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DNA barcoding of Antarctic marine zooplankton for species identification and recognition

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Abstract Polar zooplankton are particularly sensitive to climate change, and have been used as rapid-responders to indicate climate-induced changes in the fragile Antarctic ecosystem. DNA barcoding provides an alternative approach for rapid zooplankton species identification. Ninety-four specimens belonging to 32 Antarctic zooplankton species were barcoded to construct a comprehensive reference library. An 830 to 1 050 base-pair region of the mitochondrial cytochrome c oxidase subunit I (mtCOI) gene was obtained as DNA barcodes. The intraspecific variation of the gene ranged from 0 to 2.6% (p-distance), with an average of 0.67% (SD=0.67%). The distance between species within the same genera ranged from 0.1% (*Calanus*) to 29.3%, with an average of 15.3% (SD=8.4%). The morphological and genetic similarities between *Calanus propinquus* and *C. simillimus* raise new questions about the taxonomic status of *C. simillimus*. With the exception of the two *Calanus* species, the intraspecific genetic divergence was much smaller than the interspecific divergence among congeneric species, confirming the existence of a barcode gap for Antarctic zooplankton. In addition, species other than *Calanus* sp. formed a monophyletic group. Therefore, we have confirmed DNA barcoding as an accurate and efficient approach for zooplankton identification in the Antarctic area (except for Hydromedusa, Tunicata, and other gelatinous zooplankton). Indicator vector analysis further confirmed this conclusion. The new primer sets issued here may facilitate the study of Antarctic marine zooplankton species composition by environmental metagenetic analysis.

Keywords **Southern Ocean, DNA barcode, Calanus, zooplankton, high-throughput analysis**

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1 Introduction

The Antarctic area is considered the most sensitive climatic zone in terms of global climate change because of its distinctive geographical position and ecology. Within the general trend of global temperature increase, warming on the Antarctic Peninsula has been remarkable[1]. Circulation and sea ice cover are the most important biogenic elements affecting the Southern Ocean ecosystem; even a small temperature shift significantly affects the ice cover and its melting period. Thus, the marine ecosystem of the polar region is extraordinarily sensitive, making the Southern Ocean a global climate change research hot spot[2].

Zooplankton have short life cycles, strong metabolic activity, and weak motility. They are very sensitive to environmental change and respond intensely to environmental disturbance. The population succession of zooplankton living in the polar region is closely linked with sea ice cover variation. On account of these distinguishing features, zooplankton are important indicators of the effects imposed by global climate change to polar region ecosystems[3]. According to species composition, zooplankton living in the Antarctic area can be divided into pelagic ocean, nearshore, and krill communities. The structure of a zooplankton community is significantly related to current, ice cover, temperature, salinity, and other environmental elements[4-5]. Therefore, long-term observation of zooplankton community change is important in understanding the effects of global climate change on polar ecosystems. Precise species

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identification is a prerequisite for polar zooplankton community research. Even sibling species succession alters the community structure[6].

Zooplankton include various taxonomic groups, so accurate identification often involves the cooperation of specialists. Traditional identification methods based on zooplankton morphology require specialist experience, a lot of time, and great effort. Zooplankton identification often creates a bottle neck in zooplankton community change studies[7]. Species with different names and sibling species are universal, thereby increasing the difficulty of identification[8]. Moreover, zooplankton larval identification is even more difficult[9].

DNA barcoding uses mitochondrial cytochrome c oxidase subunit I (mtCOI) gene sequences to identify species[10]. The technique has proven to be an effective tool for zooplankton species identification in Chinese coastal waters[11-14]. A comparative study in the Arctic sea has confirmed the reliability of DNA barcoding in identifying polar zooplankton[15]. DNA barcoding has been convenient in related studies of other zooplankton taxa, with the exception of Ctenophores. It is a powerful tool allowing for quick species and sibling species identification, stomach content analysis, invasive species monitoring, community history evolution inversion, population genetic structure, and biogeography studies, among others[16]. Other studies have investigated zooplankton larval biology based on DNA barcoding[9,17], and the results indicated that quite a lot of larvae were not accurately identified because a comprehensive database is lacking[17]. Although the Census of Antarctic Marine Life (CAML) has collected the DNA barcodes of many marine animals, a considerable amount of the data remain unpublished[18], which seriously restricts the application of this technique in other investigations. Previous reports have only summarized CAML progress, but not systematically analyzed the zooplankton barcodes.

This research investigates samples collected in Prydz Bay and the sea area around the Antarctic Peninsula in 2008 and 2011, constructing a database of common zooplankton species. The research comparatively analyzed 124 mtCOI gene sequences of 35 common species in the survey area, and validated the use of DNA barcodes in identifying Antarctic zooplankton. In addition, we have designed an appropriate universal primer pair based on published sequences, establishing a foundation for future research aimed at monitoring zooplankton species composition and food web nutrition relationships with the help of DNA barcodes.

2 Materials and methods

Samples were collected with a North Pacific standard plankton (NORPAC) net (45-cm mouth and 333-μm mesh size) and a high-speed plankton collector (0.5-mm mesh aperture) during the ocean survey voyages of the 25th and 28th Chinese National Antarctic Research Expeditions (CHINARE). The sampling stations are shown by the red dots in Figure 1. Station coordinates are given in Table 1. The samples were preserved in 95% alcohol after nekton were removed. The volume of alcohol was four to five times the sample volume and was replaced regularly. The preserved samples were kept at –20℃ and subsequently identified microscopically. The identified samples were split into two groups: One was preserved in formaldehyde solution, and the other stored at –80℃. The samples stored at –80℃ were used for molecular analysis and the others were voucher samples.

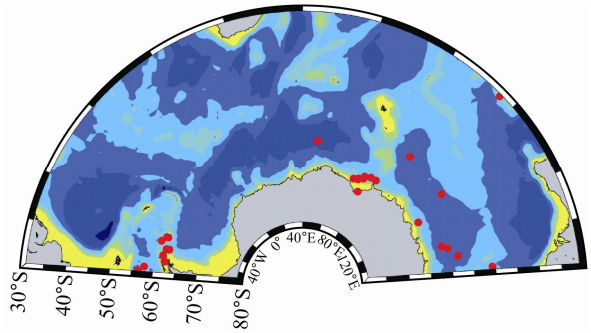

Figure 1 Regions sampled and collection locations for samples barcoded in this study.

Genomic DNA was extracted using a QIAamp DNA Mini Kit (made by Qiagen Co., Art. No: 56304). Two sets of universal primers were designed; the forward primer was coxf: GGTCCTGTAATCATAAAGAYATYGG, the reverse primers were coxr1: GCGACTACATAATAAGTRTCRTG and coxr2: TCTATCCCAACTGTAAATATRTGRTG, and the primers were 830 bp and 1 050 bp long, respectively. PCR reactions were carried out in a 50 μL volume reaction mixture, and the PCR conditions were as follows: One step at 94℃ for 5 min, 94℃ for 1 min, 42℃–47℃ for 30 s, 72℃ for 1.5 min, one step of 72℃ for 10 min. The amplified products were confirmed by electrophoresis(1.5%) and sent to the Shanghai Southgene Company for bi-directional sequencing.

CLC Main Workbench, using the default parameters, was used for sequence assembly; all of the sequences acquired were submitted to NCBI GenBank[18]. The accession numbers are shown in Table 1. Furthermore, 30 sequences from 13 species obtained from GenBank were added to the analysis for species identification with DNA barcode validation. The sequences were aligned by MUSCLE under default parameters, the pairwise *p*-distances of the DNA barcodes were calculated by PAUP*v4b10. Perl scripts were developed to compare the genetic divergence at different taxonomic levels, and were further analyzed by SPSS v18. The neighbor-joining (NJ) tree (500 bootstraps) was constructed by MEGA 5.0. Following Sirovich et al.[19], indicator vector analysis was employed for the Antarctic zooplankton DNA barcodes to show the genetic similarity for each taxa and the phylogenetic affinity between them.

The DNA barcoding sequences obtained in this study and the mtCOI gene sequences from GenBank were compared. Based on the results, the universal primers were generated by oligo 7 for a better amplification performance.

The primers were: ICO140U: TCAACAAATCATAARGAYATHGG and ICO820L: CACTTCNGGGTGACCRAARAAYCA. The primers used to amplify the mtCOI gene sequences of 32 Antarctic zooplankton species were synthesized by Shanghai Southgene Company. The amplicons were checked by gel electrophoresis (1.5%).

Table 1 Antarctic zooplankton species analyzed in this study, with specimen voucher numbers (voucher), collection information (location), and GenBank accession numbers (acc.)

No.	Group and species	Voucher	Location	Acc.
	Chaetognatha			
1-1	*Eukrohnia hamata*	GenBank	61.82 S 63.97 W	JX880227
1-2	*Eukrohnia hamata*	GenBank	64.17 S 65.3 W	JX880229
2-1	*Sagitta sp.*	euha1	66.50 S 72.99 E	KC754460
2-2	*Sagitta sp.*	euha	66.50 S 72.99 E	KC754461
	Hyperiidea			
3-1	*Themisto gaudichaudii*	pagabi1	58.30 S 135.51 E	KC754379
3-2	*Themisto gaudichaudii*	pagabi2	58.30 S 135.51 E	KC754380
4	*Themisto antarctica*	GenBank	NA	HM053514
5-1	*Cyllopus lucasii*	cylu	64.91 S 81.80 E	KC754388
5-2	*Cyllopus lucasii*	cylu	66.67 S 73.04 E	KC754389
5-3	*Cyllopus lucasii*	cylu4	64.17 S 113.75 E	KC754390
6-1	*Hyperia macrocephala*	hyma	64.17 S 113.75 E	KC754383
6-2	*Hyperia macrocephala*	GenBank	NA	EF989666
7-1	*Vibilia antarctica*	vian2	61.44 S 129.49 E	KC754385
7-2	*Vibilia antarctica*	vian	64.17 S 113.75 E	KC754386
7-3	*Vibilia antarctica*	vian	61.44 S 129.49 E	KC754387
8-1	*Hyperiella dilatata*	hydi	69.17 S 76.29 E	KC754384
8-2	*Hyperiella dilatata*	hyan	64.91 S 78.76 E	KC754381
9-1	*Hyperiella antarctica*	hydi	66.67 S 73.04 E	KC754382
9-2	*Hyperiella antarctica*	eusu3	64.17 S 113.75 E	KC754451
	Euphausiacea			
10-1	*Euphausia superba*	eusu	62.88 S 50.18 W	KC754458
10-2	*Euphausia superba*	eucy1	61.48 S 44.68 W	KC754459
11	*E. crystallorophias*	GenBank	NA	AF177183
12-1	*Thysanoessa macrura*	thma3	61.79 S 53.68 W	KC754454
12-2	*Thysanoessa macrura*	thma4	61.79 S 53.68 W	KC754455
12-3	*Thysanoessa macrura*	thma2	61.79 S 53.68 W	KC754456
12-4	*Thysanoessa macrura*	thma1	61.79 S 53.68 W	KC754457
12-5	*Thysanoessa macrura*	GenBank	66.81 S 70.38 W	DQ003709
	Polychaeta			
13-1	*Tomopteris sp.*	fc	65.45 S 75.58 E	KC754377
13-2	*Tomopteris sp.*	masp	65.45 S 75.58 E	KC754378
	Gastropoda			
14-1	*Limacina retroversa*	lire3	55.58 S 87.72 E	KC754466
14-2	*Limacina retroversa*	21lire	60.60 S 47.18 W	KC754467
15	*Clione antarctica*	spau	60.60 S 47.18 E	KC754462
17-1	*Limacina helicina*	31lihe	61.79 S 47.14 W	KC754468
17-2	*Limacina helicina*	GenBank	71.17 S 109.86 W	GQ861824
17-3	*Limacina helicina*	GenBank	73.97 S 107.42 W	GQ861825
17-4	*Limacina helicina*	GenBank	NA	GU227113
		Copepoda		
18	*P. grandispina*	pagr	61.79 S 47.14 W	KC754469
19-1	*Pleuromamma antarctica*	plan	50.74 S 141.93 E	KC754402
19-2	*Pleuromamma antarctica*	plan	31.69 S 95.76 E	KC754403
20-1	*Stephos longipes*	stlo1	67.0 S 70.5 E	KC754441
20-2	*Stephos longipes*	stlo3	67.0 S 70.5 E	KC754442
20-3	*Stephos longipes*	stloa	67.0 S 70.5 E	KC754443
20-4	*Stephos longipes*	stlo2	67.0 S 70.5 E	KC754444
20-5	*Stephos longipes*	GenBank	NA	AF531752
21-1	*Pleuromamma borealis*	plbo2	50.74 S 141.93 E	KC754408
21-2	*Pleuromamma borealis*	plbo3	50.74 S 141.94 E	KC754409
21-3	*Pleuromamma borealis*	plbo1	50.74 S 141.95 E	KC754410
21-4	*Pleuromamma borealis*	plbo4	50.74 S 141.96 E	KC754411
21-5	*Pleuromamma borealis*	plbo5	50.74 S 141.97 E	KC754412
21-6	*Pleuromamma borealis*	GenBank	NA	HM045331
22-1	*Metridia lucens*	melu1	50.74 S 141.93 E	KC754391
22-2	*Metridia lucens*	melu2	50.74 S 141.94 E	KC754392
22-3	*Metridia lucens*	melu3	50.74 S 141.95 E	KC754393
22-4	*Metridia lucens*	GenBank	NA	HM045328
22-5	*Metridia lucens*	GenBank	44.43 S 7.33 E	JN588605
22-6	*Metridia lucens*	GenBank	44.98 S 6.81 E	JN588601
22-7	*Metridia lucens*	GenBank	44.43 S 7.33 E	JN588606
22-8	*Metridia lucens*	GenBank	44.43 S 7.33 E	JN588604
23-1	*Clausocalanus laticeps*	cllaa	56.25 S 108.28 E	KC754413
23-2	*Clausocalanus laticeps*	clla2	60.6 S 47.18 W	KC754414
23-3	*Clausocalanus laticeps*	clla	60.6 S 47.18 W	KC754415
23-4	*Clausocalanus laticeps*	clla3	58.30 S 135.51 E	KC754416
24-1	*Calanoides acutus*	caac1	61.79 S 53.68 W	KC754417
24-2	*Calanoides acutus*	caac4	61.79 S 53.68 W	KC754418
24-3	*Calanoides acutus*	caac2-3	61.79 S 53.68 W	KC754419
24-4	*Calanoides acutus*	olsp1	61.79 S 44.70 W	KC754420
24-5	*Calanoides acutus*	olsp2	61.79 S 44.7 W	KC754421
24-6	*Calanoides acutus*	caac5	61.79 S 53.68 W	KC754422

(To be continued on the next page)

(Continued)

No.	Group and species	Voucher	Location	Acc.	No.	Group and species	Voucher	Location	Acc.
16	*Clio pyramidata*	32clpy	66.67 S 73.04 E	KC754465	24-7	*Calanoides acutus*	caac6	61.79 S 53.68 W	KC754423
24-8	*Calanoides acutus*	caac2	61.79 S 53.68 W	KC754424	29-8	*Rhincalanus gigas*	GenBank	47.77 S 4.14 E	JN663359
24-9	*Calanoides acutus*	caac3	61.79 S 53.68 W	KC754425	29-9	*Rhincalanus gigas*	GenBank	56.56 S 0.00 E	JN663360
25-1	*Oithona similis*	oisi1	58.30 S 135.51 E	KC754452	29-10	*Rhincalanus gigas*	GenBank	62.56 S 63.47 W	JN663361
25-2	*Oithona similis*	oisi2	58.30 S 135.52 E	KC754453	29-11	*Rhincalanus gigas*	GenBank	61.17 S 64.21 W	JN663362
26	*Haloptilus ocellatus*	haoc	56.58 S 62.24 W	KC754404	29-12	*Rhincalanus gigas*	GenBank	62.15 S 63.84 W	JN663363
27-1	*Paraeuchaeta antarctica*	paan	60.23 S 131.25 E	KC754445	30-1	*Metridia gerlachei*	mega2	61.79 S 53.68 W	KC754394
27-2	*Paraeuchaeta antarctica*	paan2	69.17 S 76.29 E	KC754446	30-2	*Metridia gerlachei*	mega5	61.79 S 53.68 W	KC754395
27-3	*Paraeuchaeta antarctica*	paan	69.17 S 76.30 E	KC754447	30-3	*Metridia gerlachei*	mega4	61.79 S 53.68 W	KC754396
27-4	*Paraeuchaeta antarctica*	paan9	66.0 S 75.49 E	KC754448	30-4	*Metridia gerlachei*	mega8	61.79 S 53.68 W	KC754397
27-5	*Paraeuchaeta antarctica*	paan3	69.17 S 76.29 E	KC754449	30-5	*Metridia gerlachei*	mega6	61.79 S 53.68 W	KC754398
27-6	*Paraeuchaeta antarctica*	paan2	66.0 S 75.49 E	KC754450	30-6	*Metridia gerlachei*	mega3	61.79 S 53.68 W	KC754399
27-7	*Paraeuchaeta antarctica*	GenBank	51.48 S 29.00 W	JQ819804	30-7	*Metridia gerlachei*	mega1	61.79 S 53.68 W	KC754400
27-8	*Paraeuchaeta antarctica*	GenBank	51.48 S 29.00 W	JQ819805	30-8	*Metridia gerlachei*	mega7	62.47 S 56.58 W	KC754401
27-9	*Paraeuchaeta antarctica*	GenBank	51.48 S 29.00 W	JQ819806	31-1	*Calanus simillimus*	casi3	57.94 S 60.37 W	KC754438
28-1	*Calanus propinquus*	33capr	60.6 S 47.18 W	KC754431	31-2	*Calanus simillimus*	casi2	57.94 S 60.37 W	KC754439
28-2	*Calanus propinquus*	capr1	62.23 S 50.36 W	KC754432	31-3	*Calanus simillimus*	casi1	57.94 S 60.37 W	KC754440
28-3	*Calanus propinquus*	capr3	62.23 S 50.36 W	KC754433	31-4	*Calanus simillimus*	GenBank	56.58 S 66.47 W	JN663365
28-4	*Calanus propinquus*	capr2	62.23 S 50.36 W	KC754434	31-5	*Calanus simillimus*	GenBank	61.82 S 63.97 W	JN663367
28-5	*Calanus propinquus*	capr4	62.23 S 50.36 W	KC754435	32-1	*Pleuromamma piseki*	plbi1	50.74 S 141.93 E	KC754405
28-6	*Calanus propinquus*	capr5	62.23 S 50.36 W	KC754436	32-2	*Pleuromamma piseki*	plbi2	50.74 S 141.94 E	KC754406
28-7	*Calanus propinquus*	capr6	62.23 S 50.36 W	KC754437	32-3	*Pleuromamma piseki*	plbi3	50.74 S 141.95 E	KC754407
29-1	*Rhincalanus gigas*	rhgi2	61.79 S 53.68 W	KC754426	33-1	*Ctenocalanus citer*	GenBank	NA	AF332789
29-2	*Rhincalanus gigas*	rhgi4	61.79 S 53.68 W	KC754427	33-2	*Ctenocalanus citer*	GenBank	NA	FJ960446
29-3	*Rhincalanus gigas*	rhgi3	61.79 S 53.68 W	KC754428			Ostracoda		
29-4	*Rhincalanus gigas*	rhgi1	61.79 S 53.68 W	KC754429	34-1	*Alacia belgicae*	albe3	66.0 S 75.49 E	KC754464
29-5	*Rhincalanus gigas*	24rhgi	66.67 S 73.04 E	KC754430	34-2	*Alacia belgicae*	albe1	NA	KC754470
29-6	*Rhincalanus gigas*	GenBank	55.24 S 0.00 E	JN663356	35	*Alacia hettacra*	albe2	66.0 S 75.49 E	KC754463
29-7	*Rhincalanus gigas*	GenBank	56.58 S 66.47 W	JN663358					

Notes: No.=the species serial number; Voucher=the serial number of specimen used for morphological confirmation; Acc.=GenBank the accession number.

3 Results

3.1 Sequence signatures

Ninety-four mtCOI gene sequences belonging to 32 species, 24 genera, 19 families, and 5 phyla were obtained (accession number: KC754377-KC754470). The arthropod zooplankton, Copepoda, krill, Ostracoda, and Hyperiidea were identified. Because gelatinous zooplankton were damaged in the preservation alcohol and their DNA ran off in the replacing of alcohol process, no DNA barcode sequences for Medusa or Tunicata were obtained. The amplified sequence lengths ranged from 830 bp to 1 050 bp, depending on the primers being used. The consensus sequences were adjusted to 800 bp for comparison. All 12 indels appeared in the mollusk sequences, and the average nucleotide content percentages of A, C, G, and T were 25.6%, 16.5%, 20.9%, and 37.0%, respectively[20]. The content of A+T was higher than that of G+C, which was coincident with the mitochondrial genome bias. The differences in nucleotide content between species were obvious, as the third codon changed frequently.

3.2 Barcode gap test

A gene with a suitable variation rate would be the ideal marker of species identification. When the intraspecific divergence is much less than the interspecific divergence, the intra- and interspecific genetic differences of two taxa

do not overlap. This is known as the barcode gap. The mtCOI gene sequences of 35 zooplankton species were compared to identify barcode gaps. A wide range of mtCOI genetic differences was observed, from 0 to 48.8%, with the average value being 27.6% (SD=8.5%). The intraspecific genetic divergence was generally small, from 0 to 2.6% (Figure 2), the average value being 0.67% (SD=0.67%). The maximum intraspecific genetic divergence of 94% of the species was less than 1%. The genetic difference between congeneric species ranged from 0.1% to 29.3% (Figure 2), with the average value being 15.3% (SD=8.4%). In Calanus, *C. propinquus* and *C. simillimus* were similar in mtCOI gene sequence; the minimum divergence was 0.64%, while the average interspecific genetic difference of other congeneric species was greater than 9.5%. In general, the intra- and interspecific genetic difference was remarkably divergent though there was little overlap (*t* test, F=469.1, df=442, p=0), and the barcode gap was evident. Within a family, genetic divergence between different genera ranged from 9.2% to 27.1%, with an average value of 20.9% (SD=2.7%). The scope overlapped with the intraspecific divergence of congeneric species. The interspecific genetic divergence of 93% of the species, belonging to different orders or higher taxa, was > 29%.

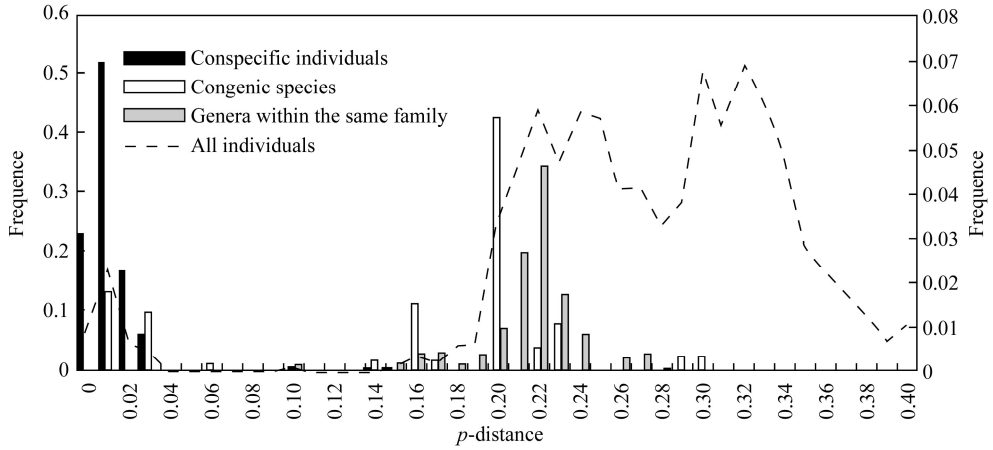

Figure 2 Frequency distribution of genetic divergence (p-distance) of mtCOI genes for pairwise comparisons between all individuals, conspecific individuals, congenic species, and between genera within the same family.

3.3 The phylogenetic tree based on DNA barcode sequences

Thirty-four monophyletic groups, linked by short branches, were generated in the phylogenetic tree according to the DNA barcode sequences of Antarctic zooplankton. Each one represented a taxonomic unit; the bootstrap value was > 99. Each monophyletic group denotes a species, except for *C. propinquus* and *C. simillimus*, which were on the same branch. In accordance with the genetic difference results, because the genetic divergence of the mtCOI gene sequences at the family and genus levels were not significant, zooplankton phylogeny could not be resolved by mtCOI gene sequences. The mutation rate trended towards saturation. Not all species of the same family or genus could be assembled in the tree. However, in higher taxa, high level genetic differences among different taxa showed great divergence. The monophyly of seven main zooplankton taxa (copepods, chaetognaths, krill, ostracods, hyperiidea, mollusks, and polychaetes) were resolved. However, low bootstrap values were observed on the branches where the main taxa are located because of background noise (Figure 3).

3.4 Indicator vector analysis of Antarctic zooplankton DNA barcodes

Tracing vector analysis is a new method used to analyze DNA barcodes. The results of the analyses based on the DNA barcodes of Antarctic zooplankton, which coincided with the NJ tree and barcoding gap analysis results, are shown in Figure 3. The color of the blocks representing each taxon indicates the genetic similarity. Not including *C. propinquus* and *C. simillimus*, the crimson blocks represent all of the species. Copepoda, krill, Ostracoda, and Hyperiidea showed high genetic similarities, therefore the blocks representing them are a light tone. The heredity divergence of the Mollusca and Polychaeta were higher, producing dark corresponding blocks. The extent of the color is concordant with the length of the branches representing the taxon shown in Figure 4. The evolutionary velocity of the mtCOI gene sequences was different among taxa.

3.5 Amplification rate of new primers for Antarctic zooplankton

The gel electrophoresis results, using redesigned primers, are shown in Figure 5. Amplification products of expected lengths were generated with all of the zooplankton DNA templates, and their concentration was sufficient for sequencing acquisition. By contrast, the amplification rate using Folmer primers was less than 60%, and produced indistinct bands.

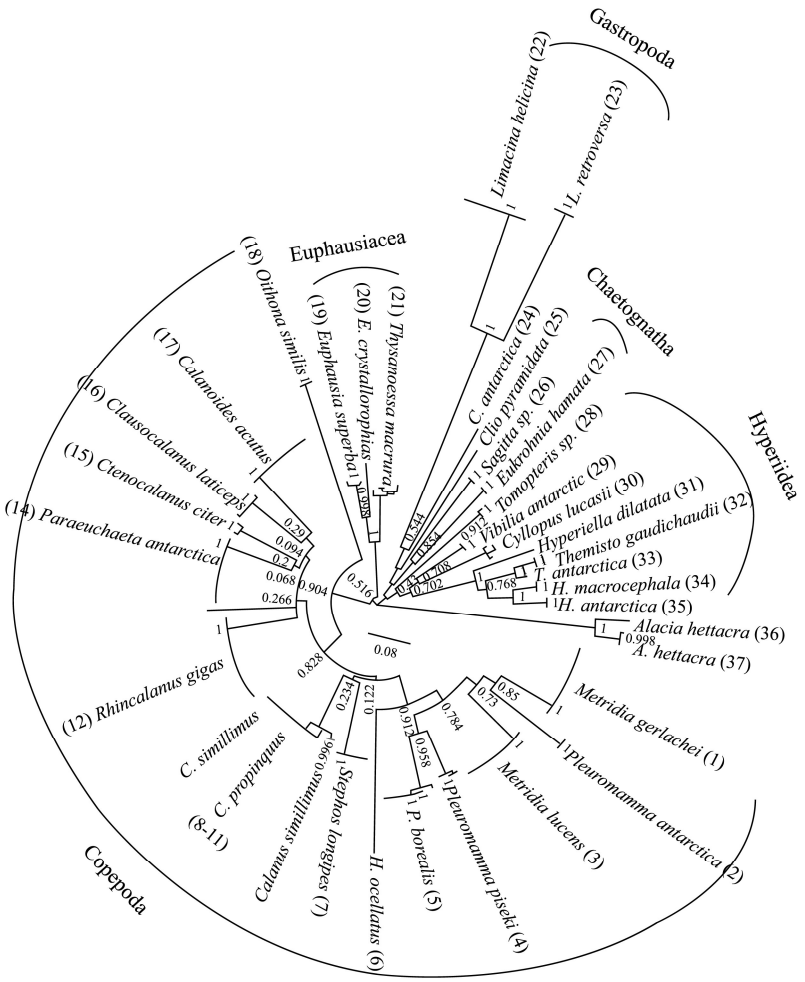

Figure 3 Unrooted mtCOI gene tree for 124 specimens belonging to 35 Antarctic zooplankton species reconstructed by neighbor-joining with maximum-likelihood distances; bootstrapping was done for 1 000 replications.

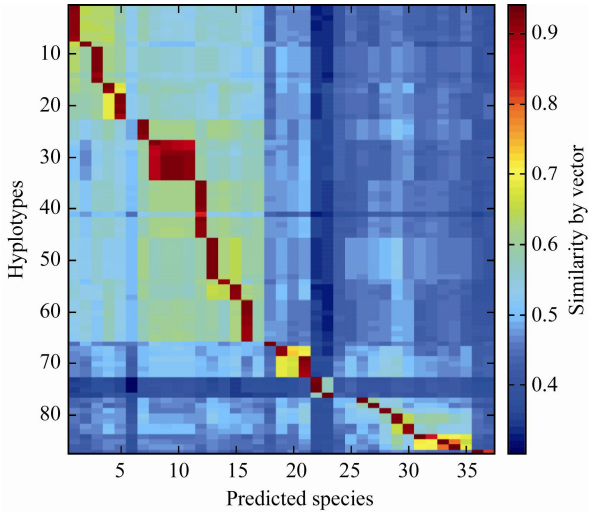

Figure 4 Klee diagram of the vector analysis of 124 barcodes belonging to 35 species. Higher similarity is visualized by warmer colors. Species names, represented by the numbers on the x-axis, are given in Figure 3.

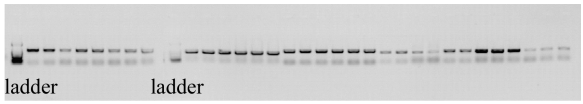

Figure 5 Representative agarose gel electrophoresis of PCR products amplified from 32 Antarctic zooplankton samples using a degenerate primer set. Ladder represents a D2000 marker.

4 Discussion

4.1 Low genetic difference between *C. propinquus* and *C. simillimus*

The mtCOI gene was chosen as the zooplankton barcoding sequence in our study. There are many advantages to using this sequence, e.g. appropriate maternal inheritance, moderate sequence change velocity, and relatively large quantities of copies in cells of organisms[7]. The validity of the gene for use in identifying zooplankton species of different taxa has been confirmed in previous reports[15,21-24].

Our results indicate that DNA barcode analysis based on the mtCOI gene sequence database can identify most

Antarctic sea copepods. According to these studies, the interspecies genetic divergence is usually greater than 10%; moreover, it is generally greater than 5% between sibling species. However, intraspecies genetic divergence is generally less than 4%[13,15,21]. In copepods, the interspecies genetic divergence is generally greater than 5%, while the intra- species genetic divergence is usually lower than 4%. The intraspecies genetic divergence of 95% of copepod species is lower than 2.5%[11]. In this study, the interspecies genetic divergence between *C. propinquus* and *C. simillimus* ranged from 0.2% to 2.9%. In previous reports, it ranged from 7% to 25%[25]. Besides, both the NJ tree and tracing vector analysis indicated that no differentiation occurred in the mtCOI gene of *C. propinquus* and *C. simillimus*.

C. propinquus and *C. simillimus* are important Antarctic Ocean copepod community species. Although their distribution ranges overlap, *C. propinquus* mainly lives in polar seas of higher latitudes[26], while *C. simillimus* mainly lives in sub-Antarctic and polar frontal seas[27]. These records are in accordance with the location of our sampling stations. The chief differences are conspicuous: *C. propinquus* is bigger than *C. simillimus*, and the structure of the inside of their fifth leg differs (http://copepodes.obs-banyuls.fr/). Apart from these differences, they are very similar and their larvae are indistinguishable[28]. Until 1902, they were considered to be the same species. Ensuring the accuracy of identification, sexually mature female individuals were chosen for the experiment, and examined for differences in the fifth legs.

Our study suggests that *C. propinquus* and *C. simillimus* share the same haplotype according to their mtCOI gene. The intraspecies genetic divergence of *C. propinquus* was smaller than the interspecies genetic divergence between it and *C. simillimus*. Our results indicate that there is insufficient interspecific genetic differentiation between these species, with apparently different morphological characters. The *C. simillimus* mtCOI gene sequence from GenBank was highly consistent with that obtained in this study. Furthermore, less than 10 *C. simillimus* and *C. propinquus* specimens were used in our study, and the sampling scope did not cover the majority of their distribution area. Thus, we cannot conclude that they are the same species without further confirmation based on multiple molecular markers.

The genus *Calanus* has been intensely studied because of their ecological importance. Several disputes about species division of the genus have arisen. *C. helgolandicus* and *C. euxinus* are probably the same species, based on studies of the mtCOI gene[29] and 16S rRNA[30]. The status of *C. agulhensis* has also been questioned because of its genetic similarity to *C. sinicus*. Kozol et al. surveyed genetic differentiation among individuals of two species from different populations using mtCOI, 18S rRNA, and 28S rRNA[31], and found that genetic differentiation in these gene sequences was small. The results indicated that *C. agulhensis* and *C. sinicus* might be the same species. Therefore, low interspecific genetic differentiation between species of *Calanus* might be common. Our results bring into question the taxonomic status of *C. simillimus*. Further investigation, combining the divergence of various genes and life-history data, is required to confirm their status.

4.2 DNA barcodes, useful tools for identifying Antarctic zooplankton

The intraspecific genetic divergence of Antarctic zooplankton was remarkably smaller than the interspecific genetic divergence, with the exception of *C. simillimus* and *C. propinquus*. The barcode gaps were evident. The results indicate that using mtCOI gene sequences as DNA barcodes to interpret species composition in Antarctica is feasible. Similar studies have been published in the Arctic[15], in Jiaozhou Bay of China[13], and in warm temperate oceans[21]. Sibling species can also be distinguished using mtCOI gene sequences as markers. In this study, two pairs of zooplankton, *Themisto antarctica* and *T. gaudichaudii*, *Alacia belgicae* and *A. hettacra*, were identified by mtCOI. These sibling species are often not accurately identified in conventional surveys[27,32].

Zooplankton species diversity information can be acquired through DNA barcoding methods without considering morphological characteristics. These methods could make studies on the identification of larvae and stomach contents possible[9,17]. Although the background database was not complete and a lot of larval species could not be identified, the accuracy was still substantially improved compared with traditional morphological methods. For example, Janosik et al. acquired further knowledge on the larval history of *Labidiaster annulatus* through DNA barcoding methods[33]. Studies aimed at determining Antarctic zooplankton diets have been carried out with the help of DNA barcoding techniques. Tobe et al. explored the prey component of *Euphausia superba* using a molecular probe, and identified the genus *Oithona* (copepod) as being the main *E. superba* food source[34]. With an increase in DNA barcode research focused on Antarctic zooplankton, the database will become more comprehensive, promoting the application of this technique in Antarctic zooplankton research[8]. Because of the popularization of high-throughput techniques, quick zooplankton detection methods based on metagenetic analysis has become practical[17].

In fact, the intraspecific genetic divergence of *Calanus propinquus/simillimus*, *Thysanoessa macrura*, *Metridia gerlachei*, and *Pleuromamma borealis* were all higher than 1.5% in our study. The intraspecific genetic divergence of *Pleuromamma borealis* revealed geographical differentiation. The biggest genetic divergence observed was between specimens sampled in westerly areas and the Prydz Bay. These results suggest that mtCOI divergence might provide information on intraspecific genetic divergence. The mtCOI gene could be used to explain the genetic divergence of zooplankton species in different Antarctic sea areas, which would contribute to the elucidation of zooplankton popula-

tion genetic structure produced by currents and sea ice. Work concentrating on zooplankton genetic divergence using mtCOI has moved forward in several taxa. For example, studies of *Euphausia superba* and *Orchomene sensu* indicate that gene flow was less than expected in the Antarctic area[35-36]. Genetic divergence of different populations and the emergence of sibling species might be common occurrences.

4.3 The new primers provide powerful tools for the metagenetic analysis of Antarctic zooplankton

The universal primers devised by Folmer greatly enhanced DNA barcode research[11]; nevertheless, they contain no degenerate bases and their efficiency varies significantly among taxa[12]. The mtCOI gene sequences of some species were not amplified[17]; bringing bias to the experimental results, thus leading to a loss of accuracy in zooplankton community analysis. In our preliminary experiment, only about 60% of the species produced distinctive bands, indicating that the Folmer primers underestimated species abundance. Because the divergence of different taxa was taken into consideration, the degenerate bases developed in our study were more efficient and the amplification success rate was higher. Metagenomic analysis is, appropriately, based on a single gene. Degenerate bases cannot be sequenced by conventional methods. They need to be sequenced after the construction of a library, which requires more time and labor. However, this high-throughput technique can circumvent this obstacle. Species abundance estimates based on the amount of DNA barcode genes, as an indicator of the amplification efficiency of the high-throughput technique, were more uniform and regular.

5 Conclusions

Based on abundant previous DNA barcoding studies involving mtCOI of marine zooplankton, it was concluded that barcoding gap existed in most zooplankton taxa. Expansion of the barcodes database of different sea areas will facilitate the identifying work, regardless of integrity and stages of the specimen. In some research fields, the barcoding approach will be the preferable methods. The barcodes acquired by our study should provide an alternative method to identify species living in Antarctic areas. Furthermore, it also can help to reveal sibling species and other taxonomic problems. Problems and main points are proposed by our results below, and should be emphasized in future:

(1) The common species, *C. propinquus* and *C. simillimus*, shared similar morphological characteristics, and the interspecies genetic difference between them was small. The results indicate that it is necessary to investigate whether or not *C. simillimus* is a distinct geographical population of *C. propinquus*, rather than a species in its own right. To do this, a combination of genomic information, morphological characteristics, and life-history data is necessary.

(2) Our study validates the use of the DNA barcoding technique in identifying Antarctic zooplankton. The high level of intraspecific genetic divergence in some species suggests that mtCOI gene sequences can be used as markers to interpret the population genetics of these species. The genetic differences in *Pleuromamma borealis* suggest that there was substantial divergence between populations from the polar and westerly sub-polar areas. Antarctic sea area fronts might be the barriers responsible for isolating zooplankton populations.

(3) The amplification efficiency of the new primers containing degenerate bases was more stable and had a higher success rate than those in the published literature. Used in high-throughput techniques, the new primers are powerful tools for metagenetic analysis on the basis of DNA barcodes.

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