antarctic fish collected from the Prydz Bay during the 29th CHINARE and homologous sequences downloaded from NCBI

ID	Scientific name from morphological identification	NCBI accession Nos	Sort by identification /%	Species name from NCBI	NCBI accession Nos
S1	*Bathyraja* sp.		99	*Bathyraja spinicauda*	FJ164384, JF895081
S2, S3	*Trematomus scotti*		100, 100	*Trematomus scotti*	HQ713283, JN641171
S4, S5	*Artedidraco lonnbergi*		99, 100	*Artedidraco lonnbergi*	HQ712811, HQ712827
S6	*Dolloidraco* sp.		100	*Histiodraco velifer*	HQ713027, JN640978
S7, S7-1	*Chionodraco hamatus*		100, 100	*Chionodraco hamatus*	HQ712912, JN640841, KT921282
S8, S8-1	*Pogonophryne* sp.		99, 100	*Pogonophryne scotti*	HQ713180, JN641119
S9, S10	*Prionodraco evansii*		100, 100	*Prionodraco evansii*	EU326416, HQ713203, JN641128
S11	*Chaenodraco wilsoni*		100	*Chaenodraco wilsoni*	HQ712902, JN640813
S12	*Trematomus* sp.		99	*Trematomus eulepidotus*	EU326425, HQ713263, JN641141
S13	*Dieidolycus* sp.		100	*Lycodichthys antarcticus*	HQ713053, HQ713056
S14, S14-1	*Cryodraco antarcticus*		100, 100	*Cryodraco antarcticus*	HQ712949, JN640867
S15	*Gerlachea australis*		100	*Gerlachea australis*	HQ713006, JN640928
S16	*Pachycara brachycephalum*		100	*Pachycara brachycephalum*	HQ713113, JN641050
S17	*Pachycara brachycephalum*	HQ713113, JN641050	99	*Ophthalmolycus amberensis*	HQ713104, HQ713105, HQ713106
S18	*Dolloidraco longedorsalis*		100	*Dolloidraco longedorsalis*	HQ712976, JN640907
S19	*Lycodichthys antarcticus*		99	*Lycodichthys antarcticus*	HQ713053, HQ713056
S20	*Bathydraco macrolepis*	EU326324, JN640779, JN640780	99	*Akarotaxis nudiceps*	HQ712805, HQ712806
S21	*Racovitzia glacialis*		100	*Racovitzia glacialis*	HQ713223, JN641132
S22, S23	*Trematomus pennelli*	EU326430, GU997445, HQ713272	99, 99	*Trematomus* cf. *lepidorhinus/loennbergi*	HQ713319, HQ713348, HQ713349
				Trematomus loennbergii	GU997426, JN641157
				Trematomus lepidorhinus	GU997424, JN641151
S24	*Artedidraco* sp. 1		100	*Artedidraco shackletoni*	HQ712858, HQ712859, HQ712860
S25, S26	*Artedidraco* sp. 2		100, 100	*Artedidraco shackletoni*	HQ712858, HQ712859, HQ712860
S27, S28	*Chionobathyscus dewitti*		99, 100	*Chionobathyscus dewitti*	HQ712909, JN640826
S29, S30	*Dacodraco hunteri*		100, 100	*Dacodraco hunteri*	HQ712963, JN640896

K digestion and purified by standard phenol-chloroform extraction (Sambrook et al., 1989). The extracted DNA was evaluated using 1.5% agarose gel electrophoresis and was then stored at –20°C before PCR amplification. A partial fragment of the COI gene was amplified using universal barcoding primers, F1: 5′-TCAACCAACCACAAAGACATTGGAC-3′ (forward) and R1: 5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′ (reverse) (Ward et al., 2005). The PCR reactions were carried out in a 25 μL reaction mixture containing 17.25 μL of ultrapure water, 2.5 μL of 10×PCR buffer (including $MgCl_2$) (Takara), 2 μL of dNTPs (Takara), 1 μL of each primer (5 μmol/L), 0.25 μL of Taq polymerase (Takara), and 1 μL of DNA template. The thermal cycling program consisted of an initial step for 4 min at 95°C; 35 cycles of 0.5 min at 94°C, 0.5 min at 52°C, and 0.5 min at 72°C; and a final step for 10 min at 72°C. Negative controls were included in all PCR reactions to confirm the absence of contaminants. Successful amplifications were purified, and both strands were sequenced by Personal Biotechnology Co., Ltd.

2.3 *Data analysis*

Sequences were edited and aligned using the DNASTAR Lasergene software package (Madison, WI, USA) and refined manually. All of the aligned sequences were translated into amino acids to confirm the accuracy of the sequences and to detect the presence of nuclear DNA pseudogenes. Other genetic indices, such as polymorphic sites, transitions, transversions, insertions/deletions, and nucleotide composition, were calculated with ARLEQUIN 3.0 (Excoffier et al., 2005). Pairwise genetic distance calculations and NJ tree analysis were implemented using MEGA 5.0 (Tamura et al., 2011) based on the K2P model with 1 000 bootstrap replicates. The DNA "barcoding gap", which is the maximum intraspecific distance of each species compared with its minimum distance to the nearest neighbor and is defined as the average interspecific distance at least 10-fold greater than the average intraspecific genetic distance, was calculated for all species (Hebert et al., 2004). All specimen sequences were aligned through a BLAST search in NCBI to evaluate the accuracy of morphological identification. Almost all currently barcoded Antarctic fish were collected during the CEAMARC survey in the winter of 2007–2008 (Dettai et al., 2011a, b). We used a general rule that defined a sequence similarity of at least 97% as a top-matched species and 3% sequence similarity as a relatively loose criterion for matched species (Wong and Hanner, 2008).

3 Results

Ninety-nine specimens were collected during the 29th CHINARE. Most of them were adults and could be easily sorted, but there were also some juvenile stages. Some specimens were damaged and could not be identified morphologically; these were termed "sp.", for example, *Dolloidraco* sp., *Pogonophryne* sp., *Bathyraja* sp., *Trematomus* sp., *Dieidolycus* sp. and *Artedidraco* sp. Thirty-three specimens were used for molecular analysis; the number of specimens per species used for the molecular analysis ranged from one to three, but most species were represented by only one specimen.

3.1 *Amplification and sequencing*

Low-quality sequences (double peaks, short fragments, and background noise), which may represent pseudogenes, were not detected. The aligned sequences contained no insertions, deletions or stop codons, indicating that all amplified sequences were functional mitochondrial COI sequences. A 652 bp fragment was successfully amplified and sequenced in this study, and the alignment revealed 256 polymorphic sites (including 239 parsimony-informative sites and 6 singleton sites), with 256 transitions and 119 transversions. The content of A, T, G, and C was 21.22%, 30.18%, 19.04% and 29.56% on average, respectively, with a slight base against G and C.

3.2 *Species identification by phylogenetic analysis of COI sequences*

Because there are limited Antarctic fish taxonomic references, mistakes were unavoidable during morphological identification. Our results indicated that six families were correctly identified: Channichthyidae (5 species, 15 specimens), Artedidraconidae (6 species, 26 specimens), Bathydraconidae (4 species, 17 specimens), Zoarcidae (3 species, 3 specimens), Nototheniidae (3 species, 37 specimens) and Rajidae (1 species, 1 specimen) (Table 1). The consensus strength of all sequences was determined by alignment through a BLAST search in NCBI. Most morphological identification results matched the BLAST annotations of the NCBI databases with at least 97% similarity, supporting that they were the same species (Murphy et al., 2017), except for five species (*H. velifer*, *L. antarcticus*, *O. amberensis*, *A. nudiceps*, and *Trematomus* cf. *lepidorhinus*/*loennbergi*) that matched with their sister species.

Based on our species sequences and vouchered data from NCBI as a reference, an NJ tree was constructed to assign species identity (Fig. 1), which depicted relationships between morphologically identified species and vouchered references. Twenty-two species from six families (Channichthyidae, Artedidraconidae, Bathydraconidae, Zoarcidae, Nototheniidae and Rajidae) were characterized by DNA barcoding. Based on the NJ tree, all species formed distinct clusters, and all individuals were associated with their conspecifics in the monophyletic clades with high bootstrap support values. However, all species from the same family clustered together, except for Bathydraconidae. In total, five Artedidraconidae species, five Channichthyidae species, four Nototheniidae species, three Zoarcidae species and one Rajidae species clustered together in the NJ tree. The NJ analysis also recovered the family Bathydraconidae as paraphyletic, consistent with recent molecular phylogenies (Dettai et al., 2012; Murphy et al., 2017), while the other families were monophyletic. *Gerlachea* was sister to other genera in Bathydraconidae.

The results of the NJ tree showed that not all of the morphologically identified species were in agreement with the vouchered references (Fig. 1); however, the majority was identified successfully, with at least 99% similarity (Table 1). S17 was distinctly different from S16, both of which were identified as *P. brachycephalum* morphologically but shared 99% nucleotide sequence similarity with the reference *O. amberensis*. S20 was identified as *B. macrolepis* morphologically but was 99% similar to the reference *A. nudiceps*. S22 and S23 were distinctly different from *T. pennelli* references but shared 99% identity with the reference *Trematomus* cf. *lepidorhinus*/*loennbergi*. Some damaged individuals and juvenile fish were also identified accurately based on barcoding but had been previously unidentified and considered as "sp." S1 (*Bathyraja* sp.) shared 99% nucleotide sequence similarity with the reference *B. spinicauda*, indicating that it was *B. spinicauda*. S6 (*Dolloidraco* sp.) was identified as *H. velifer* with 100% nucleotide sequence similarity; thus, this species was distinctly different from genus *Dolloidraco* species. S8 and S8-1 (*Pogonophryne* sp.) shared 99% and 100% nucleotide sequence identities with the reference *P. scotti*, respectively. S12 (*Trematomus* sp.) shared 99% nucleotide sequence identity with the reference *T. eulepidotus*. S13 (*Dieidolycus* sp.) was identified as *L. antarctic-*

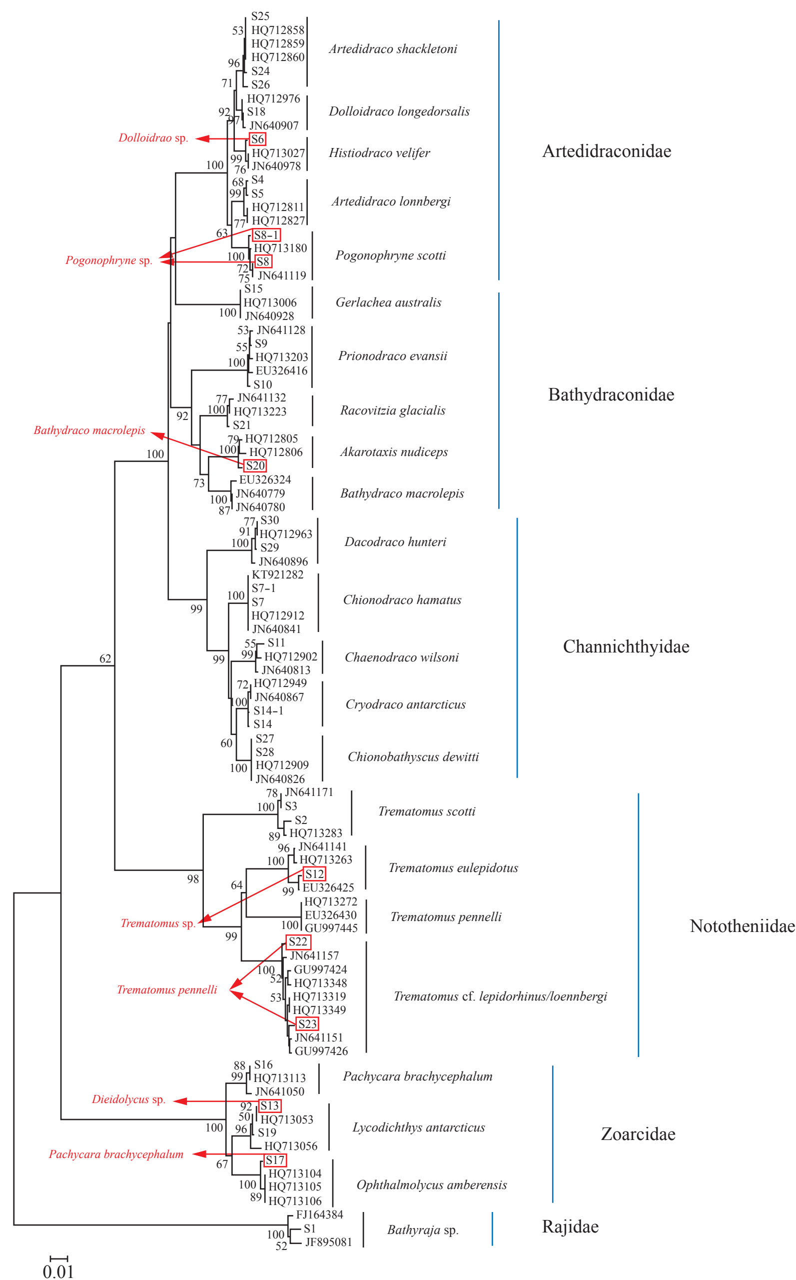

Fig. 1. NJ tree constructed with MEGA based on the K2P model. Bootstrap values higher than 50 are indicated along the branches. The species listed on the left in red text were identified based on morphology.

us with 100% nucleotide sequence similarity. Both *Artedidraco* sp. 1 (S24) and *Artedidraco* sp. 2 (S25, S26) were identified as *A. shackletoni* with 100% nucleotide sequence similarity, suggesting that they were the same species. The majority of the problematic species were, in fact, due to erroneous primary identification with poor references.

3.3 *Barcoding gaps*

Intraspecific variability was generally similar across species, and the range of interspecific differences was much more variable depending on the group (Dettai et al., 2011b). The use of the means for intraspecific and interspecific divergence comparison does not allow the detection of problematic cases (Meier et al., 2008); therefore, we instead compared minima for interspecific divergences to maxima for intraspecific divergences. If the entire dataset was considered, there would be a clear overlap between intraspecific and interspecific variabilities, as the smallest interspecific divergences were well below 2%, but the largest intraspecific divergences exceeded 2% (Hebert et al., 2003a; Dettai et al., 2011b; Shen et al., 2016).

The intraspecific K2P distances exhibited considerable heterogeneity and ranged from 0% to 1.24%, with a mean value of 0.29%. The minimum interspecific distances of all species were greater than 2%. The species discrimination power of DNA barcoding was demonstrated by the barcoding gaps that were drawn for all species on the basis of the K2P distances shown in Fig. 2. Because the latter value was always higher than the former, overlaps were not detected in all species.

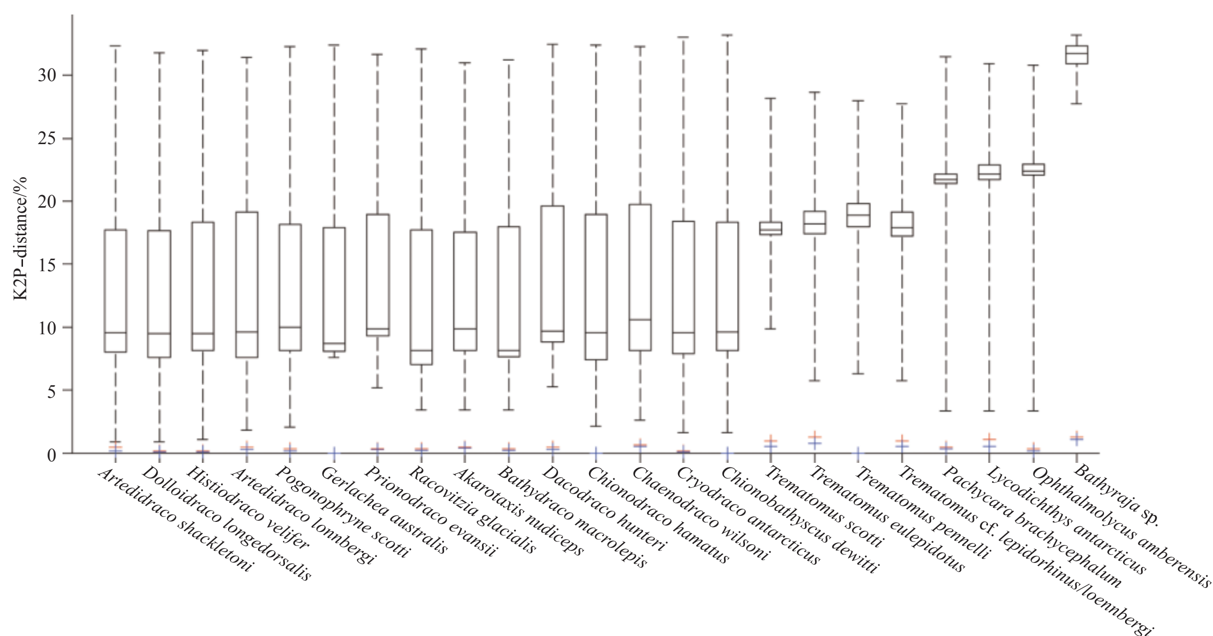

Fig. 2. DNA barcoding gaps for all of the species based on the K2P model. Median interspecific distances with maximum and minimum values are represented by the upper and lower bars, respectively. Red cross: maximum intraspecific distance; blue cross: mean intraspecific distance.

4 Discussion

Our study represents the first comprehensive molecular assessment of fish in the Prydz Bay. In the current study, DNA barcoding was effective for identifying species and provided a straightforward identification system when a perfect match existed between the morphology-based taxonomy and genetic divergence (Chen et al., 2015). A few specimens that could not be identified at the species level could therefore be placed with confidence within species clusters by the molecular analysis. The results showed nearly unanimous (99%–100%) species group support for the unknown species with their vouchered references in the NJ tree (Fig. 1), providing strong confidence for the accuracy of the species assignment.

Of the twenty-two species investigated in this analysis, only thirteen species were identified correctly, five species were identified correctly at the genus level, and unfortunately, four species were only identified to their close sister species based on morphological characteristics. However, all problematic species were entirely resolved with COI sequences. Identifications were successfully made due to reference sequences of vouchered species and the selection of COI as the gold standard barcode gene. Furthermore, a 10-fold sequence divergence between the average interspecific and the average intraspecific difference was detected; this divergence was suggested to be the standard COI threshold for species identification (Hebert et al., 2003a, b). Unfortunately, there was slight uncertainty regarding *T. lepidorhinus* and *T. loennbergi*, which did not form distinct clusters according to the morphologically identified species in the NJ tree. *Sillago analis*, *S. ciliate* (Krück et al., 2013), *Thryssa mystax* and *T. vitrirostris* (Ma et al., 2015) had different morphological characteristics but also clustered together in the phylogenetic tree. However, Krück et al. (2013) previously presented a multigene barcoding approach to successfully discriminate the two *Sillago* species.

Recent molecular phylogenies indicated that the family Nototheniidae was paraphyletic (Dettai et al., 2012; Murphy et al., 2017), but it was monophyletic in this study. Because all individuals of Nototheniidae were represented by only one genus, the congeners were closely related and easily formed a monophyletic clade. In Antarctic coastal waters, notothenioids constitute over 70% of the species diversity and 91% of catch biomass (Eastman and Hubold, 1999; Murphy et al., 2017), and five families (Artedidraconidae, Bathydraconidae, Channichthyidae, Harpagiferidae, and Nototheniidae) are traditionally recognized (Gon, 1990). Moreover, notothenioid *Trematomus* fishes (family Nototheniidae) were overwhelmingly dominant, as reported by previous studies (Eastman and DeVries, 1982; Murphy et al., 2017), accounting for 100% of all collected specimens. In contrast, the dominance of Nototheniidae species was not evident in the Prydz Bay and was less than that reported in previous studies; species from the other five families were also caught at higher frequencies than notothenioids. The low species diversity and catch biomass in this survey were caused by the severe constraints of the limited fishing methods and localized sites, which led to a biased underestimation.

This study demonstrated that DNA barcoding is a useful and effective tool and can provide further insight into the identification of species in addition to morphological characterization. The identification and distribution of Antarctic species should be an integral component in understanding Antarctic fish biodiversity and biogeography. The overall Prydz Bay diversity (21 species) represents only a small subset of Antarctic fauna. Therefore, further morphological characterization and multigene barcoding of specimens from around the Antarctic Continent is necessary.

5 Conclusions

This study elucidates fish species and their phylogenetic relationships in the Prydz Bay on the Antarctic continental shelf based on the 29th CHINARE. All of the study results show that DNA barcoding is an effective tool for accurate species identification and could play a supporting role in species diversity surveys.

Twenty-two species from six families were characterized; only thirteen species were identified correctly, five species were identified correctly at the genus level, and four species were merely identified at the close sister species level. This work is important; in particular, the partial list of fish species in the prospected waters provided in this paper represents a resource relevant to the structure of the Antarctic community. More specimens should be collected from the Prydz Bay in the future. In subsequent studies, morphological identification should be combined with DNA barcoding to identify species because morphological identification alone may not be sufficiently robust.

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