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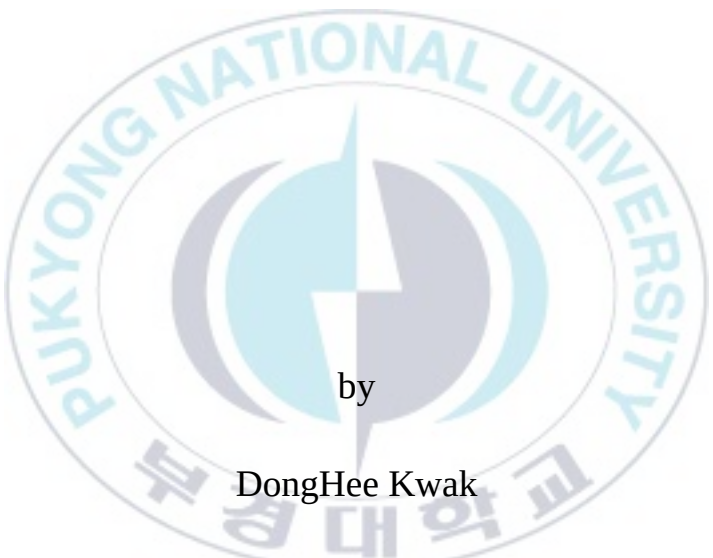
Thesis for the Degree of Master of Engineering

Design of Universal Primers and Diversity Analysis of Eucarid Species in Korean Waters



by

DongHee Kwak

Interdisciplinary program of Biomedical, Mechanical and Electrical Engineering,

The Graduate School

Pukyong National University

August 2018

Design of Universal Primers and Diversity Analysis of Eucarid Species in Korean Waters

(Eucarid 종들에 대한 유니버셜 프라이머 디자인 및 한국 해역에서의 다양성 분석)

Advisor: Prof. Hyun-Woo Kim

by

DongHee Kwak

A thesis submitted in partial fulfillment of the requirements
for the degree of

Master of Engineering

in Interdisciplinary program of Biomedical, Mechanical and Electrical Engineering,
The graduate School, Pukyong National University

August 2018

Design of Universal Primers and Diversity Analysis of Eucarid Species in Korean Waters

A dissertation

by

DongHee Kwak

Approved by :

(Chairman) Dr. Young-Mog Kim

(Member) Dr. Won-Gyu Park

(Member) Dr. Hyun-Woo Kim

August 24, 2018

PUKYONG NATIONAL UNIVERSITY 부경대학교

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Design of Universal Primers and Diversity Analysis of Eucarid Species in Korean Waters

DongHee Kwak

Interdisciplinary program of
Biomedical Mechanical & Electrical Engineering
The Graduate School, Pukyong National University

Abstract

Superorder Eucarida consists of three orders including Decapoda, Euphausiacea, and Amphionidacea and many of their species have a high economic value. Spatiotemporal distributions of their larvae provide the important ecological information such as reproduction or population, which is useful for the scientific management of their resources. However, it is hard to analyze them from the zooplankton net samples due to the difficulty in the morphological identification and their relatively smaller numbers in the zooplankton samples, which are often dominated by copepods. Here, we developed a universal primer set (EUC), which specifically amplify eucarid species from the mixture of the zooplankton sample. In addition, low amount of decapod species were also presented by blocking *Euphausia pacifica*. The reliability of EUC was tested by next generation sequencing (NGS) analysis of zooplankton net samples collected from Korean waters in 2016 using MiSeq system.

Eucarid 종들에 대한 유니버셜 프라이머 디자인 및 한국 해역에서의 다양성 분석

곽 동 희

부경대학교 대학원 의생명기계전기융합공학협동과정

요약

상목에 속하는 Eucarida 는 Decapoda, Euphausiacea, Amphionidacea 의 3 개의 목을 포함한다. Eucarida 에 속하는 종들은 대부분 높은 경제적 가치를 가진다. Eucarid 종 및 유생들에 대한 시공간적인 분포는 번식 및 개체군에 대한 중요한 생태학적 정보가 될 수 있으며, 이러한 정보는 체계적인 관리를 가능하게 한다. 그러나, 동물플랑크톤 내에서는 Copepoda 가 우점하며, Eucarida 는 적은 비율을 차지하는 특성과 Eucarid 종들에 대한 형태학적인 동정의 어려움 때문에 분석이 힘든 실정이다. 이 논문에서는 혼합된 동물플랑크톤 시료에서 Eucarid 종들을 특정적으로 증폭시키는 프라이머(EUC)를 개발하였다. 또한 낮은 비율을 차지하는 Decapod 종들은 *Euphausia pacifica* 에 대한 블로킹 프라이머를 사용하여 비율을 높였다. MiSeq 차세대 염기서열 분석기법을 통해 EUC 프라이머의 신뢰성을 검정 및 확인하였으며, 2016 년의 한국 해역 동물플랑크톤 시료들을 이용하여 시공간적 분포를 분석하였다.

INTRODUCTION

Superorder Eucarida consists of three orders including Decapoda, Euphausiacea, and Amphionidacea (Horton et al., 2018). Approximately 15,000 decapod crustacean species are currently known and Decapod species are divided into two suborders: Pleocyemata which include crayfish, crabs, lobsters, shrimp and Dendrobrachiata which include most prawns (Sammy De Grave, Pentcheff, & Ahyong, 2009). The production and value of species in Decapoda have increased every year, especially in Asia, reaching 6.5 million tons and $ 33 million in 2015 (FAO, 2017). Studies on the planktonic decapods are important to understand its relationship to their production and distribution. Euphausiacea known as krill occupies a considerably high biomass in the ocean and they are considered to mediate important trophic level connections (Atkinson, Siegel, Pakhomov, Jessopp, & Loeb, 2009; Doney et al., 2011; Teschke, Wendt, Kawaguchi, Kramer, & Meyer, 2011). From the reason, their spatio-temporal distributions have been survey by zooplankton net.

However, considering outnumbered copepods in the zooplankton net samples it has not been easy to study those taxa (Stanton & Chu, 2000). It is worse to study decapod larvae in the net samples for their low

numbers and difficulties to identify them to the species level (Lovrich, 1999). In the same vein, morphological identification of traditional taxonomists is time-consuming and complicated, so DNA barcoding can be the alternative method (Bucklin et al., 2010). In addition, the recent development of high-throughput sequencing (HTS) technologies has enabled the molecular barcoding of bio-monitoring to be more efficient because it processes large volumes of sequences at once. (Aylagas, Borja, Irigoien, & Rodríguez-Ezpeleta, 2016). Despite the promise of HTS, it has been hard to analyze the low numbered eucarid species using the typical universal primers designed for metazoan species, which shows high degree of cross-reactivity to other animal phyla especially to outnumbered copepods (Chain, Brown, MacIsaac, & Cristescu, 2016). For these reasons, we designed and tested specific metabarcoding primers for Eucarida and tried to show the spatiotemporal distribution of Eucarid species in Korean water net sample in 2016.

MATERIALS & METHODS

Designing Eucarida-specific universal primer

Eucarida-specific universal primers were designed based on 59 mitochondrial cytochrome oxidase subunit I (COI) nucleotides from GenBank database (https://www.ncbi.nlm.nih.gov/genbank), which include 37 complete COI from order Decapoda and 2 from order Euphausiacea (Table S1). In order to exclude the non-target crustaceans, 5 Hexanauplia, 1 Ostracoda, 1 Branchiopoda, 1 Cephalocarida, 1 Remipedia, and 8 other non-crustacean species were aligned together (Table S1). The conserved region for degenerated universal primers for Eucarida were identified by the multiple alignment using ClustalW of MEGA software (Kumar, Stecher, & Tamura, 2016). Due to its high degree of degeneracy in the conserved region, primers were designed to conduct nested PCR strategy (Fig. 1). Integrity and quality of primers were analyzed by OligoAnalyzer® Tool, which is the web-based program (https://sg.idtdna.com/calc/analyzer). The expected PCR product generated by the designed eucarida-specific universal primer set would be

355 bp in length (Fig. 1A, Table 1.).

In order to suppress second highest amount of euphausid sequences from zooplankton net sample DNA mixture, its blocking primer (Eupbl) was designed (Fig. 1B). Eupbl primer was modified to compete to Eup F2 to suppress euphausid sequences. For the second nested PCR, we used half of universal primer, added 1 μl of blocking primer Eupbl 100 μM and performed PCR under the same conditions as described above.

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Table 1. Primer used in this study

Primer label	Sequence (5' - 3')
EucF1	ACNGTNTAYCCNCCHYTNNCNG
EucF2	TTCTTTTTTNCCNGAYTCYTG
EucR1	GGDSCHTCNGTDGAYHTNGG
EucR2	ATRTGDGARAYTATNCCRAADG
EucF2nex	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTCTTTTTTNCCNGAYTCYTG
EucR2nex	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATRTGDGARAYTATNCCRAADG
Eupbl	ATAGGAATCTTTTCACTACATA-(C3)

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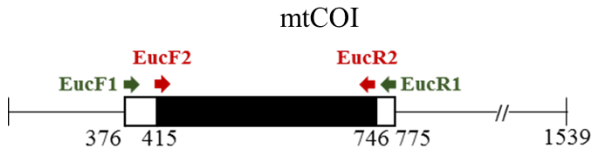
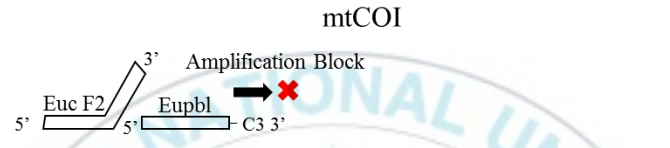
(A)



Eucarida-specific universal (Euc) primers (355 bp)

(B)



***Euphausia pacifica* blocking primer**

Fig. 1. Illustrative diagram for Eucarida-specific universal primers
(A) The Euc nested primer target region in COI (B) *Euphausia pacifica* specific primer after Euc F2 tailed with 3'-spacer C3

Sample collection and DNA extraction

The zooplankton samples were collected with the bongo net (333 μm in mesh size) as a part of regular annual survey by National Fisheries Research Institute (NIFS) in 2016. Samples were collected four times (February, April, August, and November) from all three waters of Korean peninsula including East Sea (E1-E8), South Sea (S1-S8), and Yellow Sea (W1-W8) (Figure 2 and Table. S2). The zooplankton net samples were stored in 95 % Ethanol (SK chemicals, Republic of Korea) immediately after collection. After bring them to the laboratory, samples were filtered with a 200 μm sieve and rehydrated with tap water. The wet weight of each sample was measured and its 6 times volume of lysis buffer (Bioneer, Republic of Korea) was added. After homogenizing with Tissuelyzer II (Qiagen Korea, Republic of Korea), samples were stored at -80 ℃ before DNA extraction. DNA purification was conducted according to the protocol of AccuPrep® Genomic DNA Extraction Kit (Bioneer, Republic of Korea). The extracted DNA was stored at -80 ℃ after qualification and quantification by NanoDrop spectrophotometer ND-1000 (Thermoscientific, Waltham, USA) (T.-H. Yoon et al., 2017).

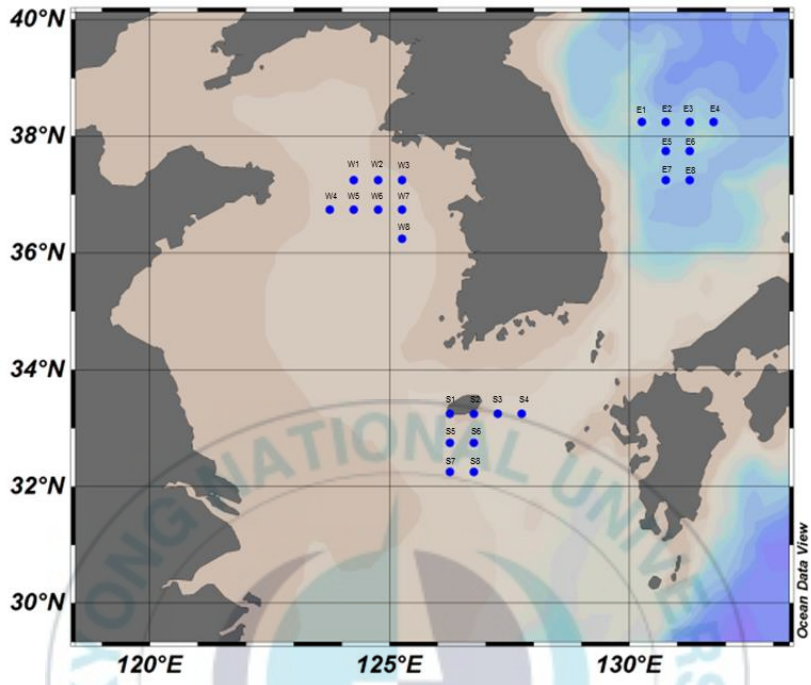


Figure 2. Sample stations in this study

Sample collection locations were drawn by Ocean Data View (ODV). Eight stations were chosen from each region of three Korean waters.

Library construction and MiSeq sequencing

Primary PCR mixture (20 μl) was composed of 1 μl of EucF1 and EucR1 primer (100 μM), template DNA 100 ng, dNTP mix (2.5 mM each) 2 μl, 10x Ex Taq buffer, Ex Taq hot start 0.2 μl (Takara Bio inc., Japan), Dimethyl sulfoxide (DMSO) 0.6 μl (Wako, Japan), and DNase/RNase free water adjusting to final volume (GeneAll, Republic of Korea). PCR conditions begin with the early denaturation 3 m at 94 ℃ followed by 12 cycles of denaturation for 30 sec at 94 ℃, annealing for 30 sec at 45 ℃, and extension for 30s at 72 ℃, and the final extension for 3 min at 72 ℃. The amplified PCR products with expected size (350-450 bp) were separated by 1.5% agarose gel electrophoresis and purified using AccuPrep Gel Extration Kit (Bioneer, Republic of Korea). A Nextera adapter sequence was included in EucF2nex, EucR2nex for application to Miseq Sequencing (2018 Illumina, Inc) (Table 1.). The nested PCR was performed as a triplicate PCR mixtures which were same to those of first PCR mixture except for 4 μl of purified PCR products as template and the use of blocking primer. PCR conditions begin with the early denaturation 3 m at 94 ℃ followed by 23 cycles of denaturation for 30 sec at 94 ℃, annealing for 30 sec at 45 ℃, and extension for 30s at 72 ℃, and the final

extension for 3 min at 72 ℃. The amplified nested PCR products (approximately 439 bp) were purified and used for the indexing PCR for sequencing. The 3rd Index PCR was performed in triplicate as following composition, template 4 μl, 10 μM Nextera index forward and reverse each 1 μl, dNTP mix (10 mM each) 0.5 μl, 5X Phusion HF Reaction Buffer, Phusion Hot Start Flex DNA polymerase (New England Biolabs, UK), DNase/RNase free water up to 20 μl. The conditions are as follows : Early denaturation 3 m at 94 ℃, 15 cycles : denaturation 30 s at 94 ℃, annealing 30s at 55 ℃, extension 30s at 72 ℃, and late extension 3m at 72 ℃ for enough amplification time. The final library amplicon was purified with the AccuPrep® Gel Extration Kit. The library concentration was measured by Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA) followed by MiSeq sequencing at a concentration of 2 μM. Finally, constructed libraries were loaded with a MiSeq Reagent Kit v3 (600-cycle) (Illumina, SanDiego, CA, USA) to perform 300-bp paired-end sequencing on a MiSeq instrument.

Bioinformatic analysis

Sequences with low quality (under QV 20 and below 100 bp) were eliminated from further analysis using CLC Genomic Workbench V.8.0 (CLC Bio, USA). Primer removal and merging reads were made by Mothur software (Schloss et al., 2009). OTUs were constructed by *de-novo* clustering with 97 % and 99.6 % identity using UPARSE (Edgar, 2013).

Taxonomic assignment was performed using Basic Local Alignment Search Tool (BLASTn, https://blast.ncbi.nlm.nih.gov). Malacostraca mitochondrial DNA from Ref-Seq was used as reference database. OTUs with sequence identity of 97% or more were assigned to species, and sequences of 90 to 97% were assigned to genus sp. Sequences clustering at 99.6% were numbered according to the OTU ratio of the assigned species, and OTUs with less than 10% were removed to construct the representative OTU database. 97% clutered OTUs were assigned with Representative OTU database and the assigned sequences were marked as stadard OTUs and denoted as genus_species_genotype_identity. OTUs with fewer than 9 sequence leads were excluded from the analysis. OTUs with an identity of less than 99% when matched with a representative

database are marked as unidentified. OTU table was created based on representative OTU database using USEARCH and imported into QIIME 2 (Caporaso et al., 2010) for alpha and beta diversity analysis.

RESULTS

Evaluation of eucarida-specific universal Primers

In order to evaluate the newly designed eucarida-specific universal primers, we compared NGS data between commonly used metazoan universal primer, COIMISQ and Euc primers (Table 2). We obtained 57,638 reads from COIMISQ, 327,566 reads from Euc primers, and 416,913 reads from application of blocking primer (Table S3). Although only 14 OTUs were obtained by COIMISQ, 38 OTUs were generated by Euc primer and 62 OTUs were found by Euc primers with the blocking primer, Eupbl (Table 2). In COIMISQ, 65.85 % of total reads accounted for copepod species including *Calanus sinicus* (60.02 %), *Centropages abdominalis* (5.83 %) whereas, only 1.21 % were eucarid species including *Euphausia pacifica* (1.06 %), *Oregonia gracilis* (0.1 %) and *Crangon sp.* (0.05 %). By contrast, in Euc primers, *Oregonia gracilis* accounted for 48.03%, followed by *Euphausia pacifica* by 46.80%. However, except for the two species, the proportion of other species is generally less than 2%. When Eupbl was applied, blocking primer target species, *Euphausia pacifica* significantly decreased by 38.62%, while the

other decapod species, *Oregonia gracilis* increased by 26.91 %, *Hyas sp.* by 3.93 % and *Oregonia sp.* by 2.43 %. Overall, the rank abundance of species except *Euphausia pacifica* increased. In the case of using the blocking primer, 17 more species were found than those not used, including *Pagurus ochotensis*, *Chionoecetes japonicas* and *Chionoecetes opilio*. Compared with the metazoan primers, the Euc primer products showed no cross reactivity with other taxa of Eucarida. However, The total OTUs obtained using the COIMISQ primer showed a low amount, which could underestimate the amplification pattern of COIMISQ. In addition, the large number of the singleton sequence leads were removed from unique sequences during sequence processing.

Table 2. Comparson with Metazoan universal primer COIMISQ and Euc primer designed in present study
Assigned species, species proportions, identity to BLASTn query are listed. The OTUs under 90% identitiy are named as *Unidentified_Species*.

COIMISQ (Yoon et al. 2017)				Euc (present study)				Eupbl (present study)			
Species	Sequnece reads	Species proportion	Identity	Species	Sequnece reads	Species proportion	Identity	Species	Sequnece reads	Species proportion	Identity
Calanus sinicus	2325	60.02%	100%	*Oregonia gracilis*	43785	48.03%	99%	*Oregonia gracilis*	78746	74.94%	99%
Invertebrate environmental sample	848	21.89%	97%	*Euphausia pacifica*	42667	46.80%	100%	*Euphausia pacifica*	8593	8.18%	100%
Engraulis japonicus	353	9.11%	100%	*Hyas sp.*	1643	1.80%	90%	*Hyas sp.*	6023	5.73%	90%
Centropages abdominalis	226	5.83%	100%	*Oregonia sp.*	1325	1.45%	96%	*Oregonia sp.*	4078	3.88%	96%
Hysterothylacium aduncum	57	1.47%	98%	*Unidentified_Charybdis natator*	923	1.01%	84%	*Unidentified_Charybdis natator*	3725	3.55%	84%
Euphausia pacifica	41	1.06%	99%	*Crangon sp.*	325	0.36%	91%	*Crangon sp.*	1539	1.47%	91%
Delftia sp.	6	0.15%	98%	*Unidentified_Lucilia ampullaceal*	165	0.18%	83%	*Unidentified_Lucilia ampullacea*	739	0.70%	83%
Oregonia gracilis	4	0.10%	98%	*Unidentified_Rutilia goerlingiana*	73	0.08%	84%	*Unidentified_Upogebia yokoyai*	399	0.38%	85%
Unidentified_Hippocampus jayakari	3	0.08%	89%	*Unidentified_Pseudoseioptera demonstrans*	58	0.06%	84%	*Unidentified_Rutilia goerlingiana*	305	0.29%	84%
Unidentified_Sphingobium chlorophenolicum	3	0.08%	80%	*Unidentified_Lysianasoidea 36*	33	0.04%	85%	*Unidentified_Lysianasoidea 36*	178	0.17%	85%
Unidentified_Polychaeta nb-po509	2	0.05%	82%	*Lucifer intermedius*	28	0.03%	99%	*Lucifer intermedius*	120	0.11%	99%
Crangon sp.	2	0.05%	91%	*Unidentified_Eualus avinus*	25	0.03%	86%	*Unidentified_Nematobrachion sexspinosum*	111	0.11%	85%
Sagitta sp.	2	0.05%	96%	*Unidentified_Anopheles cruzii*	22	0.02%	86%	*Unidentified_Anopheles cruzii*	69	0.07%	86%
Sphingomonas sp.	2	0.05%	91%	*Unidentified_Nematobrachion sexspinosum*	18	0.02%	85%	*Unidentified_Anopheles interruptus*	67	0.06%	84%
				Unidentified_Eualus barbatus	16	0.02%	87%	*Unidentified_Mycodrosophila neoprojectans*	66	0.06%	84%
				Unidentified_Atya scabra	12	0.01%	83%	*Unidentified_Eualus avinus*	58	0.06%	86%
				Unidentified_Anopheles interruptus	11	0.01%	84%	*Unidentified_Eualus barbatus*	49	0.05%	87%
				Unidentified_Damithrax spinosissimus	10	0.01%	83%	*Unidentified_Monoplistes nlg-2014*	25	0.02%	84%
				Unidentified_Drosophila bipatita	6	0.01%	84%	*Unidentified_Cancer jordani*	22	0.02%	86%
				Unidentified_Mycodrosophila neoprojectans	5	0.01%	84%	*Pagurus ochotensis*	19	0.02%	99%
				Unidentified_Anopheles laneanus	2	0.00%	83%	*Unidentified_Drosophila bipatita*	16	0.02%	84%
				Unidentified_Aegla platensis	2	0.00%	81%	*Unidentified_Damithrax spinosissimus*	16	0.02%	83%
				Pagurus brachiomastus	2	0.00%	99%	*Lucifer sp.*	14	0.01%	96%
				Euphausia sp.	2	0.00%	95%	*Unidentified_Lepidoptera bold:aaf7489*	10	0.01%	83%
				Unidentified_Crangon crangon	2	0.00%	89%	*Unidentified_Longpotamon anyuanense*	10	0.01%	85%
				Unidentified_Cancer jordani	2	0.00%	86%	*Chionoecetes opilio*	9	0.01%	100%
				Unidentified_Aegla uruguayana	2	0.00%	84%	*Euphausia sp.*	8	0.01%	92%
				Unidentified_Simulium burtoni	2	0.00%	83%	*Pagurus brachiomastus*	7	0.01%	99%
				Unidentified_Bactrocera cucurbitae	2	0.00%	81%	*Chionoecetes japonicas*	7	0.01%	100%
								Unidentified_Anopheles laneanus	7	0.01%	83%
								Unidentified_Sagmariasus verreauxi	6	0.01%	83%
								Unidentified_Atya scabra	5	0.01%	83%
								Unidentified_Rhabdamia cypselura	5	0.01%	78%
								Invertebrate sp.	4	0.00%	91%
								Unidentified_Bulinus globosus	3	0.00%	85%
								Unidentified_Nebria spatulata	3	0.00%	83%
								Unidentified_Mithrax hispidus	3	0.00%	77%
								Unidentified_Oedignathus inermis	3	0.00%	89%
								Unidentified_Panorpodes paradoxus	3	0.00%	82%
								Unidentified_Pagurus bernhardus	2	0.00%	89%
								Unidentified_Eualus macilentus	2	0.00%	87%
Total	3874	100%			91168	100%			105074	100%	

Spatio-temporal distribution in Korean waters

A standard OTU database was constructed from the total sequence data (Table 3). Genus or species names were assigned to 72 out of 125 OTUs, which showed higher than 90 % identity to the database. Fifty three OTUs were assigned as 'unidentified' species, which showed less than 90 % identity (Table 3). The spatio-temporal distributions of OTUs assigned to genus and species level are shown with proportions (Fig. 3). In case of Euphausiacea, *Euphausia pacifica* type 3 occupied 26.12 % of those from April of the South Sea, and *E. pacifica* type 7 showed the highest (39.72 %) in February of the East Sea and decreased by August and bounced again to 8.65 % in November of the South Sea, showed comparatively the low proportion and in the West sea, showed the low proportion 4.373% in February, increased to 33.138% in April, decreased in August, and increased again to the high proportion 77.151% in November. *Euphausia tenera* type 1 showed 1.101% in November of the South Sea. *Euphausia sp.* type 1 showed the more appears in February and April of the South Sea, and type 2 showed the tendency to appear in November of the South Sea. *Nematoscelis atlantica* type 1 was 1.265%, *Nematoscelis sp.* Type 1 was 3.486% in the same in April of South Sea and *Pseudoeuphausia sinica*

type 1 was 1.235% in February. *Stylocheiron affine* type 2 was 4.258% in November of the East Sea. *Thysanoessa gracilis* type 1 appeared in November of the East sea, and *Thysanoessa longipes* type 1 remained low from 2.402% in February to 62.742% in April and decreased in August and recovered 9.077% in November.

In the case of Pleocyemata of Decapoda, individual species did not occupy a large proportion except for *Eualus biunguis* type 1 in February of the East Sea, which was high as 55.03 %. *Charybdis bimaculata* type 1 is more distributed in the South Sea, and *Crangon hakodatei* seems to be distributed in the whole regions. In case of Dendrobranchiata, *Lucifer intermedius* type 1 occupied 7.62 % in August of the East Sea and *Metapenaeopsis dalei* type 1 was 4.45 % and 6.34 % in August of the East Sea and the West Sea. *Parapenaeus lanceolatus* type 1 appeared 1.364% in April of the South Sea, and *Pleoticus robustus* type 1 showed 2.07 % in November of South Sea. Overall, the proportions of unidentified species were high.

Table 3. Standard OTU profiles of spatio-temporal analysis

The sequences assigned over 90% identity listed on the left, under 90% identity listed on the right side.

OTU	Assigned species	Type	Identity	Unidentified_OTU	Assigned species	Type	Identity
OTU1	*Acetes japonicus*	1	98%	Unidentified1	*Aegla uruguayana*	1	84%
OTU2	*Acetes japonicus*	2	98%	Unidentified2	*Alpheus sp.*	1	86%
OTU3	*Alpheus japonicus*	1	100%	Unidentified3	*Alpheus distinguendus*	1	82%
OTU4	*Alpheus japonicus*	2	98%	Unidentified4	*Alpheus formosus*	1	85%
OTU5	*Alpheus sp.*	1	92%	Unidentified5	*Alpheus lobidens*	1	83%
OTU6	*Alpheus sp.*	2	91%	Unidentified6	*Atya innocous*	1	80%
OTU7	*Calappa sp.*	1	90%	Unidentified7	*Atya scabra*	1	82%
OTU8	*Charybdis bimaculata*	1	100%	Unidentified8	*Austinixa chacei*	1	82%
OTU9	*Chionoecetes japonicus*	1	100%	Unidentified9	*Charybdis natator*	1	84%
OTU10	*Chionoecetes opilio*	1	100%	Unidentified10	*Charybdis natator*	2	84%
OTU11	*Crangon hakodatei*	1	100%	Unidentified11	*Eualus avinus*	1	86%
OTU12	*Eualus biunguis*	1	99%	Unidentified12	*Eualus avinus*	2	88%
OTU13	*Eualus sp.*	1	95%	Unidentified13	*Eualus avinus*	3	87%
OTU14	*Euphausia mutica*	1	97%	Unidentified14	*Eualus macilentus*	1	87%
OTU15	*Euphausia pacifica*	1	99%	Unidentified15	*Euphausia pacifica*	1	89%
OTU16	*Euphausia pacifica*	2	98%	Unidentified16	*Euphausia pacifica*	2	89%
OTU17	*Euphausia pacifica*	3	99%	Unidentified17	*Fenneropenaeus penicillatus*	1	82%
OTU18	*Euphausia pacifica*	4	99%	Unidentified18	*Harpiosquilla harpax*	1	86%
OTU19	*Euphausia pacifica*	5	99%	Unidentified19	*Hemigrapsus takanoi*	1	79%
OTU20	*Euphausia pacifica*	6	99%	Unidentified20	*Heterocarpus gibbosus*	1	81%
OTU21	*Euphausia pacifica*	7	100%	Unidentified21	*Iberobathynella parasturiensis*	1	81%
OTU22	*Euphausia pacifica*	8	100%	Unidentified22	*Longpotamon anyuanense*	1	83%
OTU23	*Euphausia pacifica*	9	99%	Unidentified23	*Longpotamon anyuanense*	2	84%
OTU24	*Euphausia pacifica*	10	99%	Unidentified24	*Longpotamon depressum*	1	84%
OTU25	*Euphausia pacifica*	11	99%	Unidentified25	*Macrobrachium faustinum*	1	82%
OTU26	*Euphausia pacifica*	12	99%	Unidentified26	*Melicertus latisulcatus*	1	88%
OTU27	*Euphausia pacifica*	13	99%	Unidentified27	*Metopaulias depressus*	1	81%
OTU28	*Euphausia sp.*	1	94%	Unidentified28	*Munida gregaria*	1	84%
OTU29	*Euphausia sp.*	2	95%	Unidentified29	*Munida taenia*	1	84%
OTU30	*Euphausia sp.*	3	92%	Unidentified30	*Neoglyphea inopinata*	1	83%
OTU31	*Euphausia sp.*	4	93%	Unidentified31	*Neostylodactylus amarynthis*	1	89%
OTU32	*Euphausia sp.*	5	94%	Unidentified32	*Penaeus monodon*	1	82%
OTU33	*Euphausia sp.*	6	96%	Unidentified33	*Penaeus notialis*	1	80%
OTU34	*Euphausia tenera*	1	99%	Unidentified34	*Processa nouveli*	1	89%
OTU35	*Euphausia tenera*	2	97%	Unidentified35	*Processa nouveli*	2	89%
OTU36	*Gaetice depressus*	1	100%	Unidentified36	*Pseudeuphausia sinica*	1	86%
OTU37	*Idotea metallica*	1	98%	Unidentified37	*Pseudeuphausia sinica*	2	87%
OTU38	*Iiella pelagica*	1	99%	Unidentified38	*Rhithropanopeus harrisii*	1	82%
OTU39	*Lucifer dnz105*	1	99%	Unidentified39	*Sadayoshia savali*	1	84%
OTU40	*Lucifer intermedius*	1	99%	Unidentified40	*Scyra acutifrons*	1	82%
OTU41	*Lucifer sp.*	1	96%	Unidentified41	*Scyra acutifrons*	2	82%
OTU42	*Lucifer sp.*	2	95%	Unidentified42	*Sinopotamon sp.*	1	84%
OTU43	*Lucifer typus*	1	98%	Unidentified43	*Stylocheiron carinatum*	1	88%
OTU44	*Metapenaeopsis dalei*	1	100%	Unidentified44	*Thenus orientalis*	1	83%
OTU45	*Nematoscelis atlantica*	1	98%	Unidentified45	*Thysanoessa macrura*	1	82%
OTU46	*Nematoscelis atlantica*	2	97%	Unidentified46	*Trichoniscus pusillus*	1	82%
OTU47	*Nematoscelis gracilis*	1	97%	Unidentified47	*Uca rapax*	1	83%
OTU48	*Nematoscelis sp.*	1	94%	Unidentified48	*Upogebia major*	1	85%
OTU49	*Oratosquilla sp.*	1	93%	Unidentified49	*Upogebia major*	2	87%
OTU50	*Oratosquilla sp.*	2	94%	Unidentified50	*Upogebia paraffinis*	1	84%
OTU51	*Oregonia gracilis*	1	99%	Unidentified51	*Upogebia yokoyai*	1	83%
OTU52	*Parapenaeus lanceolatus*	1	99%	Unidentified52	*Upogebia yokoyai*	2	84%
OTU53	*Parapenaeus lanceolatus*	2	98%	Unidentified53	*Xiphocaris elongata*	1	80%
OTU54	*Parapenaeus sp.*	1	95%				
OTU55	*Pleoticus robustus*	1	100%				
OTU56	*Plesionika sp.*	1	90%				
OTU57	*Plesionika sp.*	2	90%				
OTU58	*Pseudeuphausia sinica*	1	99%				
OTU59	*Pseudeuphausia sinica*	2	98%				
OTU60	*Pseudeuphausia sp.*	1	95%				
OTU61	*Squilloides leptosquilla*	1	98%				
OTU62	*Stylocheiron affine*	1	100%				
OTU63	*Stylocheiron affine*	2	99%				
OTU64	*Stylocheiron sp.*	1	92%				
OTU65	*Stylocheiron sp.*	2	93%				
OTU66	*Stylocheiron suhmii*	1	99%				
OTU67	*Stylocheiron suhmii*	2	98%				
OTU68	*Thysanoessa gregaria*	1	98%				
OTU69	*Thysanoessa longipes*	1	100%				
OTU70	*Thysanoessa sp.*	1	96%				
OTU71	*Thysanopoda aequalis*	1	99%				
OTU72	*Thysanopoda sp.*	1	93%				

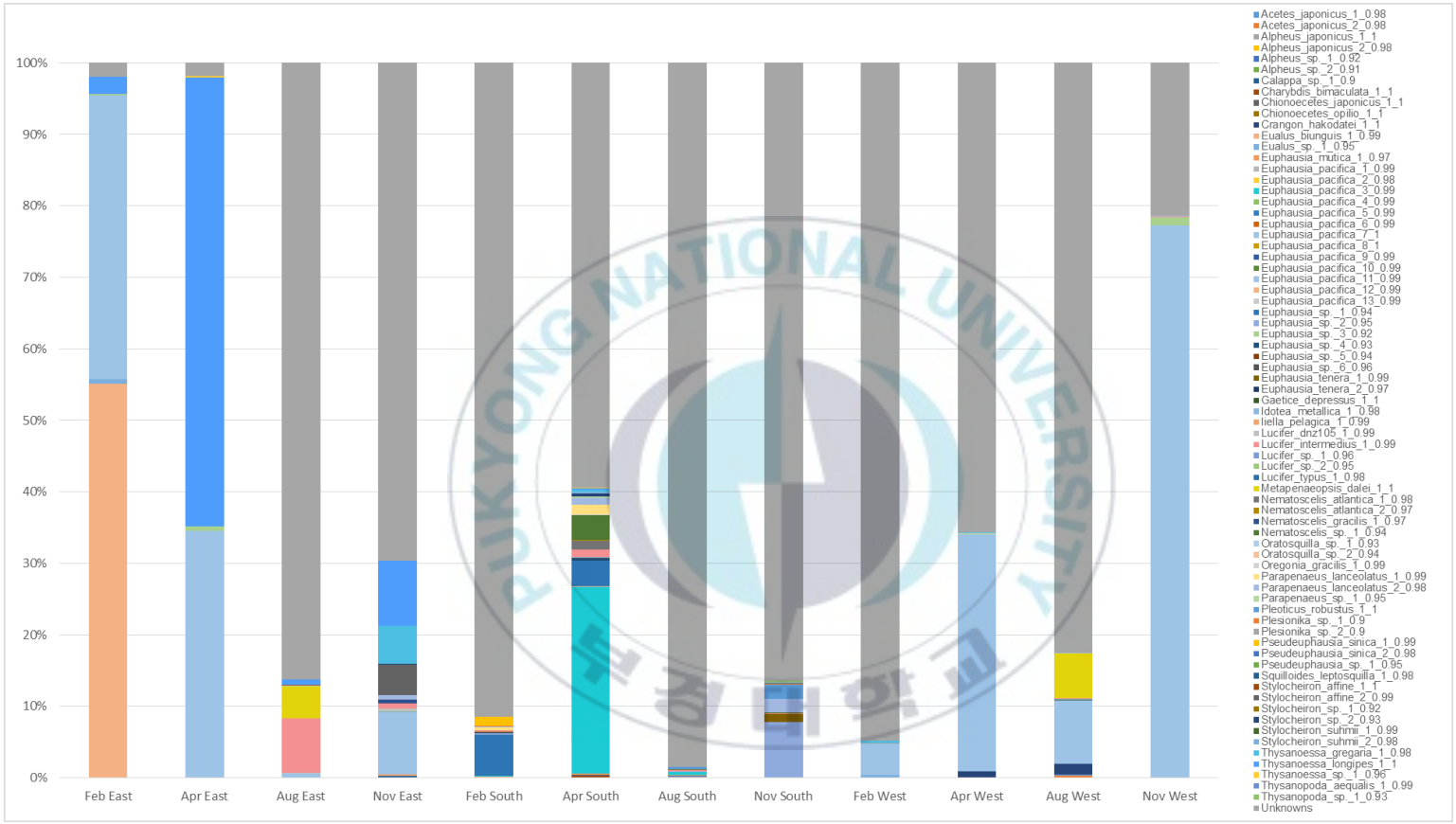


Fig 3. Spatio-temporal distridution of Eucarid species.
OTU level distributions In February, April, August, and November of 2016.

Alpha and beta diversity were analyzed to see the overall biodiversity of the samples (Fig. 5, Table 4). Alpha diversity represented by Shannon index which showed a biological diversity and the evenness of Pielou which measure how OTU proportions evenly distributed within a sample sites. Both Shannon index and Pielou's evenness index were lowest in April of the East Sea, but highest in August of the East Sea. In the South Sea, two indices the lowest in August, but the highest in April and The lowest in February of the West Sea, and the highest in August of the West Sea. The South Sea and the West Sea were significantly different in species evenness, but the others did not show any significant difference. The Jaccard dissimilarity, which represents a qualitative difference for the OTUs between samples, was shown using PCoA. (Fig. 5b, c) When analyzed regionally (Fig. 5b), there was a tendency to form clusters separated from the West Sea, the East Sea, and the South Sea from left to right, based on Axis 1. Seasonally, it seems that the clusters were formed from August to November, April and February based on Axis 2, but they were not clear.

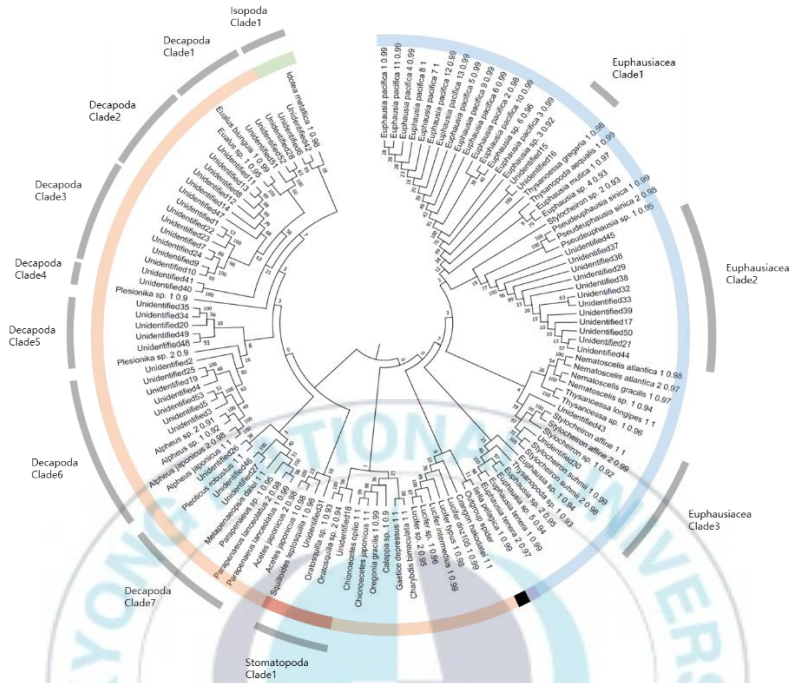


Fig 4. Phylogenetic tree of standard OTUs including clade assignments.

Unidentified species under 90% identity are merged as order clades. Inner circle represents the orders inferred from branches. Outer arcs means order clades divided by branch clusters.

Table 4. Alpha diversity indices.
Shannon's diversity index, Pielou's Evenness were measured.

	East				South				West			
	Feb	Apr	Aug	Nov	Feb	Apr	Aug	Nov	Feb	Apr	Aug	Nov
shannon	1.311	1.083	1.753	1.607	1.947	2.867	1.528	2.139	0.667	1.350	1.528	1.138
pielou_e	0.303	0.251	0.406	0.324	0.389	0.546	0.308	0.408	0.150	0.303	0.314	0.237

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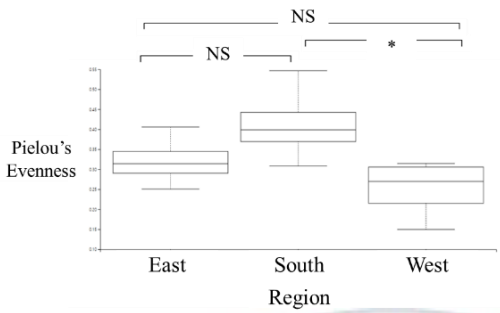
a.



b.

c.

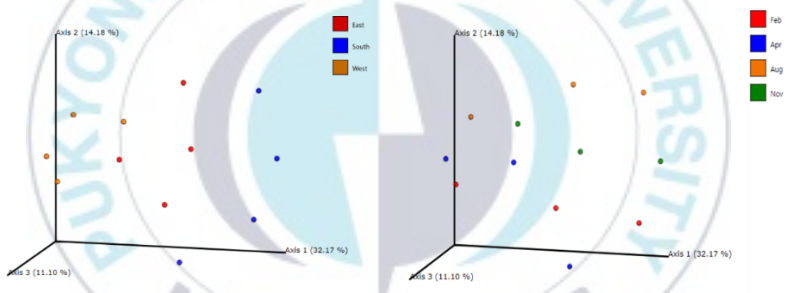


Fig. 5 Kruskal-Wallis one-way ANOVA box plot of Evenness group alpha-diversity and Jaccard beta-diversity (a) Regional evenness. $P \leq 0.05$: * (b) PCoA plot of Jaccard indices labelled with regions (c) PCoA plot of Jaccard indices labelled with seasons.

DISCUSSION

Assessments of designed primers

Recently, COI universal primers specific to marine metazoan have been developed which can implement the metabarcoding analysis (T.-H. Yoon et al., 2017), but there is a limitation to inspect the biodiversity of numerous eucarid species. Alternatively, Eucarida COI universal primer designed here could detect eucarid species more intensively. The distributions of zooplankton in the Korean waters were known as highest in Copepoda, following Chaetognatha, Cladocera, fish larvae, and Euphausiacea (An-Thanh, Jeong-Hoon, Jung-Wha, Won-Gyu, & Ki-Won, 2017; W. D. Yoon, Cho, Lim, Choi, & Lee, 2000). The Euc primers did not show the cross reactivity with other abundant planktons including outnumbered copepods in the zooplankton net. The samples amplified by Euc primers tended to occupy the higher proportion of *E. pacifica* (Table 2), which is likely to reflect the biomass actually present in the Korean waters.

The newly designed primers contained large amounts of ambiguous nucleotides, because they were intended to maximally reflect

complementarity with the priming region of target species. This can cause the possibility that the PCR biases arise (Polz & Cavanaugh, 1998). To solve this problem, it is necessary to study the primer bias for each composition of nucleotides using quantitative PCR and optimize the PCR condition for minimizing bias (Aird et al., 2011). Nowadays, the methods using universal primers with blocking primer which prevent the amplification of a specifically dominated species have been developed to increase the diversity of hidden taxa (Tan & Liu, 2018). By the use of blocking primer, we could increased the level of Decapoda proportions and diversities which were obscured by dominant species *E. pacifica* (Table. 2). We decided to employ the blocking primer to investigate eucarid species diversity in Korean waters.

Spatio-temporal distribution of eucarid species in Korean waters

According to the studies on Euphausiid in Korea, it was known that there are a large number of *Euphausia pacifica* in the East Sea and the West Sea (Im & Suh, 2016; W. D. Yoon et al., 2000). It is also indicated that there are a large number of *Thysanoessa longipes* in the East Sea and *Stylocheiron affine* in the southern sea (Hong, 1969). *Nematoscelis atlantica* and *Nematoscelis gracilis* also appeared in areas including the Sea of Japan (Taki, 2007). As a result of metacarcoding, *Euphausia pacifica* type 7 was a dominant genotype in the East Sea and the in the West Sea and another dominant genotype, *Euphausia pacifica* type 3 was found in April of the South Sea. *Pseudeuphausia sinica* was found in the southern and western seas, consistent with other distribution data (Horton et al., 2018).

In case of decapod species, *Acetes japonicas* and *Alpheus japonicas* appeared at the same time in the West Sea and the South Sea. There is a study that *Eualus Biunguis* appears in the East Sea (Chun et al., 2011; Koh, An, Baeck, & Jang, 2014). The larvae of *Charybdis bimaculata* and

Crangon hakodatei are distributed all over the Korean Water (Kim & Kim, 2016). In case of *Chionoecetes japonicus* and *Chionoecetes opilio*, there is a tendency similar to the timing of emergence of the larvae mentioned in (Lee & Park, 2012). Lucifer species distributed at high seawater temperatures (Xu, 2010) have been studied in the South Sea (Park, Ma, Hong, & Lee, 2009), and these can be seen mainly in August. The study about *Metapenaeopsis dalei* indicated that there are larvae in the summer of the west sea, and our data also appeared in August of the East Sea. There is information that the genus *Parapenaeus* is distributed in the West Sea and the South Sea. In the above data, species including *Parapenaeus lanceolatus* are mainly distributed in the South Sea, and small amounts are also found in the East Sea and the West Sea. *Plesionika* and *Pleoticus* are distributed in the South Sea (Shrimps of the Korean Waters, NFRDI).

Despite the use of blocking primer and the risk of unproven PCR bias, the patterns of contig number changes of eucarid species which respond to spatio-temporal conditions in Korean waters were corresponded with the preliminary studies. The metabarcoding analysis using the primers developed in this study suggests a potential for a quantitative approach. The diversity indices used here represent qualitative analysis. In the alpha diversity analysis, the community

richness and community evenness of the South Sea are the highest, which means the ecosystem of the South Sea have more diverse species and evenly distributed characteristics than other seas. As shown in Fig. 5, in the axis 1, it appears in the order of the West Sea, the East Sea and the South Sea. In the West Sea, there is more clustered pattern than others and the East Sea and the South Sea are more dispersed. This shows that the West Sea showed simple biodiversity and the South sea represents a dynamic biodiversity correspond with alpha diversity (Table 4). Diversity analysis shows that Eucarid species communities are tend to be more effected by regional differences than seasonal differences.

Unidentified groups with an identity of less than 90% tend to occupy the large portions in most of regions and seasons. In particular, unidentified 26 had a high ratio of 36.44% in August, while unidentified 46 had a high ratio of 4.47% in February and 31.3% in April. These belong to Decapoda clade 7, which is presumed to be a clade of Dendrobranchiata. It can be assumed that most of these unidentified OTUs are the eucarid species that have not yet been identified in the sequences (Lindeque, Parry, Harmer, Somerfield, & Atkinson, 2013). Compared with the Folmer region (Vrijenhoek, 1994), which is well known as the invertebrate COI barcode region, the primers prepared here were about 100 bp longer than

Folmer's. To examine whether the surplus region change the sequence identities, the rear part was trimmed using the COI primer for metabarcodes, jgHCO2198 (Leray et al., 2013). However, These trials did not increase the identity of the OTUs (data not shown). Therefore, the unidentified species in this study can be predicted by the lack of sequencing data of the target, Eucarida. This also suggested that there were a large parts of hidden biodiversity of unidentified species of eucarid species, especially Decapoda, in Korean waters. The phylogenetic tree shown in Fig. 4 was drawn with similar relationship patterns to Eumalacostraca COI Phylogeny (Jenner, Dhubhghaill, Ferla, & Wills, 2009), indicating that Eucarida and Stomatopoda are genetically close. This result stem from the evolution of Eumalacostraca which has been progressed primitively and radically (Jenner et al., 2009). For more accurate metabarcoding analysis, it is necessary to solve their intricate evolutionary relationships and to establish taxonomic unification. .

In conclusion, The Euc primers were more proper than metazoan universal primer to detect miscellaneous eucarid diversities. We increased the level of Decapoda OTU diversity by using specific blocking primers and by using this, we investigated the distribution and diversity analysis of eucarid species in Korean waters. The unidentified species in this study

appear to be due to the lack of sequencing data of Eucarida. Therefore, the database of eucarid species needs to be expanded.

Supplementary information

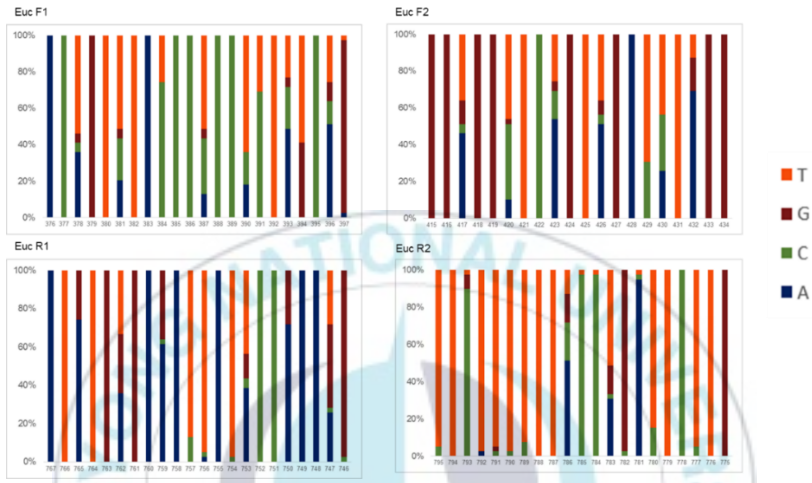


Fig S1. Primer nucleotide compositions

Table S1. Taxa information for primer design

Class	Subclass	Order	Suborder	infraorder	Accession No.	Scientific name
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Brachyura	KY785879	*Longpotamonyangtsekiense*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Brachyura	KR153996	*Portunus pelagicus*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Brachyura	MF285241	*Charybdis natator*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Brachyura	AB735678	*Chionoecetes japonicus*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Caridea	KU641481	*Crangon hakodatei*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Caridea	LC341266	*Pandalus borealis*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Caridea	KM978916	*Palaemon serenus*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Caridea	HQ830201	*Macrobrachium nipponense*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Caridea	KP276147	*Alpheus lobidens*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Anomura	KU521508	*Munida gregaria*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Anomura	JX944381	*Paralithodes camtschaticus*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Anomura	LN626968	*Clibanarius infraspinatus*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Anomura	AF150756	*Pagurus longicarpus*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Achelata	GQ223286	*Panulirus ornatus*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Achelata	LK391947	*Thenus orientalis*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Achelata	AB859775	*Sagmariasus verreauxi*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Astacidea	LN611668	*Metanephrops sibogae*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Astacidea	KM453741	*Tenuibranchiurus glypticus*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Astacidea	HQ402925	*Homarus americanus*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Astacidea	KJ573469	*Cherax destructor*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Axiidea	KU350630	*Callianassa ceramica*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Axiidea	KC107812	*Calocaris macandreae*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Axiidea	KM501040	*Trypaea australiensis*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Axiidea	NC022938	*Cherax monticola*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Gebiidea	JN897377	*Upogebia major*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Gebiidea	LC006054	*Austinogebia wuhsienweni*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Gebiidea	JN897378	*Thalassina kelanang*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Glypheidea	KT984196	*Neoglyphea inopinata*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Glypheidea	KU500619	*Laurentaeglyphea neocaledonica*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Polychelida	KC107818	*Polycheles typhlops*
Malacostraca	Eumalacostraca	Decapoda	Pleocyemata	Stenopodidea	JN399096	*Stenopus hispidus*
Malacostraca	Eumalacostraca	Decapoda	Dendrobranchiata		KX462904	*Fenneropenaeus indicus*
Malacostraca	Eumalacostraca	Decapoda	Dendrobranchiata		EF584003	*Litopenaeus vannamei*
Malacostraca	Eumalacostraca	Decapoda	Dendrobranchiata		AF217843	*Penaeus monodon*
Malacostraca	Eumalacostraca	Decapoda	Dendrobranchiata		KU899137	*Solenocera crassicornis*
Malacostraca	Eumalacostraca	Decapoda	Dendrobranchiata		JN689221	*Acetes chinensis*
Malacostraca	Eumalacostraca	Decapoda	Dendrobranchiata		LC368254	*Sergia lucens*
Malacostraca	Eumalacostraca	Euphausiacea			EU587005	*Euphausia pacifica*
Malacostraca	Eumalacostraca	Euphausiacea			EU583500	*Euphausia superba*
Malacostraca	Eumalacostraca	Amphipoda			KY197961	*Gammarus fossarum*
Malacostraca	Eumalacostraca	Isopoda			KF704000	*Limnoria quadripunctata*
Malacostraca	Eumalacostraca	Mysida			NC027510	*Neomysis japonica*
Malacostraca	Hoplocarida	Stomatopoda			KR095170	*Squilloides leptosquilla*
Hexanauplia	Copepoda	Calanoida			JX678968	*Calanus hyperboreus*
Hexanauplia	Copepoda				EU288200	*Lepeophtheirus salmonis*
Hexanauplia	Copepoda	Cyclopoida	Poecilostomatoida		KR263117	*Sinergasilus polycolpus*
Hexanauplia	Thecostraca	Sessilia	Balanomorpha		KM660676	*Balanus balanus*
Hexanauplia	Thecostraca	Sessilia	Balanomorpha		KJ434948	*Tetraclita serrata*
Ostracoda	Podocopa	Podocopida	Cypridocopina		KP063117	*Cypridopsis vidua*
Branchiopoda	Sarsostraca	Anostraca			KP273593	*Streptocephalus sirindhornae*
Cephalocarida		Brachypoda			AY456189	*Hutchinsoniella macracantha*
Remipedia		Nectiopoda			AY456190	*Xibalbanus tulumensis*
Cephalopoda	Coleoidea	Octopoda	Incirrata		HQ638215	*Octopus minor*
Actinopterygii		Beloniformes			NC011180	*Conger japonicus*
Actinopterygii		Gadiformes			KR131863	*Pollachius pollachius*
Actinopterygii		Salmoniformes			FR751400	*Salmo salar*
Actinopterygii		Osteoglossiformes			U12143	*Heterotis niloticus*
Actinopterygii		Clupeiformes			AP009498	*Clupea pallasii*
Mammalia	Theria	Cetartiodactyla	Cetancodonta	Cetacea	KC777291	*Neophocaena phocaenoides*
Mammalia	Theria	Cetartiodactyla	Cetancodonta	Cetacea	AJ554063	*Phocoena phocoena*

Table S2. Station information

Station	Latitude	Longitude	Station	Latitude	Longitude
E1	38.25	130.25	S5	32.75	126.25
E2	38.25	130.75	S6	32.75	126.75
E3	38.25	131.25	S7	32.25	126.25
E4	38.25	131.75	S8	32.25	126.75
E5	37.75	130.75	W1	37.25	124.25
E6	37.75	131.25	W2	37.25	124.75
E7	37.25	130.75	W3	37.25	125.25
E8	37.25	131.25	W4	36.75	123.75
S1	33.25	126.25	W5	36.75	124.25
S2	33.25	126.25	W6	36.75	124.75
S3	33.25	127.25	W7	36.75	125.25
S4	33.25	127.75	W8	36.25	125.25

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Table S3. Sequence read counts according to sequence processing

	raw read	paired reads	reads matched with primer	unique sequences	removed singletons	97% OTUs
COIMISQ	57638	57638	23541	13859	12328 (89.0%)	14
Euc	327566	327565	160468	19797	15836 (80.0%)	38
Eupbl	416913	416913	212392	31662	25035 (79.1%)	62

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