Geographical differences in the diet of *Dissostichus mawsoni* revealed by metabarcoding

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The diet of the Antarctic toothfish (*Dissostichus mawsoni*) was analyzed using metabarcoding to determine whether spatiotemporal factors are related to its feeding ecology. A diet metabarcoding analysis was conducted for five years from 2016 to 2020 using 1,777 samples collected from two distantly located subareas, 88.3 and 58.4.1. Metabarcoding results revealed 105 prey haplotypes (29 families, 45 genera) in the stomach contents of *D. mawsoni*, which can serve as valuable genetic information for the accurate identification of piscine species inhabiting the Southern Ocean. Most of the stomach contents of *D. mawsoni* consisted of fish taxa, comprising 99.61% of read count, which is consistent with other studies indicating that *D. mawsoni* is piscivorous. The prey compositions were highly different between the two subareas (88.3 and 58.4.1) regardless of the year, indicating that the diet of *D. mawsoni* strongly reflects the fish assemblages in geographically different habitats. These results strongly suggest that the stomach contents are good ecological indicators for monitoring any changes in the marine ecosystem caused by either the fishery of *D. mawsoni*, the most voracious piscine predator in its habitat, or environmental changes. In addition, quantitative polymerase chain reaction results of the two most abundant *Macrourus* prey species of Antarctic toothfish, *M. caml* and *M. whitsoni*, showed that the distribution of these two species may be related to the dynamics of gyres, which flow along the Antarctic continent.

KEYWORDS

feeding ecology, Antarctic toothfish, metabarcoding, CCAMLR, Antarctica

Introduction

The Antarctic toothfish *Dissostichus mawsoni* Norman, 1937, is a species of the *Nototheniidae* family that is native to the Southern Ocean. Owing to its ecological and economic importance, its fishery has been strictly managed by the Commission for the Conservation of Antarctic Marine Living Resources since 1997. One of the main concerns regarding *D. mawsoni* fishery is the lack of accurate information about the impact of fishing on the ecosystem. Since its diet reflects the status of the food web, many studies on the feeding ecology of *D. mawsoni* have been conducted in various sites of Antarctica, including Bouvet Island (Petrov, 2011), Cosmonaut Sea (Pakhomov and Tseytlin, 1993), Elephant Island (Grohsler, 1992), Lazarev Sea (Petrov and Tatarnikov, 2011), McMurdo Sound (Calhaem and Christoffel, 1969; Eastman, 1985), Pacific sector (Yukhov, 1971), Ross Sea (Yukhov, 1982; Fenaughty and Stevens, 2003; Kokorin, 2010; Stevens et al., 2014), South Sandwich Islands (Roberts et al., 2011), South Shetland Islands (Takahashi and Iwami, 1997), Area 58.4 (Park et al., 2015; Seong et al., 2021), and Weddell Sea (Petrov and Gordeev, 2015). Previous studies have shown that *D. mawsoni* mainly preys on benthic fish and cephalopods. However, prey species or their compositions vary in each study, and accurate species names often remain unknown for partially digested preys (Fenaughty and Stevens, 2003; Petrov and Tatarnikov, 2011; Roberts et al., 2011; Stevens et al., 2014; Seong et al., 2021). Identifying species inhabiting the Antarctic waters has been challenging, mainly because of the morphological similarity or phenotypic plasticity of various species (Piacentino and Barrera-Oro, 2009; Lautredou et al., 2010; Convey and Peck, 2019). In addition, it is challenging to identify partially digested preys in the stomach (Hashimoto, 1974). Besides the difficulty in species identification, the digestion time and rates for each prey are also different, which may produce biased results that are inappropriate for quantitative and morphological analyses (Amundsen and Sánchez-Hernández, 2019). Most importantly, analysis takes a long time and requires a high budget and labor to obtain statistically reliable data. Therefore, most studies on the diet of *D. mawsoni* have focused a single research term and area.

Diet studies using molecular techniques can be good alternatives to conventional methods. Owing to its cost efficiency and high sensitivity, metabarcoding has been used for diet studies in various animals (Guillerault et al., 2017; Mata et al., 2019; Mychek-Londer et al., 2020). A previous study reported the diet of *D. mawsoni* by metabarcoding its stomach contents, in which proportions of its prey were identified with high accuracy (Yoon et al., 2017). However, a single survey could not explain the reason for the variability in the prey species or their compositions in each survey of different geographical regions. A long-term study of the feeding ecology of *D. mawsoni* using metabarcoding would provide a more comprehensive understanding of its life cycle, migration, food web structure, and the impacts of its fishery on the ecosystem of the Southern Ocean. In fact, the Antarctic toothfish fishery has been prevalent since 1997, and its sustainability remains controversial. Here, we report the results of metabarcoding for the stomach contents of Antarctic toothfish, which was conducted for several years (2016–2020). Based on metabarcoding, *D. mawsoni* was identified to prey in subareas 88.3 and 58.4, which were compared to determine whether the stomach contents reflected the geographical food web structures. Finally, we used quantitative polymerase chain reaction (qPCR) to accurately quantify individual prey items. These data will contribute to the scientific management and conservation of *D. mawsoni*, providing a fast and accurate monitoring platform to detect the impact of its fishery on the marine ecosystem in the Southern Ocean.
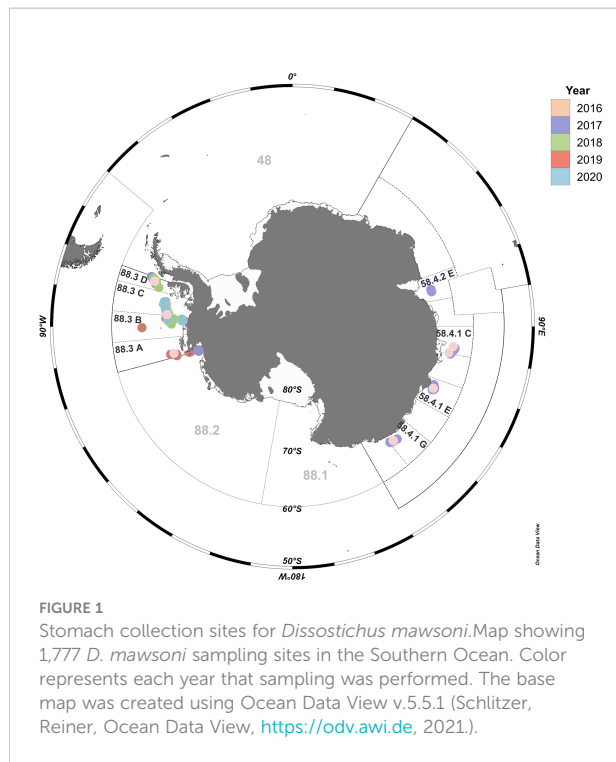
Materials and methods

Sample collection

Antarctic toothfish were collected from subareas 88.3 and 58.4 during the fishing season (from December to April each year) by Korean flagged commercial bottom longline vessels (88.3-Greenstar, 58.4-Kingstar) under the supervision of the Distant Water Fisheries Resources Division, National Institute of Fisheries Science (NIFS) in the Republic of Korea. Antarctic toothfish samples were collected for five years from 2016 to 2020 in subarea 88.3, and for two years (2016 and 2017) in subarea 58.4 (Figure 1). A trotline was used as fishing gear to collect the Antarctic toothfish, and Humboldt squid (*Dosidicus Gigas* d'Orbigny [−1834-1847], 1835) and Pacific herring (*Clupea pallasii* Valenciennes, 1847) were used as baits. The fishing depth varied from 591–2,113 m. Before stomach dissection, the total length (cm) and body weight (g) of each specimen was measured at the collection site. The dissected stomachs were individually packaged and immediately frozen at −20°C. On arrival at the port, stomach samples were transferred (frozen in dry ice) to the laboratory and stored at −70°C in a deep freezer until DNA extraction for metabarcoding analysis.

DNA extraction

Before homogenization, abiotic elements, such as hooks and stones, and baits were removed from the individual stomachs. Individual stomach contents were weighed and homogenized using an electric kitchen blender after adding an equal volume of distilled deionized water to the corresponding wet weight of the stomach contents. A blender was used for each individual, which was treated with 10% bleach for 10 min before use. Individual stomach contents from each subarea in each year were pooled into a single sample for DNA extraction in the following

FIGURE 1
Stomach collection sites for *Dissostichus mawsoni.* Map showing 1,777 *D. mawsoni* sampling sites in the Southern Ocean. Color represents each year that sampling was performed. The base map was created using Ocean Data View v.5.5.1 (Schlitzer, Reiner, Ocean Data View, https://odv.awi.de, 2021.).

manner. From each blender-homogenized stomach content, a volume of homogenate at a proportion of 1 uL to 1 g stomach content wet weight was pooled. Table 1 shows the total wet weights of all stomach contents per subarea per year and the corresponding pooled homogenate volumes. Genomic DNA was extracted using the AccuPrep Genomic DNA Extraction Kit (Bioneer, Republic of Korea) with slight modifications to the manufacturer's protocol. Six volumes of tissue lysis buffer were added to the pooled samples and homogenized using FastPrep-24 (MP Biomedicals, USA). The extracted genomic DNA was quantified using NanoDrop One (Thermo Scientific, USA) and stored in a freezer at −20 °C until further use. Individual DNA for quantitative analysis of major prey organisms was randomly selected and extracted from 50 individuals according to small-

scale research units (SSRUs). Then, 100 uL of the homogenized sample was used for DNA extraction using the AccuPrep Genomic DNA Extraction Kit (Bioneer, Republic of Korea), according to the manufacturer's protocol. For quantitative analysis, the extracted DNA was stored in a freezer at −20°C.

Library preparation

An amplicon library was constructed to analyze the Antarctic toothfish diet by metabarcoding using the universal metazoan cytochrome c oxidase subunit I (COI) primer (barcode size 463bp) (Yoon et al., 2017). The library preparation protocol used was slightly modified from Yoon et al. (2017). First PCR was performed to amplify the target gene. The PCR mixture contained 10 ng of genomic DNA, 100 μM of each primer (COIMISQF1: 5′-ATNGGNGGNT TYGGNAA-3′; COIMISQR1: 5′-TANACYTCNGGRTGNCC-3′), 2 μL of dNTPs (2.5 mM each), 0.2 μL of Ex Taq Hot Start Version (2 U) (TaKaRa Bio Inc., Japan), 2 μL of 10× buffer, and 3% dimethyl sulfoxide, and DNase/RNase-free distilled water was added to a final volume of 20 μL. Each sample was amplified in triplicate. The first PCR began with an initial denaturation at 94°C for 5 min, followed by 12 cycles (18 cycles for the second PCR) at 94°C for 30 s, 48°C for 30 s, and 72°C for 30 s, ending with a final extension at 72°C for 5 min. The first PCR products were purified using the AccuPrep PCR Purification Kit (Bioneer, Republic of Korea) and eluted with 20 μL of TE (Tris-EDTA) buffer at pH 8.0. The second PCR (approximately 570 bp) was performed using an adapter-tagging primer and the Illumina Nextera XT Index Kit (Illumina, Inc., USA). The second PCR (final volume, 20 μL) contained 6 μL of the first PCR amplicons, 100 μM of each primer for adapter-tagging (NXCOIMISQF2: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGNG GNTTYGGNAAYTG-3′; NXCOIMISQR2: 5′-GTCTCGTGG GCTCGGAGATGTGTATAAGA GACAGGGRTGNCCRAA RAAYCA-3′), 2 μL of dNTPs (each 2.5 mM), 0.2 μL of Ex Taq Hot Start Version (2 U) (TaKaRa Bio Inc., Japan), and 2 μL

TABLE 1 Stomach samples of *Dissostichus mawsoni* used in this study.

Subarea	Year	Numbers of Specimen	Averaged depth of catch (m)	Averaged body length (cm)	Averaged body Weight (kg)	Averaged wet weight of stomach contents (g)	Total wet weight of stomach contents (g)	Pooled sample (ml)
58.4	2016	158	1,321.24 ± 123.80	143.88 ± 15.89	40.43 ± 13.47	317.03 ± 392.79	50,090	50.09
	2017	620	1,393.65 ± 161.73	143.28 ± 16.44	38.69 ± 13.66	306.68 ± 363.04	190,143	190.14
88.3	2016	159	1,448.34 ± 151.18	131.67 ± 23.87	32.8 ± 16.63	347.83 ± 370.82	55,306	55.31
	2017	110	1,420.51 ± 146.85	136.67 ± 21.63	33.73 ± 13.89	259.78 ± 330.91	28,576	58.58
	2018	180	1,372.46 ± 291.73	133.24 ± 29.34	34.90 ± 18.25	364.93 ± 422.48	65,687	65.69
	2019	341	1,348.28 ± 393.61	138.93 ± 29.89	37.64 ± 17.38	286.17 ± 352.25	97,582	97.59
	2020	209	1,472.69 ± 280.87	132.38 ± 23.26	36.77 ± 17.31	413.06 ± 453.78	86,329	86.33

of 10X buffer. The expected amplicons from the second PCR were used as templates for indexing PCR (approximately 630 bp) using the Nextera XT index kit (Illumina, USA). The constructed library was quantified using a Quantus Fluorometer (Promega, USA) and sequenced using the Illumina MiSeq system (2×300 bp). For library construction, PCR without template DNA was used as a negative control.

Bioinformatics and statistical analyses

The raw reads were imported into the CLC Genomics Workbench v.8.0 (Qiagen Inc.) for adapter and index trimming using the default algorithm. Quality values < 20 sequences were removed, and under-read lengths of 100 nucleotides were filtered. The primers were removed from the reads using Cutadapt v.2.10 (Martin, 2011). The trimmed reads were inferred as correct amplicon sequence variants (ASV) using the DADA2 v.1.20.0 package in R (Callahan et al., 2016), following the DADA2 Pipeline v.1.16. The reads from which the primer was removed were filtered using 'filterAndTrim' according to the command with maxN=0, maxEE (maximum expected errors)=2, truncQ (truncate quality score)=2. After merging paired ends, a sequence other than the target length, 463 bp, were removed from the sequence table through the following command 'seqtab[,nchar(colnames(seqtab)) %in% 462:464]'. Chimeric sequences were removed with 'removeBimeraDenovo', using the default method (consensus). The generated ASVs were used for post-clustering curation using the LULU algorithm v.0.1.0 (Frøslev et al., 2017) to detect erroneous clusters, considering sequence similarity and co-occurrence patterns. The 'minimum_match' was modified to 90, and other values were performed as default following the protocol (https://github.com/tobiasgf/lulu). Taxonomic assignments were obtained from the GenBank nucleotide database using BLAST+ v.2.12.0 (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Of these, Representative sequences were assigned to species (at least 99% identity with the first hit) and genus (90%), and sequences with less than 90% identity were classified as "Unknown." The haplotypes obtained by metabarcoding were built with a maximum likelihood tree of MEGAX using a bootstrap of 1000 and Kimura 2-parameter model (Kumar et al., 2018). Analysis of similarities (ANOSIM) and similarity percentages (SIMPER) were used to show differences between the community structures of prey organisms. A sample similarity matrix was constructed using the Bray–Curtis algorithm in PRIMER v.7, using a data matrix containing square root transformed count data of relative read abundance (Clarke et al., 2014). Heat maps and non-metric

multidimensional scaling (nMDS) plots were constructed using PRIMER v.7.

Identification and quantification of two *Macrourus* species

A long COI region of genus *Macrourus* was amplified using Fish-BCL (5′-TCAACYAATCAYAAAGATATYGGCAC-3′) and Fish-BCH (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) primers (Baldwin et al., 2009). Amplicons were purified using an AccuPrep Gel Purification Kit (Bioneer, Republic of Korea), according to the manufacturer's protocol. Amplicons were sequenced using the Sanger method (Macrogen, Republic of Korea). To quantify the copy numbers of *M. caml* and *M. whitsoni* in the stomach contents of *D. mawsoni*, species-specific primers and probes were designed and commercially synthesized (Macrogen, Republic of Korea) (Supplementary Data 3). The mixture for quantitative PCR (qPCR) contained forward and reverse primers (10 pmol/mL each), Taqman probe (5 pmol/mL each), 10 µL Luna Universal Probe qPCR Master Mix (New England Biolabs Inc., UK), and 1 µL of individual stomach content DNA as a template. PCR was conducted using a Mic qPCR Cycler (Bio Molecular Systems, Australia) under the following conditions: denaturation at 95°C for 1 min, followed by 30 cycles of 95°C for 30 s, 67°C for 15 s, and 72°C for 15 s. The standard curves for both *M.caml* and *M.whitsoni* were based on the constructed pGEM-T Easy Vector plasmids (Promega, USA) containing the long COI region of each fish. The concentration of the template plasmid was 10-fold serially diluted from 10 ng to 0.01 pg (Supplementary Data 4).

Results

Comparisons of the physical parameters of fish specimens

First, 1,777 stomach contents of *D. mawsoni* were collected for five-years from 2016 to 2020. Among them, 999 and 778 specimens were collected from subareas 88.3 and 58.4, respectively (Table 1 and Supplementary Data 1). The average body length of the collected fish was 135.13 ± 26.87 cm in subarea 88.3, approximately 8.14 cm shorter than the body length of those in subarea 58.4 (143. 40 ± 16.31 cm). As for body length, a 3.29 kg higher average body weight was identified in the fish collected from subarea 58.4 (39.04 ± 13.63 kg) compared with those from subarea 88.3 (35.75 ± 17.11 kg). Furthermore, the average water depths of

the sample were similar, which are 1,402.54 ± 305.01 m and 1,378.94 ± 157.43 m in subareas 88.3 and 58.4, respectively.

Metabarcoding of the stomach contents of *Dissostichus mawsoni*

Metabarcoding of the stomach contents was conducted using seven samples pooled by year and region (Table 2). After bioinformatics analysis of the raw reads generated by MiSeq sequencing, 3,306,207 non-chimeric merged reads were obtained and used for further analysis. The average proportion of those merged reads in each pooled sample was 67.24%, ranging from 46.64–81.39%, indicating a high degree of sequencing quality. As a result of ASV analysis, 105 haplotypes covered 45 genera and 29 families (Figure 2). Among them, 88 haplotypes showed sequence identity higher than 99% with the GenBank database (https://www.ncbi.nlm.nih.gov/genbank), equivalent to 83.81% of the total haplotypes, indicating a sufficient amount of reference sequence data in the region. Eighty-eight haplotypes with high sequence identity to the database were assigned to 50 species (Table 3; Figure 2). Only genus names were assigned for nine haplotypes with identity lower than 99%, and the other eight haplotypes with less than 90% sequence identity were classified as "Unknown" (Table 3). Among eight "unknown" haplotypes, six showed 73%–77% identity with arthropods (*Neoalbionella* sp., MH748704), whereas the other two "unknown" haplotypes showed 82% and 78% identity with Annelida (Polychaeta gen. sp., KJ736517) and Mollusca (*Argopecten ventricosus* (G. B. Sowerby II, 1842), KT161261), respectively. Furthermore, 70/76 haplotypes (92.11%) belonging to fish taxa showed a high degree of sequence identity with the database (99%–100%). Among six haplotypes with low sequence identity to the database, one showed 98.69% identity with *Anotopterus vorax* Regan, 1913 (Southern Ocean daggertooth, EU148072). Because only one species in the genus is known, the haplotypes were assigned to *A. vorax* (Table 3). Two haplotypes showed 98.92% and 96.54% identity to *Magnisudis prionosa* Rofen, 1963

(Southern Barracudina, GU805997), and their occurrence was identified in both subareas. Since it was the only species in the genus that was found in the Southern Ocean, these two haplotypes were assigned to *M. prionosa* (Duhamel et al., 2014; Priede, 2017). Further studies should be conducted to accurately identify this genus. One haplotype in the genus *Lycodes*, which showed 98.27% identity with *Lycodes raridens* Taranetz & Andriashev, 1937 Marbled eelpout, NC_053740), was assigned as *Lycodes* sp. Another haplotype in the genus *Cryodraco*, which showed 97.64% identity with *Cryodraco antarcticus* Dollo, 1900 (long-fingered icefish NC_045285), was assigned as *Cryodraco* sp. (Table 3). However, this occurred only once, with a low proportion (<0.01%) in subarea 58.4. One haplotype in the Elasmobranchii showed 100% sequence identity to *Bathyraja* n. sp. "dwarf" (P.40900NIWA in BOLD systems, EU119851). Collectively, only 3.95% of fish haplotypes were not assigned to the species level, supporting the accurate diet analysis of *D. mawsoni*.

Most prey haplotypes belonged to fish taxa (76), followed by Molluscs (16), "unknown" (8), Arthropod (1), Echinoderm (1), Mammal (2), and Avian (1). Seventy-six piscine haplotypes consisted of two classes: Actinopteri (73) and Elasmobranchii (3). Additionally, 73 haplotypes in the class Actinopteri were further classified into eight orders: Perciformes (36 haplotypes with 20 species), Aulopiformes (16 haplotypes with three species), and Gadiformes (13 haplotypes with five species) (Figure 2). All 16 molluscan haplotypes belonged to cephalopods, and nine and seven belonged to Teutida and Octopoda, respectively (Table 3). One arthropod and one echinoderm haplotype were *Nematocarcinus lanceopes* (Spence Bate, 1888) (EF407630) and *Staurocucumis liouvillei* Vaney, 1914 (KP165481), respectively. The avian haplotype was identified as a *Ardenna tenuirostris* (del Hoyo and Collar 2014) (short-tailed shearwater, MK262020, 100%), whereas two mammalian haplotypes were identified as the *Balaenoptera bonaerensis* (Burmeister, 1867) (Antarctic minke whale, AP006466, 91.36%) and the *Lobodon carcinophaga* (Hombron & Jacquinot, 1842), (Crabeater seal, AM181024, 99.35%), respectively.

TABLE 2   Summarized metabarcoding results of the stomach contents of *Dissostichus mawsoni*.

Subrea	Year	Raw reads	Filtered reads	Merged reads	Non-chimeric reads [Yield (%)]
58.4	2016	899,555	899,203	816,316	553,633 (61.55)
	2017	1,585,344	1,585,099	1,356,633	1,034,271 (65.24)
88.3	2016	974,740	974,437	919,563	793,321 (81.39)
	2017	784,924	784,843	611,115	520,486 (66.31)
	2018	145,308	145,255	120,179	77,743 (53.50)
	2019	266,399	266,352	218,922	124,258 (46.64)
	2020	260,967	260,964	238,267	202,495 (77.59)
Total		4,917,237	4,916,153	4,280,995	3,306,207 (67.24)
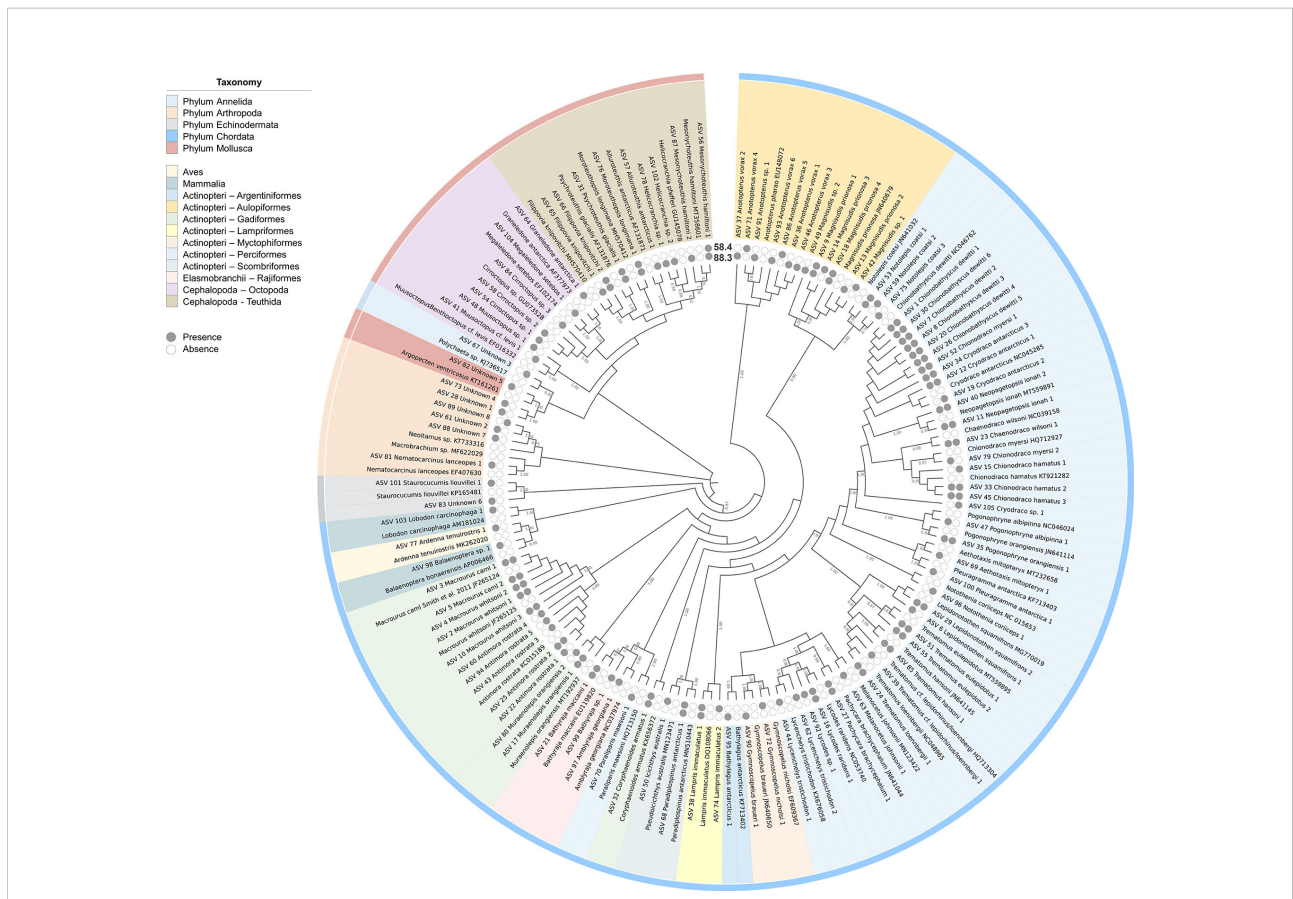
FIGURE 2
Prey haplotypes of *Dissostichus mawsoni* determined *via* metabarcoding analysis. A maximum-likelihood phylogenetic tree was generated using the prey haplotype of *D. mawsoni* and reference sequences in GenBank. A tree was created using MEGAX with a Kimura 2-parameter model and 1000 bootstrap replication. The tree was annotated in iTOL for taxonomy, haplotype presence, and bootstrap values (Letunic and Bork, 2021). Supplementary Data 2 includes information on the reference sequences used for phylogenetic analysis.

Averaged proportions of fish preys in all pooled samples were higher than 99%, ranging from 99.05–99.99%. The second most abundant prey belonged to cephalopods, with proportions ranging from 0.03–0.88% (Table 3). *Psychroteuthis glacialis* (Thiele, 1920) (glacial squid) was the most widely identified prey among the 16 haplotypes in the cephalopods. Among fish prey, those in the Elasmobranchii occupied less than 1%, whereas most fish belonged to Actionpteri (Table 3). In subarea 88.3, *Chionobathyscus dewitti* Andriashev & Neyelov, 1978 (Antarctic icefish) was the most abundant prey species, occupying 58.98% on average, followed by *Macrourus caml* (McMillan, Iwamoto, Stewart & Smith, 2012) (Caml grenadier, 18.21%) and *M. prionosa* (10.24%). In subarea 58.4, *Macrourus whitsoni* (Regan, 1913) (Whitson's grenadier) was the most abundant prey, occupying 51.27% on average (Table 3). *C. dewitti* (17.27%) and *M. caml* (10.74%) were the second- and third most abundant prey species. Collectively, fish belonging to Macrouridae and Channichthydae were among the main prey items of *D. mawsoni* (Table 3).

Molecular identification and regional differences of the two *Macrourus* species

Among the most abundant prey organisms of *D. mawsoni*, two haplotypes in the genus *Macrourus* were identified as *M. caml* and *M. whitsoni*, which were determined by both bioinformatics sequence similarity and phylogenetic analyses (Table 3; Figure 3A). However, these two species shared 99.35% sequence identity with only three base differences in barcode region, and some of sequences deposited in the GenBank did not correspond to the phylogenetic relationship, which strongly requires reconfirmation of their identification. Therefore, we re-examined the *Macrourus* haplotypes in all individual stomach contents of *D. mawsoni* using the longer COI barcodes (Fish-BCL and Fish-BCH, 652 bp) (Figure 3B). After comparing phylogenetic relationships of all the available COI barcodes obtained from the individual stomach samples and GenBank database, we reconfirmed two distinct species, *M. caml* (JF265124, 100%) and *M. whitsoni* (JF265125, 100%), respectively, which reconfirmed the previous results (Mcmillan

TABLE 3 Prey organisms of *Dissostichus mawsoni* in subareas 88.3 and 58.4.

Taxon	Haplotype	Relative read abundance (%)								
		Subarea 88.3						Subarea 58.4		
		2016	**2017**	**2018**	**2019**	**2020**	**Avg.**	**2016**	**2017**	**Avg.**
Arthropoda	**1**									
Malacostraca	1									
Decapoda										
Nematocarcinidae										
Nematocarcinus lanceopes	1								0.01	*
Chordata	**79**	**99.60**	**99.99**	**99.97**	**99.96**	**99.47**	**99.80**	**99.07**	**99.05**	**99.06**
Actinopteri	**73**									
Argentiniformes	1									
Bathylagidae										
Bathylagus antarcticus	1								*	*
Aulopiformes	16									
Alepisauridae										
Anotopterus vorax	7	0.73	0.31	0.07		0.02	**0.22**	0.01	0.01	**0.01**
Paralepididae										
Magnisudis prionosa	6	8.62	0.21	16.63	0.34	25.61	**10.28**	0.45	3.11	**1.78**
Notolepis coatsi	3					0.07	**0.01**		0.13	**0.06**
Gadiformes	13									
Macrouridae										
Coryphaenoides armatus	1				0.03		**0.01**	0.45	0.09	**0.27**
Macrourus caml	2	23.55	27.29	13.90	6.58	19.71	**18.21**	8.65	12.84	**10.74**
Macrourus whitsoni	3	2.81	12.83	0.14	0.52	0.44	**3.35**	43.43	59.12	**51.27**
Moridae										
Antimora rostrata	5	0.53	0.08			0.02	**0.13**		1.68	**0.84**
Muraenolepididae										
Muraenolepis orangiensis	2	1.17	0.20	0.03	0.03	0.04	**0.30**	0.31	0.98	**0.65**
Lampriformes	2									
Lampridae										
Lampris immaculatus	2	0.29				0.06	**0.07**		0.07	**0.03**
Lophiiformes	1									
Melanocetidae										
Melanocetus johnsonii	1			0.27			**0.05**			
Myctophiformes	2									
Myctophidae										
Gymnoscopelus braueri	1								0.01	*
Gymnoscopelus nicholsi	1					0.07	**0.01**			
Perciformes	36									
Artedidraconidae										
Pogonophryne albipinna	1					0.59	**0.12**			
Pogonophryne orangiensis	1	0.06			0.03		**0.02**	0.23	0.14	**0.19**
Channichthyidae										
Chaenodraco wilsoni	1				0.12		**0.02**	0.95		**0.47**
Chionobathyscus dewitti	6	60.41	39.53	64.63	81.30	49.05	**58.98**	21.28	13.25	**17.27**
Chionodraco hamatus	3	0.01		0.07	0.10		**0.04**	4.69	0.30	**2.49**
Chionodraco myersi	2		0.11		0.39		**0.10**	*		*
Cryodraco antarcticus	3			0.16	0.17		**0.07**	2.40	4.68	**3.54**
Cryodraco sp.	1							*		*
Neopagetopsis ionah	2	0.35		0.07	0.07		**0.10**	10.54	0.55	**5.54**
Liparidae										
Paraliparis mawsoni	1								0.01	**0.01**
Nototheniidae										
Aethotaxis mitopteryx	1				0.02	0.06	**0.02**			
Lepidonotothen squamifrons	2	0.52	19.41	2.13	9.46	0.75	**6.45**	0.34	0.02	**0.18**
Notothenia coriiceps	1								*	*
Pleuragramma antarctica	1	*					*			
Trematomus cf. lepidorhinus/loennbergi	1			0.12	0.64	0.96	**0.34**			
Trematomus eulepidotus	2	0.01		0.06	0.04		**0.02**	0.03	0.12	**0.08**
Trematomus hansoni	1				0.02		*	0.01		**0.01**

(Continued)

TABLE 3   Continued

| Taxon | Haplotype | Relative read abundance (%) | | | | | | | | |
| | | Subarea 88.3 | | | | | | Subarea 58.4 | | |
		2016	2017	2018	2019	2020	Avg.	2016	2017	Avg.
Trematomus loennbergii	1	0.03					**0.01**	0.61	0.59	**0.60**
Zoarcidae										
Lycenchelys tristichodon	2	0.10		0.34	0.04	0.50	**0.20**			
Lycodes raridens	1					1.28	**0.26**	3.14	0.37	**1.76**
Lycodes sp.	1		0.01				*			
Pachycara brachycephalum	1	0.29				0.23	**0.10**	0.78		**0.39**
Scombriformes	2									
Centrolophidae										
Icichthys australis	1			1.35			**0.27**			
Gempylidae										
Paradiplospinus antarcticus	1		0.01		0.01		*		0.01	*
Elasmobranchii	3	0.09				0.04	**0.03**	1.72		**0.86**
Rajiiformes	3									
Arhynchobatidae										
Bathyraja maccaini	1	0.09					**0.02**	1.72		**0.86**
Bathyraja sp.	1				0.02		*			
Rajidae										
Amblyraja georgiana	1				0.02		*			
Aves	1									
Procellariiformes										
Procellariidae										
Ardenna tenuirostris	1								0.01	*
Mammalia	2									
Artiodactyla										
Balaenopteridae										
Balaenoptera bonaerensis	1								*	*
Carnivora										
Phocidae										
Lobodon carcinophaga	1								*	*
Echinodermata	**1**									
Holothuroidea	1									
Dendrochirotida										
Cucumariidae										
Staurocucumis liouvillei	1							*		
Mollusca	**16**	**0.38**		**0.03**	**0.04**	**0.53**	**0.20**	**0.88**	**0.21**	**0.55**
Cephalopoda	16									
Octopoda	7									
Octopodidae										
Graneledone antarctica	1							0.03		**0.02**
Megaleledone setebos	1			0.01			*			
Muusoctopus cf. *levis*	1	0.31					**0.06**			
Muusoctopus sp.	1							0.20	0.01	**0.10**
Opisthoteuthidae										
Cirroctopus sp.	3	0.01					*	0.12	0.04	**0.08**
Teuthida	9									
Cranchiidae										
Helicocranchia sp.	2	0.01				0.01	*			
Mesonychoteuthis hamiltoni	2	0.01				*	*		0.05	**0.02**
Neoteuthidae										
Alluroteuthis antarcticus	1					0.21	**0.04**			
Onychoteuthidae										
Filippovia knipovitchi	2					0.14	**0.03**		0.01	*
Moroteuthopsis longimana	1					0.04	**0.01**		*	*
Psychroteuthis glacialis	1	0.03		0.02	0.04	0.13	**0.04**	0.54	0.10	**0.32**
Unknown	8	0.02	0.01				**0.01**	0.04	0.73	**0.38**
Total	**105**	100	100	100	100	100	100	100	100	100

*Lower than 0.01%.
Bold text indicates the phylum or class among taxa, and the bold number indicates the average value.
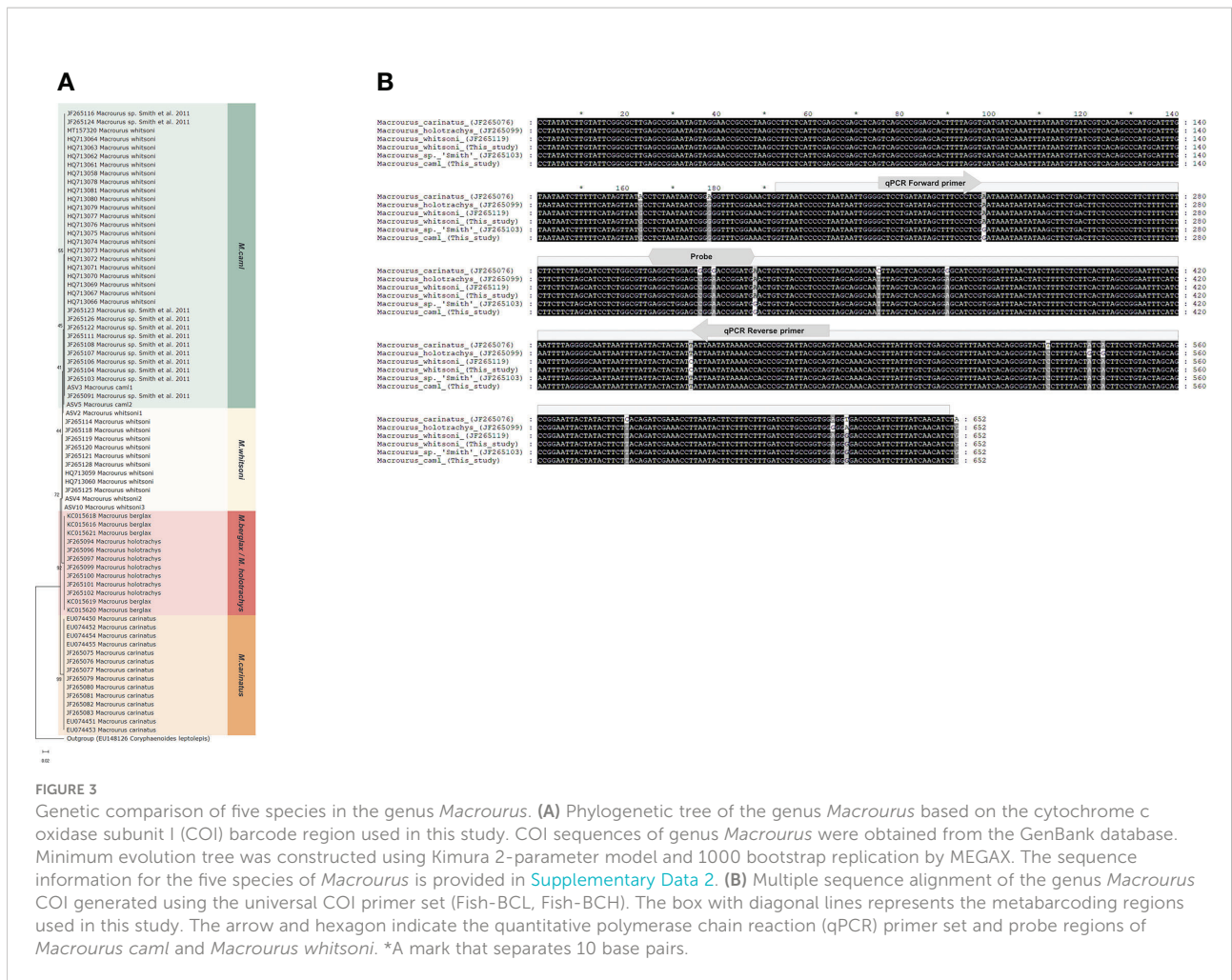
FIGURE 3

Genetic comparison of five species in the genus *Macrourus*. **(A)** Phylogenetic tree of the genus *Macrourus* based on the cytochrome c oxidase subunit I (COI) barcode region used in this study. COI sequences of genus *Macrourus* were obtained from the GenBank database. Minimum evolution tree was constructed using Kimura 2-parameter model and 1000 bootstrap replication by MEGAX. The sequence information for the five species of *Macrourus* is provided in Supplementary Data 2. **(B)** Multiple sequence alignment of the genus *Macrourus* COI generated using the universal COI primer set (Fish-BCL, Fish-BCH). The box with diagonal lines represents the metabarcoding regions used in this study. The arrow and hexagon indicate the quantitative polymerase chain reaction (qPCR) primer set and probe regions of *Macrourus caml* and *Macrourus whitsoni*. *A mark that separates 10 base pairs.

et al., 2012). The primer sets for the two *Macrourus* species were designed based on their unique sequences, without any intra-species variation.

Quantitative analysis of two *Macrourus* prey species, *D. mawsoni*, *M. caml* and *M. whitsoni*, was conducted using a quantitative PCR technique with 50 individual stomach contents randomly selected from each SSRUs. The distribution patterns of *M. caml* and *M. whitsoni* were significantly different between the subareas (88.3 and 58.4%, respectively; Figure 4). In subarea 88.3, much higher numbers of *D. mawsoni* ate *M. caml* than *M. whitsoni*. However, in subarea 88.3, *M. caml* was detected in 35% of the individual stomachs on average, ranging from 18% in 88.3D to 52% in 88.3B, whereas only 7.75% of individuals contained *M. whitsoni* (Figure 4). In contrast, in subarea 58.4, the abundance of the two *Macrourus* prey species showed a different pattern among the three SSRUs. Furthermore, 82 and 72% of individual *D. mawsoni* contained *M. whitsoni* in their stomachs at 58.4.1C and 58.4.1E, respectively, whereas *M. caml* was detected in only 4 and 6% of them (Figure 4). Unlike

the two SSRUs in 58.4, *M. caml* and *M whitsoni* were detected with similar occurrence numbers in 58.4.1. G (Figure 4).

The biomass of each *Macrourus* species showed a pattern similar to that of its detection number (Figure 4). A high degree of correlation was identified between the detection number and cumulative copy number (*M. whitsoni*: y = 7.411x − 3.5795, R^2 = 0.9996; *M. caml*: y = 6.9586x − 0.0781, R^2 = 0.9939). The highest cumulative copy number of *M. caml* was identified in 88.3B (59,919,907 copy numbers/10^4), which was 258-folds higher than that in 58.4.1E (232,644 copy numbers/10^4) (Figure 4). In contrast, the highest numbers of *M. whitsoni* were identified at 58.4.1C (179,243,756 copy numbers/10^4), which was 223-folds higher than that at 88.3D (803,149 copy numbers/10^4). The copy numbers of *M. whitsoni* and *M. caml* in *D. mawsoni* were 2,441,413 and 1,363,542 copy numbers/10^4, respectively. This may reflect the higher average biomass of *M. whitsoni* for each Antarctic toothfish as prey compared with that of *M. caml*.

In addition to the difference in abundance of the two *Macrourus* prey species, there was an additional difference in the total *Macrourus* prey between the two subareas (Figure 4).

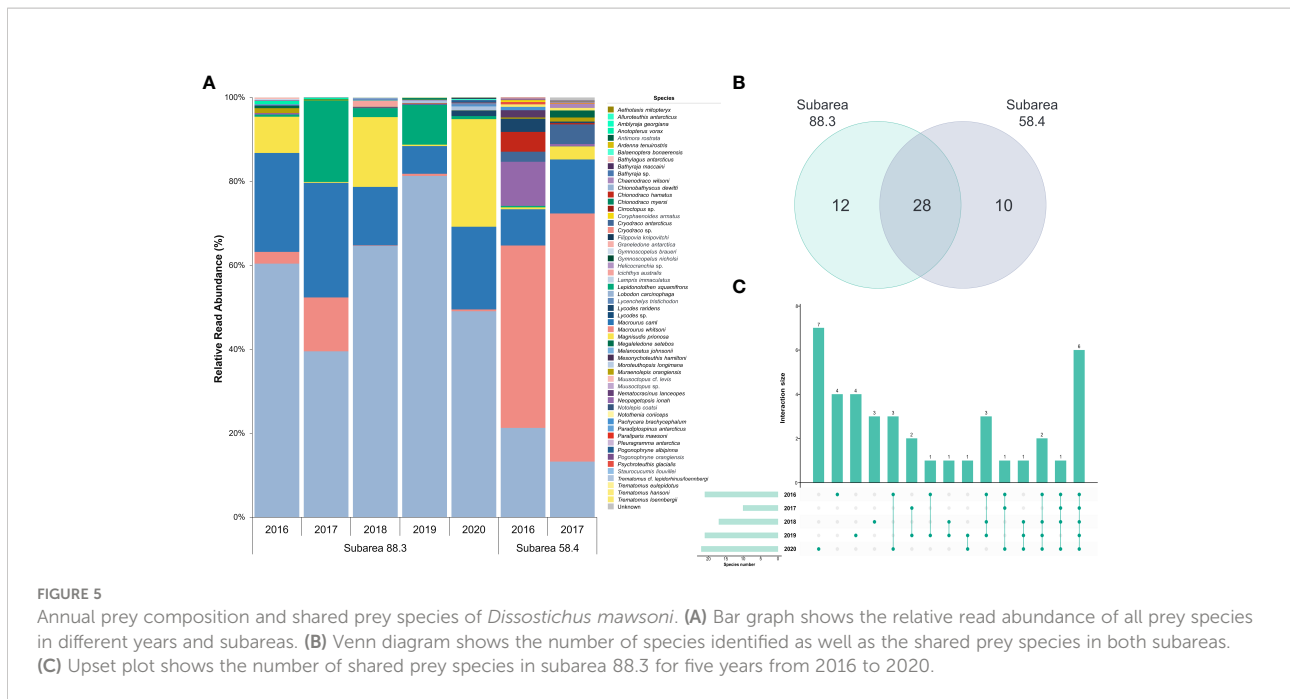**FIGURE 5**
Annual prey composition and shared prey species of *Dissostichus mawsoni*. **(A)** Bar graph shows the relative read abundance of all prey species in different years and subareas. **(B)** Venn diagram shows the number of species identified as well as the shared prey species in both subareas. **(C)** Upset plot shows the number of shared prey species in subarea 88.3 for five years from 2016 to 2020.

were exclusively identified in subarea 88.3. In contrast, four fish [*Bathylagus antarcticus* Günther, 1878 (Antarctic deepsea smelt), *Gymnoscopelus braueri* Lönnberg, 1905 (Brauer's lanternfish), *Paraliparis mawsoni* Andriashev, 1986, and *Notothenia coriiceps* Richardson, 1844 (Black rockcod)], a cephalopod (*Graneledone antarctica* Voss, 1976), and a decapod (*N. lanceurocucum*) were exclusively identified in subarea 58.4. Two mammals (*Balaenoptera bonaerensis* and *L. carcinophagus*) were identified exclusively in the 58.4. The exclusively identified prey taxa proportions were 1.12% in subarea 88.3 and 0.10% in subarea 54.4 (Figure 5).

Beta diversity analysis was performed to evaluate the differences and similarities in the structure of the prey community between the subareas. The prey of *D. mawsoni* were clustered by subarea (ANOSIM, global R = 1.000, *p* = 0.048) and not by year (ANOSIM global R= −0.263, *p* = 0.743), indicating that the diets of *D. mawsoni* are predominantly affected by geographical factors. As a result of the SIMPER analysis, 54.39% of dissimilarity in prey organisms was identified between subareas 58.4 and 88.3 (Table 4). Ten prey species contributed more than 70% to discriminating the diet composition of *D. mawsoni* between subareas 88.3 and 58.4, and which *M. whitsoni* showed the highest contribution value (20.17%), followed by *C. dewitti* (12.30%), *M. prionosa* (6.76%), *L. squamifrons* (6.23%), and *N. ionah* (6.09%). As a result of the nMDS analysis, the subareas were distinguished with 50% similarity. Notably, three surveys in 2016, 2018, and 2020 in subareas 88.3 were clustered together, distinguishing them from the other two in 2017 and 2019, with 70% similarity. According

to the Pearson correlation between the physical parameters of each year, the depth of catch contributed to distinguishing them in subarea 88.3 (Figure 6A). The heatmap shows that the prey organisms changed according to the subarea, which is consistent with the nMDS analysis (Figure 6B). Prey organisms are primarily divided into two groups: *M. caml* and *M. whitsoni*. The first group, including *M. caml*, consisted of species that were mainly detected in subarea 88.3. The second group, including *M. whitsoni*, consisted of the species mainly detected in subarea 58.4 (cophenetic correlation: 0.86716, *p* < 0.05). In addition, a distinct group of subareas 88.3 identified in nMDS that the proportion of *L. squamifrons* and *M. prionosa* contributed to distinguishing annual groups (Figure 6B). These results strongly indicate that the prey species were regionally different, reflecting the fish assemblage of each research site.

Discussion

This study analyzed the diet of Antarctic toothfish, *D. mawsoni*, using metabarcoding, indicating a high degree of reference sequences for Antarctic marine organisms in the database. Among 105 haplotypes, 88 haplotypes (83.81%) showed sequence identity higher than 99% identity in the database. Especially, 92.11% of fish haplotypes were accurately assigned, providing clear information about the diet of the piscivorous *D. mawsoni*. Compared with those for bony fish species, reference sequences for cartilaginous fish and invertebrate species should be further supplemented for

TABLE 4 Results of the analysis of similarities (ANOSIM) and similarity percentages (SIMPER) analysis of the abundance of discriminating prey items in subareas 88.3 and 58.4.

ANOSIM			SIMPER				
R statistic	P value	Average dissimilarity (%)	Species	Mean Abundance 58.4	Mean Abundance 88.3	Average dissimilarity (%)	Contribution (%)
1.000	0.048	54.39	*Macrourus whitsoni*	7.15	1.40	10.97	20.17
			Chionobathyscus dewitti	4.13	7.62	6.69	12.30
			Magnisudis prionosa	1.22	2.62	3.68	6.76
			Lepidonotothen squamifrons	0.37	2.11	3.39	6.23
			Neopagetopsis ionah	1.99	0.23	3.32	6.09
			Cryodraco antarcticus	1.86	0.16	3.24	5.95
			Chionodraco hamatus	1.36	0.14	2.28	4.20
			Macrourus caml	3.27	4.16	2.22	4.08
			Lycodes raridens	1.19	0.23	2.03	3.73
			Trematomus loennbergii	0.78	0.04	1.41	2.59

dietary studies of non-piscivorous species. Six of the nine haplotypes assigned to the genus level corresponded to cephalopods. Primarily, molluscan detection should be highly variable by geographical differences, and their reference sequences should provide useful biogeographic information about the diets of Antarctic toothfish. One haplotype was identified as *Bathyraja* n. sp. "dwarf" in Subarea 88.3. According to the Register of Antarctic Marine Species, seven species are currently reported in the genus *Bathyraja*. Further studies should be conducted to obtain accurate species identification and biogeographic information. Additionally, we identified 105 haplotypes as the prey of *D. mawsoni*, covering 29 families and 45 genera in subareas 88.3 and 58.4, respectively, from the five-year survey. Each year, the numbers of newly identified species were five on average, a proportion of only 0.94% of total prey species. This result indicated that the abundant prey species had been identified by metabarcoding for five years in subarea 88.3. However, we also identified that three to seven new species were detected each year for five years, which suggests that more stomach metabarcoding is still worth obtaining more species in each subarea.

The proportion of fish species in all annual samples was higher than 99%, which supports the previous results that *D. mawsoni* is piscivorous (Fenaughty and Stevens, 2003; Roberts et al., 2011; Stevens et al., 2014; Hanchet et al., 2015; Park et al., 2015; Yoon et al., 2017; Seong et al., 2021). However, metabarcoding analysis of five-year stomach contents provides useful data for accurate species identification by molecular methods. Compared with the three previous studies by morphological identification in the Lazarev Sea (Petrov and Tatarnikov, 2011), South Sandwich Islands (Roberts et al.,

2011), and Ross Sea (Stevens et al., 2014), we additionally identified 33 fish prey, including *Macrourus caml Magnisudis prionosa*, *Pogonophryne albipinna*, and *Trematomus eulepidotus* Regan, 1914. In addition to fish species, four molluscan species were identified: *Filippovia knipovitchi* Filippova, 1972, *Graneledone antarctica*, *Muusoctopus* (*Benthoctopus*) cf. *levis*, and *Megaleledone setebos*. Additionally, we separately analyzed two closely related species, *M. caml* and *M. whitsoni*. Although *M. caml* was reported as a new species in 2012 (Mcmillan et al., 2012), it is still challenging to discriminate using morphological analysis. Besides morphological similarities, the two species share 99.54% within the long COI region. After archiving the multiple haplotypes obtained from the five-year stomach contents, we distinguished each species based only on the difference in the nucleotide sequence. Collectively, a long-term metabarcoding analysis of stomach contents would provide genetic information about marine organisms of the habitats of *D. mawsoni*.

We designed *M.caml* and *M.whitsoni* species-specific primers with high efficiency and accuracy based on reliable haplotypes for each species obtained by metabarcoding over five years. Owing to the high degree of similarity in morphological characteristics, it has been challenging to discriminate these two *Macrourus* species from the stomach contents of Antarctic toothfish by traditional visual observation. In addition, several previously deposited reference sequences in the database were not in accordance with the phylogenetic relationship, which requires additional analysis for accurate identification of the main prey of *D. mawsoni*. Here, we reconfirmed the previous result of Mcmillan et al. (2012) and found that some of the previously deposited reference sequences
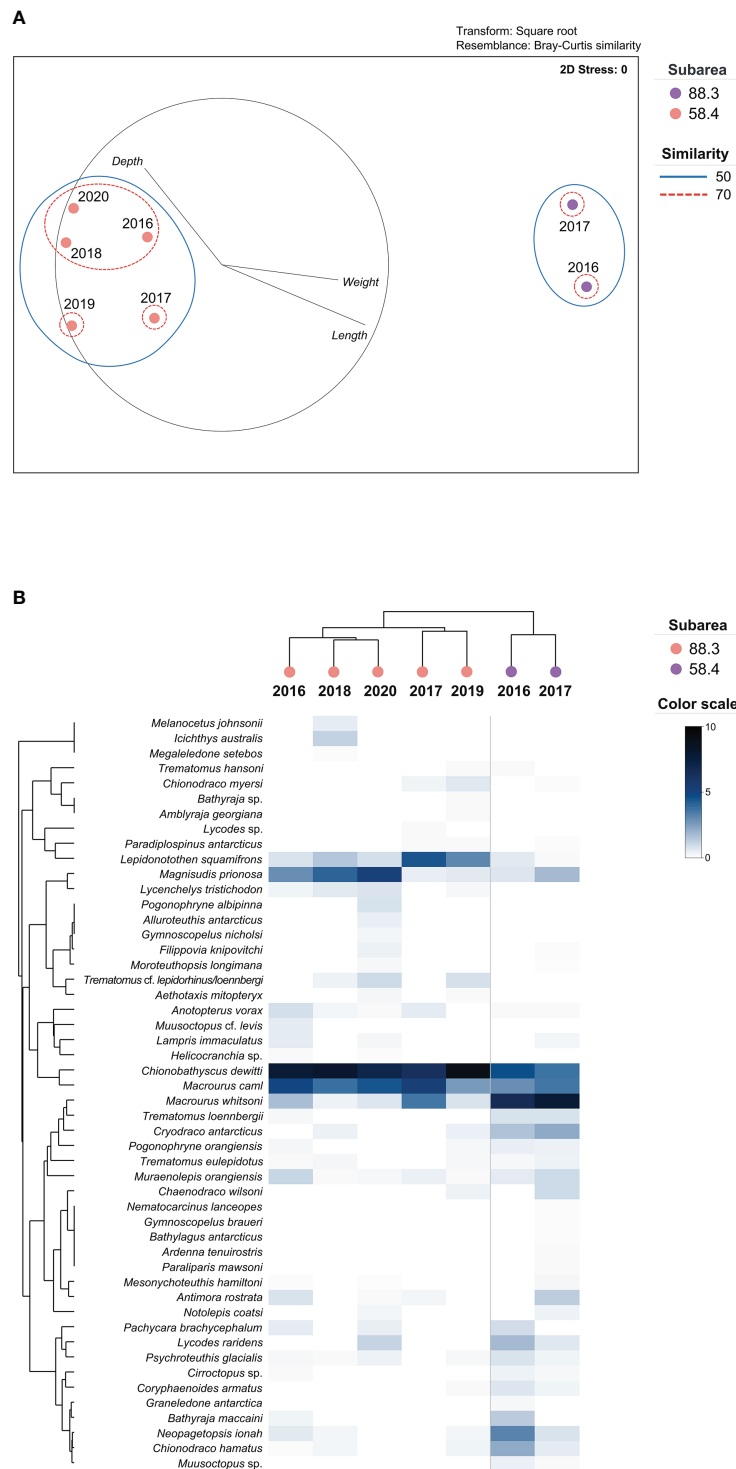
FIGURE 6

Non-metric multidimensional scaling (nMDS) plot and heatmap analyses of the prey compositions of *Dissostichus mawsoni*. **(A)** nMDS was based on Bray−Curtis similarity for the prey organism community between subareas 88.3 and 58.4. Dots represent each year in the two subareas. Blue dotted and green lines represent the hierarchical clustering similarity in the two subareas. Gray line represents the Pearson correlation between the physical parameters of each subarea. **(B)** Heatmap was based on the relative read abundance of prey organisms for each year, and a row dendrogram was generated using a hierarchical cluster based on Bray−Curtis similarity. The column dendrogram was based on cluster analysis of each species *via* cophenetic correlation.

may not be correct. The sequence difference between these two *macrourus* species was 99.35%, and some of incorrect reference sequences may cause misidentification of prey species. Therefore, an accurate reference database is essential for successful metabarcoding analysis of diet studies in Antarctic toothfish. In particular, notothenioid fish, the other main prey of *D. mawsoni*, have recently evolved as a result of adaptive radiation in stable cold water, exhibiting a high degree of sequence similarity among the species (Kim et al., 2019). Therefore, as shown in *Macrourus* species, longer barcodes of notothenioid prey should also be examined for more accurate diet analysis of *D. mawsoni*. qPCR using the two species-specific primer sets designed in this study revealed a significant spatial difference between the two *Macrourus* species in the Southern Ocean. Although metabarcoding provided quantitative information about each prey species, qPCR was highly accurate for quantitative analysis. Although read numbers obtained by metabarcoding may reflect the abundance of each prey species, it is difficult to use a simple matrix to transform them to quantify each prey species. This is due to various factors during metabarcoding, including the number of target genes, digestibility, quality of DNA, annealing bias for each target gene by universal primers, or more (Deagle et al., 2013; Manzari et al., 2020). Owing to its high feasibility and accuracy, quantitative values obtained by qPCR can be transformed into values used in traditional feeding ecological studies. Although we investigated only two *Macrourus* species in this study, additional qPCR analyses for more prey species should be conducted. These results provide precise information on the feeding ecology and spatiotemporal distribution or genetic diversity of the prey species of *D. mawsoni*.

We determined that the stomach contents of *D. mawsoni* reflected the regional fish assemblage. A previous study also showed that the dispersion range of *D. mawsoni* was less than 50–100 km, which supports our results (Hanchet and Rickard, 2008; Hanchet et al., 2015). nMDS analysis of five-year metabarcoding results showed that the stomach contents of *D. mawsoni* were strongly clustered according to each subarea by approximately 70% in similarity. Other physical parameters did not significantly contribute to the difference in the prey composition of *D. mawsoni* between subareas 88.3 and 58.4. Although slightly higher body lengths and weights were identified for those collected from subarea 58.4, they were negligible. Since its habitat is limited to the floor of the continental shelf before attaining buoyancy at approximately 100 cm in length (Fuiman et al., 2002), the average body lengths of the collected *D. mawsoni* (138.75 ± 23.22 cm) were larger than the limited stages supporting physical parameters, which may not have affected the results in this study. The water depths between subareas of 58.4 and 88.3°were also similar. Therefore, differences in the prey composition of *D. mawsoni* between 88.3 and 58.4 may have come from the food availability of each site, supporting the idea that analysis of its stomach contents would

be a good indicator to monitor any changes in fish assemblages. In this study, six and eight preys occupied more than 97.57% of subarea 88.3 and 94.40% of subarea 58.4, suggesting that a long-term study of these preys would be useful to evaluate the impact of the Antarctic toothfish fishery on the sustainability of the ecosystem.

Differences in prey composition were identified, even within each subarea. Among the five preys in subarea 88.3, two in 2017 and 2019 showed lower similarity than the other three in 2016, 2018, and 2020. *D. mawsoni* in 2017 and 2019 were caught at a shallower water depth than those in the other three years. Heatmap analysis showed that *L. squamifrons* and *M. prionosa* discriminated between the two groups in subarea 88.3. According to FishBase (www.fishbase.org), the main habitat of *L. squamifrons* ranged from 195–312 m in depth, whereas *M. prionosa* was found mainly from 300–800 m. This result suggests that higher proportions of *L. squamifrons* are due to its higher availability as prey of *D. mawsoni* caught in 2017 and 2019, whereas those in 2016, 2018, and 2020 had a high chance of preying on *M. prionosa*, which is relatively abundant in deeper water within subarea 88.3.

QPCR results of the two *Macrourus* species showed the regional prey availability of *D. mawsoni*. In addition to their regional differences between subareas 88.3 and 58.4, we identified a unique proportion in 58.4.1G, an SSRU in the subarea where *M. caml* and *M. whitsoni* were identified in a similar amount. This result indicates the difference in the distribution of the two *Macrourus* species; 58.4.1G is located at the edge region of the Kerguelen Plateau Gyre, which is a boundary to discriminate between the two species. Both 88.3C and 88.3D showed lower occurrence numbers than 88.3B, which showed deeper water depths. Long-term surveys are required to obtain reliable data along with collaborations among different research groups. We first showed that metabarcoding analysis of stomach contents can provide useful information about regional prey availability for *D. mawsoni*. Moreover, the stomach contents of *D. mawsoni* provide important information about the sustainability of toothfish fisheries in the Southern Ocean, which has been conducted for approximately 25 years since 1997. Due to its highest trophic level, the stomach contents of Antarctic toothfish mirror the fish assemblage of its habitats. Decreased numbers of high-trophic predator bycatch result in a series of knock-on effects throughout all trophic levels in a food web, upsetting the ecological balance by altering the number of different species (Ripple and Beschta, 2006). Therefore, any change in the prey composition of each designated habitat may be an important sign of the adverse impacts of *D. mawsoni* catch. In addition to the impact of human activities, ecosystems in the Southern Ocean have been facing considerable change for at least the last 30 years due to climate change, which includes increased water temperature, changes in areas, and seasonality of sea ice (Constable et al., 2014). These considerable changes may alter the fish assemblage of the ecosystem, replacing those with low tolerance to high

temperatures with lower latitude species in the long term. Therefore, the stomach contents of *D. mawsoni* are useful indicators of climate change. Moreover, long-term monitoring of the diet of *D. mawsoni* using metabarcoding analysis established in this study can aid in addressing these issues in the Southern Ocean.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA809505.

Ethics Statement

This study does not violate animal ethics, as the stomachs were collected from by-products (i.e., waste - skin, bones, entrails, or flesh) generated the commercial fishing of Antarctic toothfish.

Author contributions

SL designed and performed the experiments and analyzed the data, prepared figures and tables, and drafted the manuscript. S-GC, SC and DK supported fieldwork for Antarctic toothfish sampling. C-KK and H-WK conceived the experiments, analyzed the data, reviewed drafts of the manuscript, and approved the final draft. All authors contributed to the article and approved the submitted manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.888167/full#supplementary-material

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