

# Development of a new set of PCR primers for eDNA metabarcoding decapod crustaceans

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Academic editor: *Vasco Elbrecht* | Received 13 February 2019 | Accepted 3 April 2019 | Published 24 April 2019

## Abstract

The Decapoda is one of the largest orders within the class Malacostraca, comprising approximately 14,000 extant species and including many commercially important species. For biodiversity monitoring in a non-invasive manner, a new set of PCR primers was developed for metabarcoding environmental DNA (eDNA) from decapod crustaceans. The new primers (herein named “MiDeca”) were designed for two conservative regions of the mitochondrial 16S rRNA gene, which amplify a short, hyper-variable region (153–184 bp, 164 bp on average) with sufficient interspecific variations. With the use of MiDeca primers and tissue-derived DNA extracts, we successfully determined those sequences (154–189 bp) from 250 species, placed in 186 genera and 65 families across the suborder Dendrobranchiata and 10 of the 11 infraorders of the suborder Pleocyemata. We also preliminarily attempted eDNA metabarcoding from natural seawater collected at Banda, Tateyama, the Pacific coast of central Japan and detected 42 decapod species including 34 and 8 species with sequence identities of > 98% and 80–98%, respectively. The results suggest the usefulness of eDNA metabarcoding with MiDeca primers for biodiversity monitoring of the decapod species. It appears, however, that further optimisation of primer sequences would still be necessary to avoid possible PCR dropouts from eDNA extracts.

## Key Words

mitochondrial 16S rRNA gene, biodiversity monitoring, MiDeca, natural sea water

## Introduction

Classical methods of biodiversity monitoring have been primarily based on the collection of specimens and subsequent morphology-based identification. Such biodiversity monitoring is costly and time-consuming and requires considerable expertise for various taxonomic groups. Recent technological developments in molecular ecology have provided a novel tool for species detection using DNA present in aquatic or terrestrial environments (environmental DNA or eDNA; Taberlet et al. 2012).

There are two major approaches to applying eDNA analysis: “eDNA barcoding”, which aims at detecting a single species in the environment (species-specific approach); and “eDNA metabarcoding”, which simultaneously detects multiple species from an environmental

sample (multi-species approach). The latter approach has been developed with rapidly developed high-throughput next-generation sequencing (NGS) (e.g. Taberlet et al. 2012, Thomsen et al. 2012, Miya et al. 2015, Valentini et al. 2016). Application of eDNA is now quite wide-ranging in studies of biodiversity, aquatic ecology and conservation biology (Bohmann et al. 2014, Díaz-Ferguson and Moyer 2014).

In particular, with regard to aquatic environments, the multi-specific assessment and monitoring of the fauna using eDNA have focused mainly on vertebrates (e.g. Thomsen et al. 2012, Kelly et al. 2014, Miya et al. 2015, Andruszkiewicz et al. 2017, Ushio et al. 2017, 2018), which are known to release abundant DNA derived from faeces, body mucus, blood and sloughed tissue or scales (Bohmann et al. 2014). The applicability of the eDNA

metabarcoding for aquatic invertebrates, notably those with an exoskeleton (Crustacea), has received less attention (Thomsen et al. 2012, Rees et al. 2014). The vast majority of previous studies on crustaceans yielded a “species-specific” approach detecting invasive species (e.g. Tréguier et al. 2014, Dougherty et al. 2016, Larson et al. 2017) or monitoring seasonal migrations of particular species (Wu et al. 2018).

The Decapoda is the largest order of the crustacean class Malacostraca (Arthropoda: Pancrustacea), comprising more than 14,000 extant species worldwide (De Grave et al. 2009, Ahyong et al. 2011), with continuing discovery of new species. The great majority of decapod crustaceans are marine, but other environments have also been colonised, such as lowland freshwaters, mountain rivers, estuaries and even land. Decapods are also highly diverse in ecology and include a number of commercially important species (such as shrimp, prawn, lobster, crayfish, king crab, snow crab etc.), attracting much scientific and economic interests.

The importance of marker selection in eDNA metabarcoding has recently been emphasised (Coissac et al. 2012, Deagle et al. 2014). As there is no ideal universal metabarcode (Riaz et al. 2011), marker selection could be specific to the target taxonomic group. The aim of this study was to develop new universal PCR primers for eDNA metabarcoding of decapod crustaceans, which enable detection of multiple species for biodiversity assessment and monitoring. The performance of newly developed primers (herein named “MiDeca”) was tested using tissue-derived DNA extracts from 250 species and an eDNA sample from natural seawater collected from the near-shore environment.

## Materials and methods

### Primer development

Mitochondrial rRNA genes have been recommended for identification of animal taxa because they have a similar taxonomic resolution to the COI marker and they present conserved regions that flank variable regions, which allows the design of primers with high-resolving power for the target taxonomic group (Deagle et al. 2014). In order to identify a suitable region in the mitogenome for species identification based on eDNA, 267 whole mitogenome sequences of Decapoda registered in the databases have been downloaded from NCBI as of 17 October 2017.

Three requirements were considered for designing the new primers: 1) a target amplicon consisting of fewer than 200 bp is desirable because the eDNA will often be degraded; 2) the amplified regions include sufficient interspecific differences for all target species; and 3) conserved regions (20–30 bp) across all target species are located at both ends of the short hyper-variable region to simultaneously amplify the target sequences (Riaz et al. 2011, Miya et al. 2015, Valentini et al. 2016).

After removing problematic sequences (i.e. 18 sequences that could not be aligned with other sequences), the remaining 249 sequences from 203 species and five sequences of unidentified species of *Cherax* (Astacidea: Parastacidae) (Table 1) were subjected to multiple alignment using MUSCLE (Edgar 2004) implemented in MEGA7 (Kumar et al. 2016) with a default set of parameters. The aligned sequences were imported into MEGA7 for visual inspection of the conserved and hyper-variable regions. The visual search for a short hyper-variable region (up to 200 bp for paired-end sequencing using the Illumina MiSeq) flanked by two conservative regions (ca. 20–30 bp) was performed on the entire set of the two aligned 12S rRNA and 16S rRNA genes. Primers were designed using Primer3 (Rozen et al. 2000) accounting for G/C contents (40–60%) and melting temperature (T<sub>m</sub>: 50–60 °C).

The new universal primers for decapod eDNA were designed on the 16S rRNA gene (for details, see Results and Discussion) and were named MiDeca-F/R (F and R represent forward and reverse, respectively).

### *In silico* evaluation of variation in the target region

Interspecific differences within the amplified DNA sequences are required for accurate taxonomic assignments. To computationally evaluate levels of interspecific variations within the target region (hereafter called “MiDeca sequence”) across different taxonomic groups of decapods, 254 whole mitogenome sequences used for the primer development were subjected to calculation of pairwise edit distances (intra-species, inter-species, inter-genus and inter-family levels, respectively). The edit distance is defined as the minimum number of single-nucleotide substitutions, insertions or deletions that are required to transform one sequence into the other. (Jones and Pevzner 2004).

### Test of primers with tissue extracted DNA

In order to examine the performance of MiDeca primers, extracted DNA from a single individual of each 250 species across the suborder Dendrobranchiata and 10 of 11 infraorders of the suborder Pleocyemata was used to amplify MiDeca sequences (Table 2). Total genomic DNA was extracted from each tissue (thoracic or pleon muscle or pereopod or pleopod muscle), which was preserved in 70–99% ethanol for one to more than 10 years, using a DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) with an elution volume of 100 µl.

DNA concentrations were measured and recorded with a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, DE, USA). PCR was carried out with 30 cycles of a 8.0 µl reaction volume (divided from the original PCR mastermix) containing 2.2 µl sterile distilled water, 3.8 µl 2 × Gflex PCR buffer (Takara, Otsu, Japan), 0.4 µl of each primer (5 µM), 0.2 µl Tks Gflex DNA polymerase (Takara, Otsu, Japan) and 1.0 µl template. The thermal cycle

profile after an initial 1 min denaturation at 94 °C was as follows: denaturation at 98 °C for 10 s, annealing at 50 °C for 10 s and extension at 68 °C for 10 s with the final extension at the same temperature for 7 min. The PCR products were subjected to agarose gel electrophoresis using 2% L03 (Takara, Otsu, Japan) to confirm the amplifications. The PCR products were purified using Exo SAP-IT (USB, OH, USA) to remove redundant dNTPs and primers.

Direct sequencing of the purified PCR products was performed with the ABI 3130xl Genetic Analyzer (Life Technologies, CA, USA) and dye-labelled terminators (BigDye terminator v. 1.1; Applied Biosystems, CA, USA) following the manufacturer's protocol. The DNA sequences were edited and assembled by GENETYX-MAC v. 17 (Genetyx, Tokyo, Japan) or MEGA7 and registered in the DDBJ/EMBL/NCBI database (Table 2).

### Water sampling and filtration

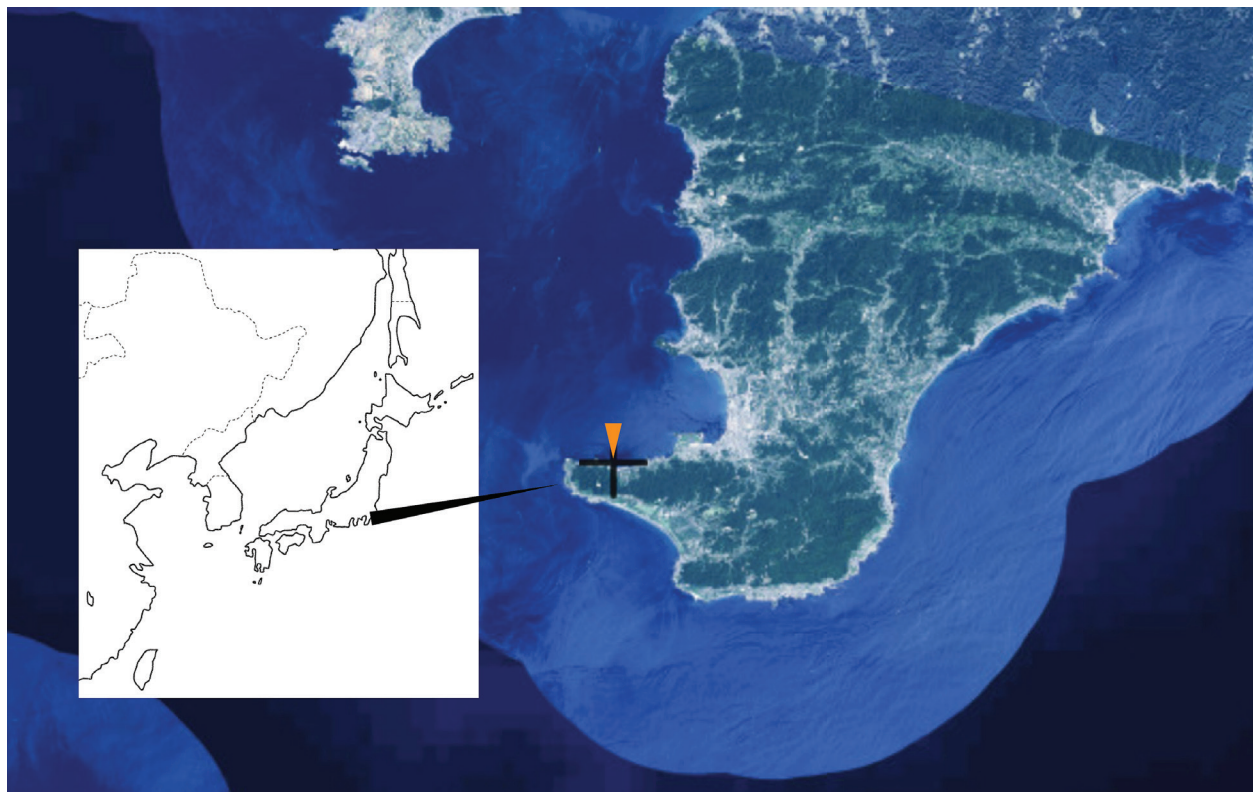
In order to test the versatility of the newly designed primers (MiDeca-F/R), we used a filtered seawater sample collected at the rocky shore of Banda, Tateyama City, Chiba Prefecture (34.9758N, 139.7675E) on 14 September 2017 (Figs 1, 2), where the decapod crustacean fauna is well documented.

An on-site filtration method was employed to collect decapod eDNA. Disposable gloves were worn and changed between each sample. The sampling equipment

(8 litre polyethylene bucket) was thoroughly decontaminated with a 10% bleach solution before use. Surface water was collected using 10 casts of the bucket fastened to a 15 m rope. In each cast, approximately 50 ml seawater was drawn into a disposable syringe with the lure lock connector (50 ml; TERUMO, Tokyo, Japan), an inlet port of the 0.45 µm Sterivex filter cartridge (Merck Millipore, MA, USA) was attached to the syringe and the seawater was filtered on to the membrane by pushing the plunger. This step was repeated twice in a single cast and the final filtration volume reached 1000 ml with 10 casts of the bucket.

After the on-site filtration, an outlet port of the filter cartridge was sealed with the Parafilm (LMS, Tokyo, Japan), RNAlater (1.6 ml; Thermo Fisher Scientific, DE, USA) was added into the cartridge using a disposable pipette to prevent eDNA degradation and an inlet port was sealed with the film. A filtration blank was made by filtering 500 ml of pure water in the same manner at the end of the water sampling. Filter cartridges were transported to the laboratory in a cooler with ice packs and then kept at -20 °C in the freezer prior to eDNA extractions.

eDNA was extracted from Sterivex cartridges using a DNeasy Blood & Tissue kit (Qiagen) following the method developed by Miya et al. (2016) with slight modifications. An inlet port of each Sterivex cartridge was connected with a 2.0 ml collection tube and the connection between the cartridge and collection tube was tightly sealed with the Parafilm. The combined unit was inserted into a centrifuge adaptor for a 15 ml conical tube and was centri-



**Figure 1.** Map of Japan, showing the location of the sea water sampling site (Banda, Tateyama, Chiba Prefecture). This map is based on the aerial photography published by Geospatial Information Authority of Japan.





**Figure 2.** A, sampling site at Banda, Tateyama, Chiba Prefecture; B, sea water sampling operation with a bucket.

fused at 6000g for 1 min to remove seawater and RNA-later for DNA extraction. In order to completely remove liquid remaining in the cartridge, an aspirator (QIAvac 24 Plus, Qiagen) was used. The Sterivex cartridge was subjected to lysis using proteinase K. Before the lysis, PBS (220  $\mu$ l), proteinase K (20  $\mu$ l) and buffer AL (200  $\mu$ l) were mixed and the mixed solution was gently pipetted into the Sterivex cartridge from an inlet port. The Sterivex cartridge was again sealed and then incubated in a 56 °C pre-heated incubator for 20 min, using a rotator (Mini Rotator ACR-100, As One) with a rotation rate of 10 rpm. After the incubation, the Sterivex cartridge connected with a 2 ml tube (DNA LowBind tube, SARSTEDT), which was placed in a 50 ml conical tube, was centrifuged at 6000g for 1 min to collect the DNA. The collected DNA solution (ca. 900  $\mu$ l) was purified using the DNeasy Blood and Tissue kit following the manufacturer's protocol.

### Library preparation and MiSeq sequencing with eDNA sample

eDNA extracted from the seawater sample collected at Banda was subjected to the first-round PCR (1<sup>st</sup> PCR) and the second-round PCR (2<sup>nd</sup> PCR) in order to append amplified sequences with three kinds of adaptor sequences: 1) primer-binding sites for sequencing; 2) dual-index sequences to distinguish amplicons; and 3) sequences for binding to the flowcells of the Illumina MiSeq (Illumina, CA, USA).

The 1<sup>st</sup> PCR was carried out with 38 cycles of a 12  $\mu$ l reaction volume containing 6.0  $\mu$ l 2  $\times$  KAPA HiFi Hot-Start ReadyMix (KAPA Biosystems, MA, USA), 1.4  $\mu$ l of each MiDeca primer (5  $\mu$ M primer F/R), 1.2  $\mu$ l sterile distilled H<sub>2</sub>O and 2.0  $\mu$ l eDNA template. In order to minimise PCR dropouts, 8 replications were performed for the 1<sup>st</sup> PCR using a 0.2 ml 8-strips tube.

The thermal cycle profile after an initial 3 min denaturation at 95 °C was as follows: denaturation at 98 °C for 20 s, annealing at 60 °C for 15 s and extension at 72 °C for 15 s with the final extension at the same tem-

perature for 5 min. The 1<sup>st</sup> PCR products from the 8 tubes were pooled in a single 1.5 ml tube and the pooled products were purified using a GeneRead Size Selection kit (Qiagen, Hilden, Germany) in order to remove dimers and monomers following the manufacturer's protocol. Subsequently, the purified products were quantified using TapeStation 2200 (Agilent, Tokyo, Japan), diluted to 0.1 ng/ $\mu$ l using Milli Q water and the diluted products were used as a template for the 2<sup>nd</sup> PCR.

The 2<sup>nd</sup> PCR was conducted with 12 cycles of a 15  $\mu$ l reaction volume containing 7.5  $\mu$ l 2  $\times$  KAPA HiFi Hot-Start ReadyMix, 0.9  $\mu$ l each primer (5  $\mu$ M), 3.9  $\mu$ l sterile distilled H<sub>2</sub>O and 1.9  $\mu$ l template. The thermal cycle profile after an initial 3 min denaturation at 95 °C was as follows: denaturation at 98 °C for 20 s, annealing and extension combined at 72 °C (shuttle PCR) for 15 s with the final extension at the same temperature for 5 min.

In order to monitor contamination during the process of PCRs, blank samples were prepared. During the 1<sup>st</sup> PCR, a filtration blank (FB), an extraction blank (EB) and a PCR blank (1B) with 2.0  $\mu$ l milli Q water instead of template eDNA were added; during the 2<sup>nd</sup> PCR, in addition to the three blanks used during the 1<sup>st</sup> PCR, one more PCR blank (2B) was added.

All the libraries containing the target region and the three adapter sequences were mixed in equal volume and the pooled libraries were size-selected from approximately 340 bp using a 2% E-Gel Size Select agarose gel (Invitrogen, CA, USA). The concentration of the size-selected libraries was measured using a Qubit dsDNA HS assay kit and a Qubit fluorometer (Life Technologies, CA, USA) and sequenced on the MiSeq platform using a MiSeq v2 Reagent Kit for 2  $\times$  150 bp PE (Illumina, CA, USA) following the manufacturer's protocol.

### Data preprocessing and taxonomic assignment

All data preprocessing and analysis of MiSeq raw reads were performed using USEARCH v10.0.240 (Edgar 2010) according to the following steps.

1) Both forward and reverse reads were merged by aligning them using the *fastq\_mergepairs* command. During this process, low-quality tail reads with a cut-off threshold set at a quality (Phred) score of 2, too short reads (< 64 bp) after tail trimming and those paired reads with too many differences (> 5 positions) in the aligned region (ca. 70 bp) were discarded; 2) primer sequences were removed from those merged reads using the *fastx\_truncate* command; 3) those reads without the primer sequences underwent quality filtering using the *fastq\_filter* command to remove low quality reads with an expected error rate of > 1% and too short reads of < 50 bp; 4) the preprocessed reads were dereplicated using the *fastx\_uniques* command and all singletons, doubletons and tripletons were removed from the subsequent analysis following the recommendation by Edgar (2010); 5) the dereplicated reads were denoised using the *unoise3* command and all putatively chimeric and erroneous sequences were separated from the subsequent OTU assignment; 6) finally all processed reads were assigned to OTU with a sequence identity of > 98% (query coverage  $\geq$  90%, 2 or 3 nucleotide differences allowed) using the *usearch\_global* command. Reads with a sequence identity of 80–98% were also assigned to “U98 OTU” and were subjected to clustering at the level of 0.98 using *cluster\_smallmem* command. All of these outputs were tabulated with read abundances.

For reference sequences, decapod crustacean mitochondrial 16S rRNA gene sequences were downloaded from NCBI as of 23 June 2018 and MiDeca sequences were extracted using custom Perl scripts and used as the reference database during taxonomic assignment. In addition to those published sequences, we independently determined MiDeca sequences from 250 decapod crustaceans (Table 2) and added those sequences to the reference database (27,236 reference sequences in total as of 26 September 2018; those sequences are represented by 4005 decapod taxa identified to species from 1135 genera of 167 families of 11 infraorders/suborders).

## Results

### MiDeca primers

By visual inspection of the two aligned mitochondrial rRNA genes from 254 sequences, two conservative regions (ca. 20 bp) that flank a hyper-variable region (154–184 bp with the exception of one extremely short sequence of 68 bp for a sergestid *Acetes chinensis*) were identified within the 16S rRNA gene. The new PCR primers for metabarcoding eDNA from decapods were designed on the basis of these two conservative regions and the primers were named MiDeca. The MiDeca-forward primer (MiDeca-F) comprised 5′-GGA CGA TAA GAC CCT ATA AA-3′ (20 mer), whereas the MiDeca-reverse primer (MiDeca-R) comprised 5′-ACG CTG TTA TCC CTA AAG T-3′ (19 mer). Melting temperatures and G/C

contents of the MiDeca-F/R primers are 51.1 °C/51.6 °C and 40.0%/42.1%, respectively. Nucleotide variation in the primer region amongst the 207 species used to design MiDeca is summarised in Table 3.

With the use of tissue extracted DNA, MiDeca primers, without adapter sequences, were able to amplify each variable region of 250 decapod species from 10 suborders/infraorders (Table 2) and those nucleotide sequences were determined using the Sanger method. The lengths of MiDeca sequences vary from 148 bp to 189 bp (mean 165.7 bp). Those sequences were deposited in DDBJ/EMBL/GenBank databases (Table 2).

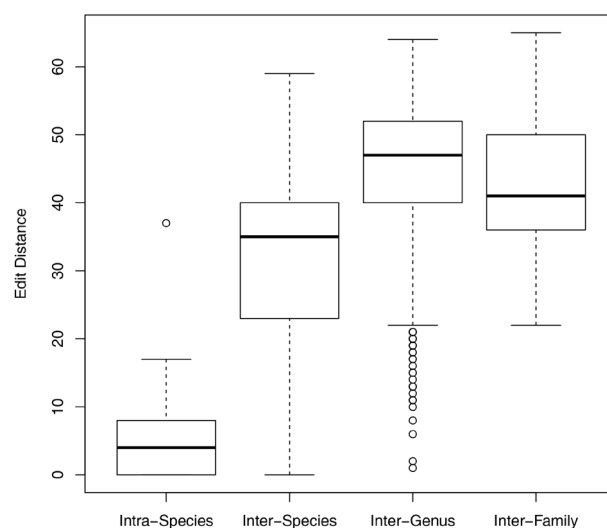
### In silico evaluation of variation in MiDeca sequence

The pairwise edit distances from MiDeca sequences were calculated for 254 sequences distributed across 10 infraorders, 56 families, 123 genera and 207 species and the results were sorted into between-families, genus, species and within species (Fig. 3). Each median of the edit distance was 41, 47, 35 and 4, respectively.

### eDNA detection from natural seawater

In total, the MiSeq paired-end sequencing yielded a total of 4,693,875 raw reads with an average of 95.2% base calls being Phred quality scores of more than or equal to 30.0 (Q30; error rate = 0.1% or base call accuracy = 99.9%). This run was highly successful considering that the quality scores specified by Illumina are more than 80% bases higher than Q30 at 2 × 150 bp (Illumina Publication no. 770-2011-001 as of 27 May 2014).

Of the 4,693,875 raw reads, our sample from Tateyama Bay comprised 185,690 raw reads and they were merged, quality-filtered, dereplicated and denoised, resulting in a total of 161,753 reads (87.1% raw reads being retained).



**Figure 3.** Summary of intraspecific, inter-species, inter-genus and inter-family edit distances of MiDeca sequences from 254 decapods used for the primer development.

**Table 1.** Mitogenome sequences used to design the MiDeca primers. Scientific names follow those as registered in GenBank database.

Suborder	Infraorder	Family	Species	Acc. No.	
Dendrobranchiata		Penaeidae	<i>Farfantepenaeus californiensis</i> (Holmes, 1900)	EU497054	
			<i>Fenneropenaeus chinensis</i> (Osbeck, 1765)	DQ518969	
			<i>Fenneropenaeus chinensis</i>	DQ656600	
			<i>Fenneropenaeus indicus</i> (H. Milne Edwards, 1837)	KX462904	
			<i>Fenneropenaeus merguensis</i> (de Man, 1888)	KP637168	
			<i>Fenneropenaeus penicillatus</i> (Alcock, 1905)	KP637169	
			<i>Litopenaeus stylirostris</i> (Stimpson, 1874)	EU517503	
			<i>Litopenaeus vannamei</i> (Boone, 1931)	DQ534543	
			<i>Litopenaeus vannamei</i>	EF584003	
			<i>Litopenaeus vannamei</i>	KT596762	
			<i>Metapenaeopsis dalei</i> (Rathbun, 1902)	KU050082	
			<i>Metapenaeus ensis</i> (De Haan, 1844)	KP637170	
			<i>Parapenaeopsis hardwickii</i> (Miers, 1878)	KU899136	
			<i>Penaeus monodon</i> Fabricius, 1798	AF217843	
				Solenoceridae	<i>Solenocera crassicornis</i> (H. Milne Edwards, 1837)
	Sergestidae	<i>Acetes chinensis</i> Hansen, 1919	JN689221		
Pleocyemata	Stenopodidea	Stenopodidae	<i>Stenopus hispidus</i> (Olivier, 1811)	JN399096	
			<i>Stenopus hispidus</i>	KC107811	
	Caridea	Alpheidae	<i>Alpheus distinguendus</i> de Man, 1909	GQ892049	
			<i>Alpheus lobidens</i> De Haan, 1849	KP276147	
		Atyidae	<i>Caridina cf. nilotica</i> HMG-2016	KU726823	
			<i>Caridina gracilipes</i> de Man, 1902	KM023648	
			<i>Halocaridina rubra</i> Holthuis, 1963	DQ917432	
			<i>Halocaridina rubra</i>	KF437502	
			<i>Halocaridina rubra</i>	KF437503	
			<i>Halocaridina rubra</i>	KF437504	
			<i>Halocaridina rubra</i>	KF437505	
			<i>Halocaridina rubra</i>	KF437506	
			<i>Halocaridina rubra</i>	KF437507	
			<i>Halocaridina rubra</i>	KF437508	
			<i>Halocaridina rubra</i>	KF437509	
			<i>Halocaridinides fowleri</i> Gordon, 1968	KX844723	
			<i>Neocaridina denticulata</i> (De Haan, 1844)	JX156333	
			<i>Paratya australiensis</i> Kemp, 1917	KM978917	
			<i>Stygiocaris lancifera</i> Holthuis, 1960	KX844714	
			<i>Stygiocaris stylifera</i> Holthuis, 1960	KX844722	
			<i>Typhlatya arfae</i> Jaume & Bréhier, 2005	KX844721	
			<i>Typhlatya consobrina</i> Botoșăneanu & Holthuis, 1970	KX844717	
			<i>Typhlatya dzilamensis</i> Alvarez, Iliffe & Villalobos, 2005	KX844719	
			<i>Typhlatya galapagensis</i> Monod & Cals, 1970	KX844711	
			<i>Typhlatya galapagensis</i>	KX844718	
			<i>Typhlatya garciai</i> Chace, 1942	KX844720	
			<i>Typhlatya iliffei</i> Hart & Manning, 1981	KX844710	
			<i>Typhlatya mitchelli</i> Hobbs & Hobbs, 1976	KX844712	
			<i>Typhlatya monae</i> Chace, 1954	KX844715	
			<i>Typhlatya pearsei</i> Creaser, 1936	KX844709	
			<i>Typhlatya</i> sp. JR2016	KX844713	
			<i>Typhlatya taina</i> Estrada & Gómez, 1987	KX844708	
			<i>Typhlopatsa pauliani</i> Holthuis, 1956	KX844716	
			Alvinocarididae	<i>Alvinocaris chelys</i> Komai & Chan, 2010	JX184903
				<i>Alvinocaris longirostris</i> Kikuchi & Hashimoto, 1995	AB821296
				<i>Alvinocaris longirostris</i>	JQ035659
				<i>Nautilocaris saintlaurentae</i> Komai & Segonzac, 2004	KF226726
		<i>Opaepele loihi</i> Williams & Dobbs, 1995		JQ035657	
		<i>Rimicaris exoculata</i> Williams & Rona, 1986		KP284529	
				<i>Rimicaris kairei</i> Watabe & Hashimoto, 2000	JQ035656
		Crangonidae		<i>Crangon hakodatei</i> Rathbun, 1902	KU641481
		Rhynchocinetidae		<i>Rhynchocinetes durbanensis</i> Gordon, 1936	KT590405
		Palaemonidae		<i>Macrobrachium bullatum</i> Fincham, 1987	KM978918
				<i>Macrobrachium lanchesteri</i> (de Man, 1911)	FJ797435
				<i>Macrobrachium nipponense</i> (De Haan, 1849)	HQ830201
				<i>Macrobrachium rosenbergii</i> (de Man, 1879)	AY659990

Suborder	Infraorder	Family	Species	Acc. No.	
Pleocyemata	Caridea	Palaemonidae	<i>Palaemon carinicauda</i> (Holthuis, 1950)	EF560650	
			<i>Palaemon gravieri</i> (Yu, 1930)	KT935323	
			<i>Palaemon gravieri</i>	KU899135	
			<i>Palaemon serenus</i> Heller, 1862	KM978916	
		Pandalidae	<i>Chlorotocus crassicornis</i> (Costa, 1871)	KY944589	
	Astacidea	Astacidae	<i>Astacus astacus</i> (Linnaeus, 1758)	KX279347	
			<i>Astacus astacus</i>	KX279348	
			<i>Astacus leptodactylus</i> Eschscholtz, 1823	KX279349	
			<i>Astacus leptodactylus</i>	KX279350	
			<i>Austropotamobius pallipes</i> (Lerebullet, 1858)	KP205430	
			<i>Austropotamobius torrentium</i> (Schränk, 1803)	KX268734	
			<i>Pacifastacus leniusculus</i> (Dana, 1852)	KX268740	
			Cambaridae	<i>Cambarus robustus</i> Girard, 1852	KX268738
				<i>Orconectes sanbornii</i> (Faxon, 1884)	KU239995
				<i>Orconectes luteus</i> (Creaser, 1933)	KX268739
		<i>Orconectes rusticus</i> (Girard, 1852)		KU239994	
		<i>Faxonius limosus</i> (Rafinesque, 1817)		KP205431	
		<i>Procambarus acutus</i> (Girard, 1852)		KX268741	
		<i>Procambarus alleni</i> (Faxon, 1884)		KT074363	
		<i>Procambarus clarkii</i> (Girard, 1852)		JN991197	
		<i>Procambarus clarkii</i>		JX316743	
		<i>Procambarus clarkii</i>		KT036444	
		<i>Procambarus clarkii</i>		KX268742	
		<i>Procambarus fallax</i> Hagen, 1870		KC107813	
		<i>Procambarus fallax</i>		KT074364	
		<i>Procambarus fallax</i>		KT074365	
		Cambaroididae	<i>Cambaroides dauricus</i> Pallas, 1773	KX268735	
			<i>Cambaroides japonicus</i> De Haan, 1841	KX268736	
			<i>Cambaroides schrenckii</i> (Kessler, 1874)	KX268737	
			<i>Cambaroides similis</i> (Koelbel, 1892)	JN991196	
		Enoplometopidae	<i>Enoplometopus debelii</i> Holthuis, 1983	KM488333	
			<i>Enoplometopus occidentalis</i> (Randall, 1840)	KC107819	
		Nephropidae	<i>Homarus americanus</i> H. Milne Edwards, 1837	HQ402925	
			<i>Homarus gammarus</i> (Linnaeus, 1758)	KC107810	
			<i>Metanephrops thomsoni</i> (Bate, 1888)	KP889215	
			<i>Nephrops norvegicus</i> (Linnaeus, 1758)	LN681403	
		Parastacidae	<i>Astacopsis gouldi</i> Clark, 1936	KM458973	
			<i>Cherax bicarinatus</i> (Gray, 1845)	KM501041	
			<i>Cherax boesemani</i> Lukhaup & Pekny, 2008	KM501042	
			<i>Cherax cainii</i> Austin & Ryan, 2002	HG942366	
			<i>Cherax cainii</i>	KF649849	
			<i>Cherax cairnsensis</i> Riek, 1969	HG799094	
			<i>Cherax crassimanus</i> Riek, 1967	HG942365	
			<i>Cherax destructor</i> Clark, 1936	AY383557	
			<i>Cherax destructor</i>	HG799087	
			<i>Cherax destructor</i>	HG799092	
			<i>Cherax destructor</i>	HG799099	
			<i>Cherax destructor</i>	HG942177	
			<i>Cherax destructor</i>	HG942178	
			<i>Cherax destructor</i>	KJ573468	
			<i>Cherax destructor</i>	KJ573469	
			<i>Cherax dispar</i> Riek, 1951	HG799093	
	<i>Cherax glaber</i> Riek, 1967		KF649852		
	<i>Cherax holthuisi</i> Lukhaup & Pekny, 2006		KM501039		
	<i>Cherax monticola</i> Holthuis, 1950		KF649851		
	<i>Cherax preissii</i> (Erichson, 1846)		HG799097		
	<i>Cherax quadricarinatus</i> (von Martens, 1868)		HG942364		
<i>Cherax quadricarinatus</i>	KF649850				
<i>Cherax quinquecarinatus</i>	HG799091				
<i>Cherax robustus</i> Riek, 1961	HG799090				
<i>Cherax</i> sp. HG-2014a	HG799095				
<i>Cherax</i> sp. HG-2014a	HG799096				
<i>Cherax</i> sp. HG-2014b	HG799098				
<i>Cherax</i> sp. HMG-2016	KX119167				



Suborder	Infraorder	Family	Species	Acc. No.		
Pleocyemata	Astacidea	Palaemonidae	<i>Cherax</i> sp. YPL-2015	KM501043		
			<i>Cherax tenuimanus</i> Smith, 1912	KP205429		
			<i>Engaeus cunicularius</i> (Erichson, 1846)	HG942173		
			<i>Engaeus lengana</i> Hortitz, 1990	KF546209		
			<i>Engaeus lyelli</i> Clark, 1936	HG799086		
			<i>Engaeus quadrimanus</i> Clark, 1936	LK391949		
			<i>Engaeus sericatus</i> Clark, 1936	LK391948		
			<i>Engaewa subcoerulea</i> Riek, 1967	KT946764		
			<i>Engaewa walpolea</i> Horwitz & Adams, 2000	KT946765		
			<i>Euastacus armatus</i> (von Martens, 1866)	KP294310		
			<i>Euastacus spinifer</i> (Heller, 1865)	KM458972		
			<i>Euastacus yarraensis</i> (McCoy, 1888)	HG942176		
			<i>Geocharax gracilis</i> Clark, 1936	HG942174		
			<i>Geocharax gracilis</i>	HG942175		
			<i>Gramastacus insolitus</i> Riek, 1972	KX148478		
			<i>Gramastacus lacus</i> McCormack, 2014	LK022684		
			<i>Paranephrops planifrons</i> White, 1842	LN681402		
			<i>Tenuibranchiurus glypticus</i> Riek, 1951	KM453741		
			Glypheidea	Glypheidae	<i>Laurentaeglyphea neocaledonica</i> (Richer de Forges, 2006)	KU500619
					<i>Neoglyphea inopinata</i> Forest & de Saint Laurent, 1975	KT984196
	<i>Neoglyphea inopinata</i>	KT984197				
	Axiidea	Axiidae	<i>Calocaris macandreae</i> Bell, 1853	KC107812		
		Callianassidae	<i>Callianassa ceramica</i> Fulton & Grant, 1906	KU350630		
			<i>Callianassa ceramica</i>	KU362925		
			<i>Corallianassa coutierei</i> (Nobili, 1904)	KC107817		
			<i>Nihonotrypaea harmandi</i> (Bouvier, 1903)	LC221567		
			<i>Nihonotrypaea thermophila</i> Lin, Komai & Chan, 2007	JN897380		
			<i>Paraglypturus tonganus</i> Ahn, Kim, Ju & Min, 2017	KJ820739		
			<i>Trypaea australiensis</i> (Dana, 1852)	KM501040		
		Strahlaxiidae	<i>Neaxius acanthus</i> (A. Milne Edwards, 1879)	KC107821		
			<i>Neaxius glyptocercus</i> (von Martens, 1868)	JN897379		
		Gebiidea	Thalassinidae	<i>Thalassina kelanang</i> Moh & Chong, 2009	JN897378	
			Upogebiidae	<i>Austinogebia edulis</i> (Ngoc-Ho & Chan, 1992)	JN897376	
				<i>Austinogebia wuhsienweni</i> (Yu, 1931)	LC006054	
	<i>Upogebia major</i> (De Haan, 1841)			JF793665		
	<i>Upogebia major</i>			JN897377		
	<i>Upogebia pusilla</i> (Petagna, 1792)			KC107815		
	<i>Upogebia yokoyai</i> Makarov, 1938			KM886610		
	Achelata	Palinuridae	<i>Palinurellus wieneckii</i> (de Man, 1881)	KC847078		
			<i>Panulirus homarus</i> (Linnaeus, 1758)	JN542716		
			<i>Panulirus japonicus</i> (von Siebold, 1824)	AB071201		
			<i>Panulirus ornatus</i> (Fabricius, 1798)	GQ223286		
			<i>Panulirus ornatus</i>	HM446347		
			<i>Panulirus stimpsoni</i> Holthuis, 1963	GQ292768		
			<i>Panulirus versicolor</i> (Latreille, 1804)	JQ320274		
			<i>Panulirus versicolor</i>	KC107808		
			<i>Sagmariasus verreauxi</i> (H. Milne Edwards, 1851)	AB859775		
			Scyllaridae	<i>Ibacus ciliatus</i> (von Siebold, 1824)	KM488334	
		<i>Scyllarides latus</i> (Latreille, 1803)		KC107814		
		<i>Thenus orientalis</i> (Lund, 1793)		LK391947		
		Polychelida	Polychelidae	<i>Polycheltes typhlops</i> Heller, 1862	KC107818	
		Anomura	Kiwaidae	<i>Kiwa tyleri</i> Thatje et al., 2015	KY423514	
			Munididae	<i>Munida gregaria</i> (Fabricius, 1793)	KU521508	
			Munidopsidae	<i>Shinkaia crosnieri</i> Baba & Williams, 1998	EU420129	
			Porcellanidae	<i>Neopetrolisthes maculatus</i> (H. Milne Edwards, 1837)	KC107816	
	<i>Petrolisthes haswelli</i> Miers, 1884			LN624374		
	Diogenidae		<i>Clibanarius infraspinus</i> (Hilgendorf, 1869)	LN626968		
	Lithodidae		<i>Paralithodes brevipes</i> (H. Milne Edwards & Lucas, 1848)	AB735677		
			<i>Paralithodes camtschaticus</i> (Tilesius, 1815)	JX944381		
	Paguridae		<i>Pagurus longicarpus</i> Say, 1817	AF150756		
	Brachyura	Bythograeidae	<i>Austinograea alayseae</i> Guinot, 1990	JQ035660		
			<i>Austinograea alayseae</i>	KC851803		
			<i>Austinograea rodriguezensis</i> Tsuchida & Hashimoto, 2002	JQ035658		
			<i>Gandalfus puia</i> McLay, 2007	KR002727		



Suborder	Infraorder	Family	Species	Acc. No.
Pleocyemata	Brachyura	Bythograeidae	<i>Gandalfus yunohana</i> (Takeda, Hashimoto & Ohta, 2000)	EU647222
			<i>Segonzacia mesatlantica</i> (Williams, 1988)	KY541839
		Dotillidae	<i>Ilyoplax deschampsii</i> (Rathbun, 1913)	JF909979
		Dynomeneidae	<i>Dynomene pilumnoides</i> Alcock, 1900	KT182070
		Eriphiidae	<i>Pseudocarcinus gigas</i> (Lamarck, 1818)	AY562127
		Geryonidae	<i>Chaceon</i> sp. BZ-2016	KU507298
		Grapsidae	<i>Pachygrapsus crassipes</i> (Randall, 1840)	KC878511
		Homolidae	<i>Homola orientalis</i> Henderson, 1888	KT182071
			<i>Homologenus malayensis</i> Ihle, 1912	KJ612407
			<i>Moloha majora</i> (Kubo, 1936)	KT182069
		Leucosiidae	<i>Pyrhila pisum</i> (De Haan, 1841)	KU343210
		Macrophthalmidae	<i>Macrophthalmus japonicus</i> (De Haan, 1835)	KU343211
		Majidae	<i>Maja crispata</i> Risso, 1827	KY650651
			<i>Maja squinado</i> (Herbst, 1788)	KY650652
		Matutidae	<i>Ashtoret lunaris</i> (Forskål, 1775)	LK391941
		Menippidae	<i>Myomenippe fornasinii</i> (Bianconi, 1851)	LK391943
		Mictyridae	<i>Mictyris longicarpus</i> Latreille, 1806	LN611670
		Mithracidae	<i>Damithrax spinosissimus</i> (Lamarck, 1818)	KM405516
		Ocypodidae	<i>Tabuca arcuata</i> (De Haan, 1835)	KX911977
			<i>Ocypode ceratophthalmus</i> (Pallas, 1772)	LN611669
			<i>Ocypode cordimanus</i> Latreille, 1818	KT896743
		Oregoniidae	<i>Chionoecetes japonicus pacificus</i> Sakai, 1978	AB735678
		Parathelphusidae	<i>Somanniathelphusa boyangensis</i> Dai, Peng & Zhou, 1994	KU042042
		Portunidae	<i>Callinectes sapidus</i> Rathbun, 1896	AY363392
			<i>Charybdis feriata</i> (Linnaeus, 1758)	KF386147
			<i>Charybdis japonica</i> (A. Milne-Edwards, 1861)	FJ460517
			<i>Charybdis natator</i> (Herbst, 1794)	MF285241
			<i>Portunus pelagicus</i> (Linnaeus, 1758)	KM977882
			<i>Portunus pelagicus</i>	KR153996
			<i>Portunus pelagicus</i>	KT382858
			<i>Portunus sanguinolentus</i> (Herbst, 1783)	KT438509
			<i>Portunus trituberculatus</i> (Miers, 1876)	AB093006
			<i>Scylla olivacea</i> (Herbst, 1796)	FJ827760
			<i>Scylla paramamosain</i> Estampador, 1949	FJ827761
			<i>Scylla paramamosain</i>	JX457150
			<i>Scylla serrata</i> (Forskål, 1775)	FJ827758
			<i>Scylla serrata</i>	HM590866
			<i>Scylla tranquebarica</i> (Fabricius, 1798)	FJ827759
		<i>Thalamita crenata</i> Rüppel, 1830	LK391945	
		Potamidae	<i>Geothelphusa dehaani</i> (White, 1847)	AB187570
			<i>Huananpotamon lichuanense</i> Dai, Zhou & Peng, 1995	KX639824
			<i>Longpotamon xiushuiense</i> (Dai, Zhou & Peng, 1995)	KU042041
			<i>Potamiscus motuoensis</i> Dai, 1990	KY285013
		Raninidae	<i>Lyreidus brevifrons</i> Sakai, 1937	KM983394
			<i>Ranina ranina</i> (Linnaeus, 1758)	KM189817
			<i>Umalia orientalis</i> (Sakai, 1963)	KM365084
		Sesarmidae	<i>Clistocoeloma sinense</i> Shen, 1933	KU589292
			<i>Metopaulias depressus</i> Rathbun, 1896	KX118277
			<i>Parasesarma tripectinis</i> (Shen, 1940)	KU343209
			<i>Sesarma neglectum</i> de Man, 1887	KX156954
			<i>Sesarmops sinensis</i> (H. Milne Edwards, 1853)	KR336554
		Varunidae	<i>Cyclograpsus granulatus</i> H. Milne Edwards, 1853	LN624373
			<i>Eriocheir sinensis</i> H. Milne Edwards, 1853	AY274302
			<i>Eriocheir sinensis</i>	KM516908
			<i>Eriocheir sinensis</i>	KP064329
			<i>Eriocheir sinensis</i>	KP126617
			<i>Eriocheir sinensis</i>	KY041629
<i>Helicana wuana</i> (Rathbun, 1931)	KX344898			
<i>Helice latimera</i> Parisi, 1918	KU589291			
<i>Helice tientsinensis</i> Rathbun, 1931	KR336555			
<i>Hemigrapsus sanguineus</i> (De Haan, 1835)	KX456205			
<i>Leptodius sanguineus</i> (H. Milne Edwards, 1834)	KT896744			
Xenograpsidae	<i>Xenograpsus ngatama</i> McLay, 2007	KY985236		
	<i>Xenograpsus testudinatus</i> Ng, Huang & Ho, 2000	EU727203		

**Table 2.** A list of decapod species for testing MiDeca primers (without adapter sequences) using extracted DNA subsequently sequenced with a Sanger method. Institutional abbreviation: AMF, Aquamarine Fukushima, Iwaki, Japan.

Suborder/ Infraorder	Family	Species	CBM reg. No.	Acc. No.	bp	
Dendrobranchiata	Penaeidae	<i>Metapenaeopsis dalei</i> (Rathbun, 1902)	15064	LC464519	168	
		<i>Metapenaeus brevicornis</i> (H. Milne Edwards, 1837)	13761	LC430751	170	
		<i>Penaeus latisulcatus</i> Kishinouye, 1896	14890	LC430718	168	
		<i>Trachysalambria curvirostris</i> (Stimpson, 1860)	14210	LC430766	169	
	Solenoceridae	<i>Haliporoides sibogae</i> (de Man, 1907)	14982	LC430749	167	
		<i>Pleoticus muelleri</i> (Spence Bate, 1888)	14981	LC430764	167	
	Sicyonidae	<i>Sicyonia laevis</i> Spence Bate, 1881	14851	LC464520	167	
		<i>Sicyonia truncata</i> (Kubo, 1949)	15110	LC464521	166	
	Sergestidae	<i>Sergia talismani</i> (Barnard, 1947)	14966	LC430728	172	
		<i>Sicyonella antennata</i> Hansen, 1919	14897	LC430716	165	
Pleocyemata/ Stenopodidea	Stenopodidae	<i>Richardina parvioculata</i> Saito & Komatsu, 2009	15048	LC430808	158	
Pleocyemata/ Caridea	Pasiphaeidae	<i>Leptochela robusta</i> Stimpson, 1860	14948	LC464522	160	
		<i>Leptochela sydniensis</i> Dakin & Colefax, 1940	14965	LC430727	161	
	Atyidae	<i>Neocaridina davidi</i> (Bouvier, 1904)	14978	LC430762	163	
	Disciadiidae	<i>Discias exul</i> Kemp, 1922	15285	LC469628	150	
	Palaemonidae	<i>Macrobrachium nipponense</i> (De Haan, 1849)	14986	LC430769	159	
		<i>Palaemon debilis</i> Dana, 1852	14835	LC464524	162	
		<i>Palaemon macrodactylus</i> Rathbun, 1902	14968	LC464526	162	
		<i>Palaemon pacificus</i> (Stimpson, 1860)	15087	LC464525	160	
		<i>Palaemon paucidens</i> De Haan, 1844	14979	LC430763	162	
		<i>Palaemon serrifer</i> (Stimpson, 1860)	15298	LC464527	160	
		<i>Palaemon orientis</i> Holthuis, 1950	13697	LC430771	160	
		<i>Phycomenes indicus</i> (Kemp, 1915)	14899	LC464528	159	
		<i>Cuapetes grandis</i> (Stimpson, 1860)	14768	LC464523	160	
		Alpheidae	<i>Alpheus hailstonei</i> Coutière, 1905	14949	LC464536	157
	<i>Alpheus pacificus</i> Dana, 1852		14688	LC464538	160	
	<i>Alpheus heeia</i> Banner & Banner, 1975		14762	LC464537	159	
	<i>Alpheus ikedosoma</i> Komai, 2015		14825	LC430798	158	
	<i>Alpheus longipalma</i> Komai & Ohtomi, 2018		14484	LC430730	159	
	<i>Alpheus</i> sp. aff. <i>pacificus</i>		15299	LC472887	160	
	<i>Arete dorsalis</i> (Stimpson, 1860)		14756	LC464539	155	
	<i>Athanas japonicus</i> Kubo, 1936		14658	LC464540	159	
	<i>Betaeus granulimanus</i> (Yokoya, 1927)		14240	LC464541	158	
	<i>Caligoneus cavernicola</i> Komai & Fujita, 2018		14136	LC464542	183	
	<i>Salmoneus brucei</i> Komai, 2009		14558	LC464543	158	
	<i>Synalpheus tumidomanus</i> (Paulson, 1875)		15089	LC464544	158	
	Ogyrididae		<i>Ogyrides orientalis</i> (Stimpson, 1860)	14824	LC430799	154
	Hippolytidae	<i>Latreutes acicularis</i> (Ortmann, 1890)	14682	LC464529	155	
		<i>Latreutes porcinus</i> Kemp, 1916	14900	LC430717	163	
		<i>Saron marmoratus</i> (Olivier, 1811)	14911	LC430713	171	
	Thoridae	<i>Eualus biungius</i> (Rathbun, 1902)	15178	LC430743	161	
		<i>Eualus kikuchii</i> Miyake & Hayashi, 1967	14516	LC430740	162	
		<i>Eualus leptognathus</i> (Stimpson, 1860)	14503	LC464530	160	
		<i>Heptacarpus acuticarinatus</i> Komai & Ivanov, 2008	14507	LC430737	161	
		<i>Heptacarpus futirostris</i> (Spence Bate, 1888)	14056	LC464531	161	
		<i>Heptacarpus geniculatus</i> (Stimpson, 1860)	14055	LC464532	161	
		<i>Heptacarpus jordani</i> (Rathbun, 1902)	14993	LC464533	163	
		<i>Lebbeus armatus</i> (Owen, 1839)	14316	LC464534	161	
		<i>Lebbeus kubo</i> Hayashi, 1992	15177	LC430742	161	
		<i>Thor paschalis</i> (Heller, 1862)	14973	LC430702	161	
		<i>Spirontocaris prionota</i> (Stimpson, 1864)	14058	LC464535	162	
		Pandalidae	<i>Heterocarpus hayashii</i> Crosnier, 1988	14983	LC430767	163
			<i>Pandalopsis glabra</i> Kobjakova, 1936	AMF-ZC 0012	LC430745	160
	<i>Pandalopsis miyakei</i> Hayashi, 1986		8898	LC430744	160	
	<i>Plesionika semilaevis</i> Spence Bate, 1888		14984	LC430768	159	
	Glyphocrangonidae	<i>Glyphocrangon formosana</i> Komai, 2004	15054	LC469627	165	
	Crangonidae	<i>Aegaeon lacazei</i> (Gourret, 1887)	14860	LC464545	159	
		<i>Crangon uritai</i> Hayashi & Kim, 1999	15084	LC464546	164	
<i>Parapontophilus demani</i> (Chace, 1983)		15038	LC430760	161		
<i>Philocheras coralliophilus</i> Komai & Kim, 2010		15027	LC430747	165		
<i>Philocheras japonicus</i> (Doflein, 1902)		15045	LC430761	159		
<i>Philocheras parvirosotris</i> (Kemp, 1916)		14831	LC430800	155		
<i>Syncrangon angusticauda</i> (De Haan, 1849)		14212	LC472888	160		

Suborder/ Infraorder	Family	Species	CBM reg. No.	Acc. No.	bp	
Pleocyemata/ Astacidea	Nephropidae	<i>Nephropsis stewarti</i> Wood-Mason, 1872	14212	LC430805	163	
Pleocyemata/ Axiidea	Axiidae	<i>Eiconaxius farreae</i> Ortmann, 1890	14870	LC464548	158	
		<i>Litoraxius boshu</i> Komai & Tachikawa, 2007	9225	LC472889	162	
	Strahlaxiidae	<i>Neaxius acanthus</i> (A. Milne-Edwards, 1878)	14990	LC430806	165	
	Callianassidae	<i>Cheramus spinicauda</i> Komai, Maenosono & Fujita, 2014	15139	LC464550	159	
		<i>Neocallichirus jousseaumei</i> (Nobili, 1904)	15026	LC430756	161	
		<i>Nihonotrypaea harmandi</i> (Bouvier, 1903)	14728	LC430796	160	
		<i>Nihonotrypaea japonica</i> (Ortmann, 1890)	15176	LC430729	160	
		<i>Nihonotrypaea petalura</i> (Stimpson, 1860)	15003	LC464551	162	
		<i>Paratrypaea bouvieri</i> (Nobili, 1904)	14812	LC464552	159	
	Eucalliidae	<i>Calliuxina novaebritanniae</i> (Borradaile, 1900)	14653	LC464549	162	
<i>Eucalliux aequimana</i> (Baker, 1904)		15028	LC430748	160		
Pleocyemata/ Gebiidea	Laomediidae	<i>Laomedia astacina</i> De Haan, 1849	13759	LC430775	163	
	Upogebiidae	<i>Tuerkayogebia kiiensis</i> (Sakai, 1971)	15218	LC472890	176	
Pleocyemata/ Achelata	Palinuridae	<i>Panulirus japonicus</i> (von Siebold, 1824)	14991	LC430807	171	
Pleocyemata/ Polychelida	Polychelidae	<i>Stereomastis helleri</i> (Spence Bate, 1878)	10076	LC430804	172	
Pleocyemata/ Anomura	Galatheidae	<i>Coralliogalatea humilis</i> (Nobili, 1905)	14896	LC430703	163	
		<i>Galatea orientalis</i> Stimpson, 1858	15014	LC430817	163	
	Munididae	<i>Munida multilineata</i> Komai, 2012	14494	LC430735	163	
		<i>Munida munin</i> Komai, 2011	15154	LC464553	166	
		<i>Raymunida elegantissima</i> (de Man, 1902)	14939	LC464554	164	
	Munidopsidae	<i>Munidopsis petalorhyncha</i> Baba, 2005	13528	LC430765	164	
	Porcellanidae	<i>Novorostrum decorocrus</i> Osawa, 1998	14801	LC464555	166	
		<i>Pachycheles hertwigi</i> Balss, 1913	15015	LC430818	161	
		<i>Pachycheles stevensii</i> Stimpson, 1858	15016	LC430813	161	
		<i>Petrolisthes asiaticus</i> Leach, 1820	14912	LC430712	167	
		<i>Petrolisthes japonicus</i> (De Haan, 1849)	15017	LC430816	166	
		<i>Pisidia dispar</i> Stimpson, 1858	14758	LC464556	163	
		<i>Polyonyx sinensis</i> Stimpson, 1858	13700	LC430772	165	
		<i>Polyonyx utinomii</i> Miyake, 1943	14360	LC464557	165	
		Lithodidae	<i>Paralomis verrilli</i> Benedict, 1894)	15179	LC430803	169
		Diogenidae	<i>Aniculus miyakei</i> Forest, 1984	14628	LC430788	167
	<i>Calcinus vachoni</i> Forest, 1954		15009	LC430811	165	
	<i>Clibanarius virescens</i> (Krauss, 1843)		15008	LC464558	170	
	<i>Dardanus pedunculatus</i> (Herbst, 1804)		14629	LC430754	165	
	<i>Diogenes avarus</i> Heller, 1865		14555	LC464559	165	
	<i>Diogenes spinifrons</i> (De Haan, 1849)		14828	LC430797	167	
	<i>Paguristes albimaculatus</i> Komai, 2001		14852	LC464560	170	
	<i>Paguristes ortmanni</i> Miyake, 1978		15246	LC464561	169	
	<i>Pseudopaguristes laurentae</i> Morgan & Forest, 1991		14853	LC430725	189	
	Paguridae		<i>Alainopaguroides andamanensis</i> McLaughlin, 2000	14954	LC464562	167
		<i>Alloeopagurodes spiniacicula</i> Komai, 1998	15173	LC430734	167	
		<i>Catapaguroides japonicus</i> de Saint Laurent, 1968	15300	LC464563	169	
		<i>Decaphyllus spiniornis</i> de Saint Laurent, 1968	14863	LC464564	165	
		<i>Pagurixus anceps</i> (Forest, 1954)	14565	LC464565	167	
		<i>Pagurus filholi</i> (de Man, 1887)	15161	LC464566	168	
		<i>Pagurus imafukui</i> McLaughlin & Konishi, 1994	14862	LC464567	167	
		<i>Pagurus japonicus</i> (Stimpson, 1858)	15011	LC464568	168	
		<i>Pagurus maculosus</i> Komai & Imafuku, 1996	15012	LC464569	168	
		<i>Pagurus nigrivittatus</i> Komai, 2003	15013	LC430810	168	
	Parapaguridae	<i>Parapagurus furici</i> Lemaitre, 1999	15037	LC430757	172	
	Pleocyemata/ Brachyura	Dromiidae	<i>Cryptodromia bullifera</i> (Alcock, 1900)	14249	LC469629	161
			<i>Metadromia wilsoni</i> (Fulton & Grant, 1902)	14251	LC469630	161
		Dynomeneidae	<i>Dynomene pilumnoides</i> Alcock, 1900	14951	LC469631	164
		Cyclodorippidae	<i>Tymolus japonicus</i> Stimpson, 1858	14864	LC469632	152
		Cyonomidae	<i>Cyonomus curvirostris</i> Sakai, 1963	14849	LC469633	167
		Homolidae	<i>Homolochunia gadaletae</i> Guinot & Richer de Forges, 1995	15039	LC469634	167
			<i>Latreillopsis bispinosa</i> Henderson, 1888	15033	LC430759	167
			<i>Yaldwynopsis saguili</i> Richer de Forges & Ng, 2007	14252	LC430792	169
		Calappidae	<i>Calappa gallus</i> (Herbst, 1803)	14630	LC430784	171
			<i>Calappa hepatica</i> (Linnaeus, 1758)	15006	LC469635	170
			<i>Mursia orientalia</i> Takeda & Galil, 2005	14513	LC430739	167
		Matutidae	<i>Ashtoret miersi</i> (Henderson, 1887)	14989	LC430704	159
<i>Izanami curtispina</i> (Sakai, 1961)			15153	LC469636	159	

Suborder/ Infraorder	Family	Species	CBM reg. No.	Acc. No.	bp
Pleocyemata/ Brachyura	Ethusidae	<i>Ethusa quadrata</i> Sakai, 1937	14492	LC430733	164
	Leucosiidae	<i>Arcania cornigera</i> Naruse, 2014	14857	LC469637	159
		<i>Ebalia tuberculosa</i> (A. Milne-Edwards, 1873)	14850	LC469638	160
		<i>Merocryptus lambryiformis</i> A. Milne-Edwards, 1873	14856	LC469639	159
		<i>Nucia speciosa</i> Dana, 1852	14261	LC469640	158
		<i>Philyra kanekoi</i> Sakai, 1934	14967	LC430795	160
		<i>Philyra syndactyla</i> Ortmann, 1892	15146	LC469641	163
		<i>Pseudophilyra intermedia</i> Ihle, 1918	14379	LC469642	161
		<i>Tokoyo eburnea</i> (Alcock, 1896)	14866	LC469643	160
	Inachidae	<i>Cyrtomaia owstoni</i> Terazaki, 1903	15040	LC469644	164
	Epialtidae	<i>Huenia heraldica</i> De Haan, 1839	14632	LC430776	165
		<i>Menaetius monoceros</i> (Latreille, 1825)	14778	LC430721	148
		<i>Naxioides taurus</i> (Pocock, 1890)	15152	LC469645	163
		<i>Oxypleurodon stimpsoni</i> (Miers, 1886)	15118	LC469646	161
		<i>Phalangipus hystrix</i> (Miers, 1886)	15080	LC469647	164
		<i>Pugettia minor</i> Ortmann, 1893	14855	LC469648	169
		<i>Pugettia nipponensis</i> Rathbun, 1932	15035	LC469649	170
		<i>Pugettia quadridens</i> (De Haan, 1839)	15018	LC430812	170
	Majidae	<i>Choniognathus reini</i> (Balss, 1924)	14867	LC469650	161
		<i>Micippa philyra</i> (Herbst, 1803)	15019	LC469651	159
		<i>Paramaia spinigera</i> (De Haan, 1837)	14636	LC430786	169
		<i>Prismatopus harmandi</i> (Bouvier, 1906)	14633	LC469651	174
		<i>Schizophrys aspera</i> (H. Milne Edwards,	14637	LC430777	166
	Oregoniidae	<i>Pleistacantha sanctijohannis</i> Miers, 1879)	14495	LC430736	164
		<i>Pleistacanthoides simplex</i> (Rathbun, 1932)	15116	LC469653	172
	Cancriidae	<i>Glebocarcinus amphioetus</i> (Rathbun, 1898)	14988	LC430701	177
		<i>Romaleon gibbosulum</i> (De Haan, 1833)	14858	LC469654	174
	Parthenopidae	<i>Rhinolambrus contrarius</i> (Herbst, 1804)	14639	LC430779	165
	Aethridae	<i>Aethra edentata</i> Edmondson, 1951	14264	LC469655	168
	Pilumnidae	<i>Actumnus setifer</i> (De Haan, 1835)	14382	LC469656	156
		<i>Actumnus squamosus</i> (De Haan, 1835)	14648	LC430781	156
		<i>Actumnus uformis</i> Takeda & Komatsu, 2017	14944	LC469657	161
		<i>Benthopanope pearsei</i> (Rathbun, 1932)	14918	LC430707	168
		<i>Calmania balssi</i> (Sakai, 1935)	14869	LC469658	162
		<i>Cryptolutea sagamiensis</i> (Sakai, 1935)	14512	LC430738	160
		<i>Harrovia japonica</i> Balss, 1921	14312	LC469659	164
		<i>Heteropilumnus ciliatus</i> (Stimpson, 1858)	11291	LC469660	177
		<i>Pilumnus longicornis</i> Hilgendorf, 1878	11133	LC469661	165
		<i>Pilumnus minutus</i> De Haan, 1835	14335	LC469662	165
		<i>Pilumnus tomentosus</i> Latreille, 1825	14310	LC430794	164
		<i>Pilumnus</i> sp.1	14941	LC469663	165
		<i>Pseudolithochira integra</i> (Miers, 1884)	14917	LC430706	160
		<i>Typhlocarcinus</i> sp.	14522	LC469664	161
		<i>Zehntneriana tadafumii</i> Lee, Kim & Ng, 2015	14916	LC430705	162
		<i>Xenophthalmodes morsei</i> Rathbun, 1932	14961	LC430724	162
	Portunidae	<i>Charybdis acuta</i> (A. Milne-Edwards, 1869)	14647	LC430785	168
		<i>Charybdis natator</i> (Herbst, 1794)	14640	LC430783	168
		<i>Carupa ohashii</i> Takeda, 1983	14269	LC469665	173
		<i>Cycloachelous granulatus</i> (H. Milne Edwards, 1834)	15095	LC469666	174
		<i>Laleonectes nipponensis</i> (Sakai, 1938)	14272	LC469667	167
		<i>Portunus pelagicus</i> (Linnaeus, 1758)	15168	LC469668	170
		<i>Scylla olivacea</i> (Herbst, 1796)	15169	LC469669	169
		<i>Thalamita chaptali</i> (Audouin, 1826)	15200	LC469670	167
		<i>Thalamita longifrons</i> (A. Milne-Edwards, 1869)	15158	LC469673	167
		<i>Thalamita picta</i> Stimpson, 1858	12359	LC469671	167
		<i>Thalamita sima</i> H. Milne Edwards, 1834	14646	LC430780	164
		<i>Thalamita seurati</i> Nobili, 1906	12692	LC469672	167
		<i>Thalamita</i> sp. aff. <i>coeruleipes</i>	15197	LC469674	165
		<i>Thranita pelsarti</i> (Mondgomery, 1931)	14650	LC430787	164
		<i>Xiphonectes brocki</i> (de Man, 1887)	14905	LC430719	172
		<i>Xiphonectes macrophthalmus</i> (Rathbun, 1906)	14276	LC469675	171
		<i>Geothelphusa dehaani</i> (White, 1847)	14231	LC430731	169
	Goneplacidae	<i>Notonyx angulatus</i> Naruse & Takeda, 2010	14314	LC469676	167
		<i>Pedroplax megalops</i> (Takeda, 1977)	14950	LC469677	174
		<i>Pycnoplax surugensis</i> (Rathbun, 1932)	15036	LC430758	170
	Xanthidae	<i>Actaea bocki</i> Odhner, 1925	14856	LC469678	168
		<i>Actaea semblatae</i> Guinot, 1976	14288	LC469679	171



Suborder/ Infraorder	Family	Species	CBM reg. No.	Acc. No.	bp	
Pleocyemata/ Brachyura	Xanthidae	<i>Alainodaeus nuku</i> Davie, 1997	14942	LC469680	170	
		<i>Atergatis floridus</i> (Linnaeus, 1767)	14641	LC430782	170	
		<i>Atergatis granulatus</i> de Man, 1889	14285	LC430793	170	
		<i>Atergatis integerrimus</i> (Lamarck, 1818)	14286	LC469681	171	
		<i>Atergatis subdentatus</i> (De Haan, 1835)	14642	LC430789	172	
		<i>Banareia subglobosa</i> (Stimpson, 1858)	14644	LC430790	169	
		<i>Cycloxanthops truncatus</i> (De Haan, 1837)	15021	LC430809	169	
		<i>Danielea noelensis</i> (Ward, 1942)	14296	LC469682	169	
		<i>Euxanthus herdmani</i> Laurie, 1906	14292	LC469683	167	
		<i>Etisus electra</i> (Herbst, 1801)	14974	LC430710	171	
		<i>Etisus frontalis</i> (Dana, 1852)	14975	LC430711	171	
		<i>Etisus laevimanus</i> Randall, 1840	14643	LC430791	170	
		<i>Forestiana granulata</i> (Krauss, 1843)	15155	LC469684	171	
		<i>Gaillardiiellus orientalis</i> (Odhner, 1925)	8363	LC469685	169	
		<i>Lachnopodus subacutus</i> (Stimpson, 1858)	14913	LC430708	168	
		<i>Leptodius affinis</i> (De Haan, 1835)	15022	LC430815	167	
		<i>Leptodius sanguineus</i> (H. Milne Edwards, 1834)	14914	LC430709	169	
		<i>Lophozozymus dodone</i> (Herbst, 1801)	14283	LC469686	170	
		<i>Liomera caelata</i> (Odhner, 1925)	15172	LC469687	169	
		<i>Liomera rugata</i> (H. Milne Edwards, 1834)	14907	LC430715	169	
		<i>Medaeops granulatus</i> (Haswell, 1882)	13757	LC430752	171	
		<i>Metaxanthops acutus</i> Serène, 1984	14308	LC469688	168	
		<i>Microcassiope orientalis</i> Takeda & Miyake, 1969	14301	LC469689	169	
		<i>Neoliomera pubescens</i> (H. Milne Edwards, 1834)	14282	LC469690	175	
		<i>Palapedia rastripes</i> (Müller, 1887)	14906	LC430714	168	
		<i>Palapedia truncatifrons</i> (Sakai, 1974)	13758	LC430753	168	
		<i>Paraxanthias elegans</i> (Stimpson, 1858)	15023	LC430814	170	
		<i>Pilodius areolatus</i> (Milne Edwards, 1834)	14290	LC469691	168	
		<i>Platypodia granulosa</i> (Rüppel, 1830)	14959	LC430722	171	
		Eriphiidae	<i>Eriphia scabricula</i> Dana, 1852	15143	LC469692	166
		Oziidae	<i>Epixanthus corrosus</i> A. Milne-Edwards, 1873	14772	LC430720	173
		Grapsidae	<i>Pachygrapsus minutus</i> (A. Milne Edwards, 1873)	15144	LC430755	171
		Sesarmidae	<i>Clistocoeloma sinense</i> Shen, 1933	14664	LC469693	179
			<i>Nanosesarma minutum</i> (de Man, 1887)	15166	LC469694	175
			<i>Parasesarma affine</i> (De Haan, 1837)	14992	LC469695	183
			<i>Parasesarma pictum</i> (De Haan, 1835)	14838	LC469696	184
		Plagusiidae	<i>Guinusia dentipes</i> (De Haan, 1835)	14645	LC430778	169
		Percnidae	<i>Percnon planissimum</i> (Herbst, 1804)	14571	LC469697	176
		Varunidae	<i>Acmaeopleura parvula</i> Stimpson, 1848	14848	LC469698	171
			<i>Chasmagnathus convexus</i> (De Haan, 1835)	14662	LC469699	171
			<i>Cyclograpsus intermedius</i> Ortmann, 1894	14847	LC469700	173
			<i>Gaetice depressus</i> (De Haan, 1835)	15024	LC469701	169
			<i>Gopkittisak angustum</i> Komai, 2011	14561	LC469702	165
			<i>Helice tridens</i> (De Haan, 1835)	13695	LC430770	172
<i>Hemigrapsus longitarsis</i> (Miers, 1879)	15174		LC469703	174		
<i>Hemigrapsus takanoi</i> Asakura & Watanabe, 2005	13696		LC430750	175		
<i>Ptychognathus barbatus</i> (A. Milne-Edwards, 1873)	15029		LC430746	172		
<i>Sestrostoma depressum</i> (Sakai, 1965)	15175		LC469704	172		
Camptandriidae	<i>Deiratonotus cristatum</i> (de Man, 1895)		13760	LC430774	171	
Macrophthalmidae	<i>Macrophthalmus brevis</i> (Herbst, 1804)	15201	LC469705	172		
	<i>Macrophthalmus convexus</i> Stimpson, 1858	14841	LC469706	172		
	<i>Macrophthalmus serenei</i> Takeda & Komai, 1991	14556	LC469707	174		
	<i>Macrophthalmus telescopicus</i> (Owen, 1839)	14384	LC469708	171		
	<i>Tritodynamia japonica</i> Ortmann, 1894	14228	LC430732	171		
Pinnotheridae	<i>Pinnotheres pholadis</i> De Haan, 1835	14225	LC430773	171		
Hexapodidae	<i>Hexapinus simplex</i> Rahayu & Ng, 2014	14393	LC430741	168		

Preprocessed reads from the four blanks (FB, EB, 1B, 2B) were minor, comprising only 7–128 reads (0.004–0.07% of the non-negative sample). We therefore considered that those reads from the four blanks were negligible and they were not used in the subsequent taxonomic assignment.

The preprocessed reads from Tateyama Bay sample were subjected to taxonomic assignment with the

custom database. Finally, these reads were assigned to 35 crustacean species with the sequence identity of 98–100%, 10 crustacean species with the sequence identity of 80–98% (Table 4) and 69 no-hit taxa. Of the 35 species with the identity of 98–100%, 34 were decapods with the exception of the amphipod *Caprella scaura*. Of those 34 decapod species, the occurrence of

**Table 3.** Nucleotide sequences of the universal primers (MiDeca) and base compositions in the selected 254 sequences (see Table 1). This forward (F) and reversal (R) primer pair amplifies the mid region of the mitochondrial 16S rRNA gene with a mean length of 164 bp (154–184 bp; except for one unusually short sequence from *Acetes chinensis*).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
MiDeca-F	5'	G	G	A	C	G	A	T	A	A	G	A	C	C	C	T	A	T	A	A	A	3'
	A	20	0	254	2	2	254	1	253	254	0	254	0	1	0	0	236	0	236	254	254	
	T	4	0	0	1	0	0	238	1	0	0	0	0	0	0	254	0	252	0	0	0	
	G	230	254	0	0	252	0	0	0	0	254	0	0	0	0	18	1	18	0	0	0	
	C	0	0	0	251	0	0	15	0	0	0	0	254	253	254	0	0	0	0	0	0	
	–	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	
MiDeca-R	3'	T	G	A	A	A	T	C	C	C	T	A	T	T	G	T	C	G	C	A	A	5'
	A	254	0	0	0	0	254	0	0	0	253	0	254	254	0	254	0	0	3	0		
	T	0	0	254	237	237	0	0	0	1	254	0	0	0	0	0	3	0	254			
	G	0	0	0	0	0	0	254	254	254	0	0	0	0	0	254	0	251	0			
	C	0	254	0	17	17	0	0	0	0	0	0	0	0	254	0	0	251	0	0		

**Table 4.** A list of malacostracan species detected from natural sea water sampled at Banda, Tateyama, Chiba Prefecture. Non-decapod taxa are marked by bold. Number of reads, degree of sequence identity, length of marker sequences and accession numbers are summarised.

Family	Species	No. of reads	Identity	bp	Acc. No.
Identity > 98%: 35 species					
Portunidae	<i>Thranita pelsarti</i> (Montgomery, 1931)	53,745	100	164	LC430787
Portunidae	<i>Thalamita sima</i> H. Milne Edwards, 1834	32,026	100	164	LC430780
Xanthidae	<i>Paraxanthias elegans</i> (Stimpson, 1858)	6,611	99.4	170	LC430814
Xanthidae	<i>Pilodius nigrocrinitus</i> Stimpson, 1859	5,737	99.4	169	KM888688
Varunidae	<i>Gaetice depressus</i> (De Haan, 1835)	5,732	100	169	LC469701
Alpheidae	<i>Betaeus granulimanus</i> Yokoya, 1927	4,778	100	158	LC464541
Calappidae	<i>Calappa gallus</i> (Herbst, 1803)	3,364	98.6	173	KU206589
Pilumnidae	<i>Pilumnus longicornis</i> Hilgendorf, 1878	3,204	100	165	LC469661
Penaeidae	<i>Metapenaeopsis lamellata</i> (Rathbun, 1902)	1,937	100	169	AF105043
Portunidae	<i>Thalamita seurati</i> Nobili, 1906	1,757	98.2	169	LC469672
Xanthidae	<i>Leptodius affinis</i> (Dana, 1852)*	1,710	100	167	KP256206
Thoridae	<i>Heptacarpus futirostris</i> (Bate, 1888)	1,676	100	161	LC464531
Upogebiidae	<i>Tuerkayogebia kiiensis</i> (Sakai, 1971)	1,391	100	174	LC472887
Majidae	<i>Micippa philyra</i> (Herbst, 1803)	1,002	99.7	159	EU682780
Xanthidae	<i>Actaea semblatae</i> Guinot, 1976	949	100	171	LC469679
Macrophthalmidae	<i>Chaenostoma orientale</i> Stimpson, 1858**	914	100	168	AB101492
Xanthidae	<i>Cycloxanthops truncatus</i> (De Haan, 1837)	779	100	169	LC430809
Majidae	<i>Tiarinia cornigera</i> (Latreille, 1825)	635	99.7	162	EU682786
Grapsidae	<i>Pachygrapsus crassipes</i> (Randall, 1840)	490	100	173	KC878511
Plagusidae	<i>Guinusia dentipes</i> (De Haan, 1835)***	419	100	169	AJ308421
Paguridae	<i>Pagurus filholi</i> (de Man, 1887)	310	100	168	LC464566
Epialtidae	<i>Pugettia quadridens</i> (De Haan, 1839)	292	99.1	170	LC430812
Alpheidae	<i>Alpheus pacificus</i> Dana, 1852	268	99.4	160	LC464538
Alpheidae	<i>Synalpheus tumidomanus</i> (Paulson, 1875)	172	100	158	LC464544
<b>Caprellidae</b>	<b><i>Caprella scaura</i> Templeton, 1836</b>	89	99.3	134	AB847634
Eriphiidae	<i>Eriphia ferox</i> Koh & Ng, 2008	79	100	150	HM637968
Diogenidae	<i>Aniculus miyakei</i> Forest, 1984	70	100	167	LC430788
Sergestidae	<i>Sergia lucens</i> (Hansen, 1919)	43	100	172	AB705159
Diogenidae	<i>Clibanarius virescens</i> (Krauss, 1843)	39	98.8	170	LC464558
Xanthidae	<i>Microcassiope orientalis</i> Takeda & Miyake, 1969	26	98.2	170	LC469689
Xanthidae	<i>Atergatis floridus</i> (Linnaeus, 1767)	20	100	170	LC430782
Paguridae	<i>Pagurus maculosus</i> Komai & Imafuku, 1996	16	100	168	LC464569
Grapsidae	<i>Pachygrapsus minutus</i> A. Milne-Edwards, 1873	11	100	171	LC430755
Thoridae	<i>Lebbeus groenlandicus</i> (Fabricius, 1775)	5	99.4	162	KJ833727
Xanthidae	<i>Gaillardiiellus orientalis</i> (Odhner, 1925)	4	100	169	LC469685
80% < Identity < 98%: 10 species					
Majidae	<i>Micippa thalia</i> (Herbst, 1803)	3,743	96.9	159	EU682780
Portunidae	<i>Thalamita</i> sp. aff. <i>coeruleipes</i>	3,677	97	164	LC469674
Alpheidae	<i>Alpheus</i> sp. aff. <i>pacificus</i>	1,321	81.3	160	LC472887
Pilumnidae	<i>Typhlocarcinus</i> sp.	938	89.4	161	LC469664
Pilumnidae	<i>Pilumnus</i> sp.	465	93.9	165	LC469663
Xanthidae	<i>Actaea polyacantha</i> (Heller, 1861)	378	96.4	167	HM798416
Varunidae	<i>Sestrostoma depressum</i> (Sakai, 1965)	225	87.4	175	LC469704
Pilumnidae	<i>Cryptolutea sagamiensis</i> (Sakai, 1935)	189	89.4	161	LC430738
<b>Maeridae</b>	<b><i>Quadrimeaera pacifica</i> (Schellenberg, 1938)</b>	179	84.1	126	AB432980
<b>Euphausiidae</b>	<b><i>Euphausia similis</i> G.O. Sars, 1885</b>	174	88.8	169	AF281274

Names in GenBank database: \**Leptodius nigromaculatus* or *L. exaratus*; \*\**Macrophthalmus bosicii* (see also the text); \*\*\**Plagusia dentipes*.

**Table 5.** A list of 90 decapod species recorded from rocky intertidal to shallow subtidal zones (< 5 m) in Tateyama Bay (including Banda) and nearby areas.

Infraorder	Family	Species	CBM-ZC voucher	16S sequence	eDNA detection
Caridea	Alpheidae	<i>Alpheus bisincisus</i> De Haan, 1849	14164	ND	–
		<i>Alpheus edwardsii</i> (Audouin, 1826)	4674	ND	–
		<i>Alpheus heeia</i> Banner & Banner, 1975	2965, 7010, 14816, 14820, 15088	✓	–
		<i>Alpheus pacificus</i> Dana, 1852	14830	✓	✓
		<i>Arete dorsalis</i> (Stimpson, 1860)	371, 8210,	✓	–
		<i>Athanas japonicus</i> Kubo, 1936	2724, 5835, 14999	✓	–
		<i>Athanas dimorphus</i> Ortmann, 1894	5836, 7989, 14163	ND	–
		<i>Athanas parvus</i> de Man, 1910	3641, 14818	ND	–
		<i>Betaeus gelasinifer</i> Nomura & Komai, 2000	5209	ND	–
		<i>Betaeus granulimanus</i> Yokoya, 1927	4673, 9481, 15239	✓	✓
		<i>Salmoneus gracilipes</i> Miya, 1972	14185	ND	–
	<i>Synalpheus tumidomanus</i> Paulson, 1875	11722, 15089	✓	✓	
	Crangonidae	<i>Syncrangan angusticauda</i> (De Haan, 1849)	4937, 9937, 15001	✓	–
	Hippolytidae	<i>Hippolyte ventricosa</i> H. Milne Edwards, 1837	6023, 9940	ND	–
	Palaemonidae	<i>Cuapetes grandis</i> (Stimpson, 1860)	14994	✓	–
<i>Palaemon pacificus</i> (Stimpson, 1860)		15087, 15245	✓	–	
<i>Palaemon serrifer</i> (Stimpson, 1860)		540	✓	–	
<i>Eualus sinensis</i> (Yu, 1931)		9479	ND	–	
Thoridae	<i>Heptacarpus futirostris</i> (Bate, 1888)	4672, 9480, 14162, 15148	✓	✓	
Achelata	Palinuridae	<i>Panulirus japonicus</i> (von Siebold, 1824)	14991	✓	–
Axiidea	Axiidae	<i>Litoraxius boshu</i> Komai & Tachikawa, 2007	9228	✓	–
	Callianassidae	<i>Nihonotrypaea petalura</i> (Stimpson, 1860)	7011, 7990, 15003	✓	–
Gebiidea	Upogebiidae	<i>Tuerkayogebia kiiensis</i> (Sakai, 1971)	9057, 15218	✓	✓
Anomura	Galatheidae	<i>Galathea orientalis</i> Stimpson, 1858	4679, 9500, 15014	✓	–
		<i>Pachycheles hertwigi</i> Balss, 1913	15015, 15091	✓	–
	Porcellanidae	<i>Pachycheles stevensii</i> Stimpson, 1858	4678, 5143, 7828, 15016	✓	–
		<i>Petrolisthes coccineus</i> Owen, 1839	11289	ND	–
		<i>Petrolisthes japonicus</i> (De Haan, 1849)	7580, 9938, 15017	✓	–
	Diogenidae	<i>Aniculus miyakei</i> Forest, 1984	2584	✓	✓
		<i>Calcinus laevimanus</i> (Randall, 1840)	690–692, 689	✓	–
		<i>Calcinus latens</i> (Randall, 1840)	698–703	✓	–
		<i>Calcinus vachoni</i> Forest, 1954	707–709, 15009	✓	–
		<i>Clibanarius virescens</i> (Krauss, 1843)	5141, 15008	✓	✓
		<i>Dardanus sanguinolentus</i> (Quoy & Gaimard, 1824)	15233	ND	–
		<i>Paguristes ortmanni</i> Miyake, 1978	15234	✓	–
		<i>Pagurus decimbranchiae</i> Komai & Osawa, 2001	5897	ND	–
	Paguridae	<i>Pagurus erythrogrammus</i> Komai, 2003	6496	ND	–
		<i>Pagurus filholi</i> (de Man, 1887)	15010, 15161	✓	✓
		<i>Pagurus japonicus</i> (Stimpson, 1858)	15011	✓	–
		<i>Pagurus lanuginosus</i> (De Haan, 1849)	4676	ND	–
<i>Pagurus maculosus</i> Komai & Imafuku, 1996		4677, 5142, 15012	✓	✓	
<i>Pagurus nigrivittatus</i> Komai, 2003		6482–6484, 15013	✓	–	
<i>Pagurus</i> sp.		15235	✓	✓	
Brachyura	Calappidae	<i>Calappa gallus</i> (Herbst, 1803)	15235	✓	✓
	Inachidae	<i>Paratymolus pubescens</i> Miers, 1879	7991	ND	–
	Epialtidae	<i>Huenia heraldica</i> (De Haan, 1837)	7992, 15094	✓	–
		<i>Menaethius monoceros</i> (Latreille, 1825)	15093	✓	–
		<i>Pugettia quadridens</i> (De Haan, 1849)	7581, 14821, 15018	✓	✓
	Hymenosomatidae	<i>Elamena truncata</i> (Stimpson, 1858)	11773	ND	–
		<i>Lucasinus coralicola</i> (Rathbun, 1909)	3632	ND	–
		<i>Neohymenicus orientalis</i> (Sakai, 1932)	4680	ND	–
		<i>Rhynchoplax messor</i> Stimpson, 1858	4681, 6024, 9939, 11774	ND	–
	Majidae	<i>Micippa philyra</i> (Herbst, 1803)	2044, 15019	✓	✓

Infraorder	Family	Species	CBM-ZC voucher	16S sequence	eDNA detection
Brachyura	Majidae	<i>Micippa thalia</i> (Herbst, 1803)	11031	✓	✓
		<i>Pseudomicippe okamotoi</i> (Sakai, 1938)	8017	ND	–
		<i>Tiarinia cornigera</i> (Latreille, 1825)	7900, 15020	✓	✓
	Eriphiidae	<i>Eriphia ferox</i> Koh & Ng, 2008	2967, 15164	✓	✓
	Portunidae	<i>Thalamita sima</i> H. Milne Edwards, 1834	895, 2653, 13448	✓	✓
		<i>Thalamita seurati</i> Nobili, 1906	13447	✓	✓
		<i>Thlanita pelsarti</i> (Montgomery, 1931)	7582	✓	✓
	Pilumnidae	<i>Benthopanope pearsei</i> (Rathbun, 1932)	4683, 5148, 15241	✓	–
		<i>Heteropilumnus ciliatus</i> (Stimpson, 1858)	11291	✓	–
		<i>Pilumnus longicornis</i> Hilgendorf, 1878	11133, 12814, 12819, 14130	✓	✓
		<i>Pilumnus minutus</i> De Haan, 1835	7997	✓	–
	Xanthidae	<i>Actaea semblatae</i> Guinot, 1976	898, 12147, 14558	✓	✓
		<i>Atergatis floridus</i> (Linnaeus, 1767)	11549, 15242	✓	✓
		<i>Cycloxanthops truncatus</i> (H. Milne Edwards, 1834)	2611, 5144, 7995, 14822, 15021	✓	✓
		<i>Danielea noelensis</i> (Ward, 1942)	15236	✓	–
		<i>Etisus laevimanus</i> (Randall, 1840)	2662	✓	–
		<i>Gaillardiiellus orientalis</i> (Odhner, 1925)	8363	✓	✓
		<i>Forestiana granulata</i> (Krauss, 1843)	15155	✓	–
		<i>Leptodius affinis</i> (Dana, 1852)	15022	✓	✓
		<i>Medaeops granulatus</i> (Haswell, 1882)	7993, 11290, 13757, 14819	✓	–
		<i>Microcassiope orientalis</i> Takeda & Miyake, 1969	5145, 5838, 5987	✓	✓
		<i>Novactaea pulchella</i> (A. Milne-Edwards, 1865)	5146	ND	–
		<i>Palapedia integra</i> (De Haan, 1835)	7996	ND	–
		<i>Palapedia truncatifrons</i> (Sakai, 1972)	13758, 15237	✓	–
		<i>Paraxanthias elegans</i> (Stimpson, 1858)	15023	✓	✓
		<i>Pilodius nigrocrinitus</i> Stimpson, 1859	4682, 5147, 15096	✓	✓
	<i>Zozymodes cavipes</i> (Dana, 1852)	15156	ND	–	
	Grapsidae	<i>Pachygrapsus crassipes</i> (Randall, 1840)	7541, 15243	✓	✓
		<i>Pachygrapsus minutus</i> A. Milne-Edwards, 1873	15157, 15165	✓	✓
	Macrophthalmidae	<i>Chaenostoma orientale</i> Stimpson, 1858	15167	✓	✓
	Plagusiidae	<i>Guinusia dentipes</i> (De Haan, 1835)	469, 564, 899, 7540	✓	✓
	Sesarmidae	<i>Chiromantes haematocheir</i> (De Haan, 1833)	2725	✓	–
		<i>Nanosesarma minutum</i> (de Man, 1887)	4685, 5149, 15166	✓	–
Varunidae	<i>Gaetice depressus</i> (De Haan, 1835)	15024, 15097	✓	✓	
	<i>Hemigrapsus sanguineus</i> (De Haan, 1835)	6762	✓	–	
	<i>Sestrostoma depressum</i> (Sakai, 1965)	7012, 10545	✓	–	
Pinnotheridae	<i>Sakaina asiatica</i> (Sakai, 1933)	6917	ND	–	
	<i>Sakaina incisa</i> Sakai, 1969	4684	ND	–	

the 31 species in the study site and its adjacent areas is confirmed by examination of the museum collection and recent field surveys (Table 5). Reads assigned to *Lebbeus groenlandicus* are considered to be cross-contamination apparently derived from previous experiments based on aquarium tank water (for details, see below).

Sequences of the 69 no-hit taxa were subjected to BLAST search on GenBank database. None of them was assigned to crustacean species. Of the 69 taxa, five were assigned to the following three known molluscan species with high sequence identity: *Patelloida saccharina*

(Lesson 1831) (Gastropoda: Lottiidae; one haplotype, sequence identity 100%); *Limnoperna fortunei* (Dunker 1857) (Bivalvia: Mytilidae; three haplotypes, sequence identity 99–100%); *Mytilus galloprovincialis* Lamarck, 1819 (Bivalvia: Mytilidae; one haplotype, sequence identity 100%). One taxon was assigned to a bryozoan *Beania klugei* Cook, 1968 (Gymnolaemata: Cheilostomatida: Beaniidae) with low sequence identity (93%). One taxon was linked to two unidentified cyanobacteria taxa with low sequence identity, i.e. *Synechococcus* sp. WH 8109 (sequence identity 95.5%) or *Synechococcus* sp. CC9605 (sequence identity 94.2%).



## Discussion

### Usefulness of eDNA metabarcoding with MiDeca primers

It has been confirmed that the newly developed MiDeca primers are able to amplify the hyper-variable region of the mitochondrial 16S rRNA gene from the tissue-derived DNA extracts. We have successfully sequenced the target segment from 250 species from 186 genera and 65 families distributed across 10 suborders/infraclasses. The edit distance between species was very high (Fig. 3), suggesting that the MiDeca sequence has sufficient interspecific variations for taxonomic assignment. A preliminary examination of eDNA from the natural seawater from Banda, Tateyama, Chiba Prefecture, detected 34 decapod species (sequence identity > 98%) (Table 4). In addition to those species, 10 unidentified species with lower identity (80–98%) were also detected (Table 4). There is little doubt that the eDNA metabarcoding with the MiDeca primers could provide information on the presence of particular decapod species without the requirement for capturing specimens or visual census.

### Taxa detected from eDNA metabarcoding

It is remarkable that as many as 34 decapod species were detected from only one sample. Of the 34 species detected, the occurrence of 32 species in the study site and nearby areas was confirmed by examination of the museum collections and our field surveys (Tables 4 and 5). Although voucher specimens have not been collected, the occurrence of *Metapenaeopsis lamellata* is still likely. This penaeid species is sublittoral and nocturnal and, thus, collection of specimens at the study site is difficult. The detection of the sergestid shrimp *Sergia lucens* is remarkable, because it is a mesopelagic species, undergoing diurnal vertical migration along the continental shelf (Omori 1969). This species sometimes occurs in coastal areas in Boso Peninsula (TK, personal observation; voucher material CBM-ZC 2053) and, thus, its detection was due either to an accidental migration to the coastal water or to transport of the eDNA from the nearby oceanic water.

The detection of the macrophthalmid crab “*Macrophthalmus boscii*” needs explanation. Recent studies have shown that more than one species were confounded under the name *M. boscii* (cf. Naderloo and Türkay 2011, Teng et al. 2016) and *M. boscii* and allied taxa were transferred to the genus *Chaenostoma*. Teng et al. (2016) showed that two species, *C. crassimanus* Stimpson, 1858 and *C. orientale* occur in the north-western Pacific, including Japan, with true *C. boscii* restricted to the western Indian Ocean. At the studied site, specimens of *C. orientale* have been collected and, thus, it is reasonable to consider that it was the eDNA of *C. orientale* that was detected and that the GenBank sequence identified as “*Macrophthalmus boscii*” has been misidentified.

On the other hand, the detection of the thorida shrimp *Lebbeus groenlandicus* is dubious, because it is a boreal, deep-sea benthic species that does not occur in or adjacent to the study area. As such, we concurrently examined eDNA from tank water in Aquamarine Fukushima, where individuals of this species had been kept [for the species identity, see Komai (2015)]. This is, thus, the suspected source of “contamination”. This again highlights the importance of safeguarding against cross-contamination during the eDNA metabarcoding (e.g. Bohmann et al. 2014).

Amongst the eight decapod taxa with a lower identity match (80–98%), e-DNA assigned to the majid crab *Micippa thalia* (identity 96.9%) was also found. It is now known that the Japanese population that has been referred to as *M. thalia* in literature is actually a separate species (P. K. L., personal communication). As such, it is not surprising that the detected sequence here does not fully match that of *M. thalia* registered in GenBank. Although we have tried to sequence specimens identified as “*M. thalia*” in the CBM collection, we have not been successful. Unfortunately, our attempts to sequence specimens identified with *M. thalia* deposited in the CBM collection were unsuccessful.

The species status of the other seven decapod taxa with lower identity (80–98%) cannot be determined with a high degree of confidence. As shown in Table 5, there are several local species for which MiDeca sequence data are still not available. On the other hand, it is highly likely that there are species for which their occurrence has not been confirmed in the study area. At the study site, the intertidal to subtidal area consists of fragile sandstone, which provides cryptic habitats for those decapod species. To reduce the number of unknown species, continuous efforts for the collection and accumulation of reference sequences are necessary.

It is fortuitous that eDNA of the other malacostracan taxa, including Amphipoda (a caprellid *Caprella scaura* and an unidentified taxon linked to the maerid *Quadrimaera pacifica*) and Euphausiacea (an unidentified taxon linked to euphausiid *Euphausia similis*), were also detected (Table 4). In addition to those, taxa other than Crustacea were also detected, as noted above. This suggests broad applicability of MiDeca metabarcoding to non-decapod Malacostraca and even to non-crustacean taxa with slight modifications of primer sequences.

A false negative (species is not detected where it is present), as well as a false positive (species is detected where it is absent), are important issues in eDNA metabarcoding, because they will cause under- or overestimation of species richness. In fact, considerable numbers of decapod species that are recorded from the study site and adjacent areas were not detected by the present metabarcoding exercise (Table 5). Two major factors could be considered for the false negatives: 1) no eDNA from those species was collected during sampling; 2) PCR amplification of eDNA from those species was not successful.

With regard to the first factor, water sampling at low tide, including various microhabitats, may be more effective for collecting more eDNA. With regard to the second

factor, exploration of an optimal method that generates a greater species richness of MiDeca sequences to avoid PCR dropouts would be necessary. As PCR dropouts might be due to PCR bias derived from primer-template mismatches, an optimal number of PCR replicates and use of multiple annealing temperatures would be alternative approaches to comprehensively detect target eDNA. In fact, in a fungal metabarcoding study, pooling multiple repeated PCRs and using multiple annealing temperatures were recommended to facilitate the recovery of more accurate species richness (Schmidt et al. 2013). Furthermore, Majaneva et al. (2018) demonstrated that choice of the DNA extraction method affects DNA metabarcoding. Clarification of the optimal DNA extraction method for the target group might be advisable.

The other important point for accurate assessment of biodiversity is completeness of the reference sequence database, which is indispensable for satisfactory taxonomic assignments. Reference sequences, used in the present analyses, were primarily derived from the GenBank database with the addition of the sequence data generated by ourselves. The process of exploration of the GenBank decapod crustacean database highlighted the lack of sufficient sequence data. During this study, we have newly sequenced the target marker from 250 decapod species (including 4 unidentified species), but the number of the currently available species known from Japanese waters is still 1054, representing 36% of the decapod species presently known from the areas (about 2,890 species placed in 950 genera distributed across 147 families). Furthermore, there are many species with uncertain taxonomy, including undescribed species and poorly defined species, for example, taxa in the snapping shrimp genus *Alpheus* (cf. Anker and De Grave 2016), swimming crabs of the genus *Thalamita* (cf. Spiridonov 2017) and several genera in the highly diverse crab family Pilumnidae (cf. Ng 1987). As with standard DNA barcoding, significantly more taxonomic work, including building of verified reference sequence databases, is necessary to optimise the effectiveness of eDNA approaches.

### Data accessibility

MiDeca sequences from the 250 decapod crustaceans are available from DDBJ/EMBL/GenBank databases (Table 2). Raw reads from the MiSeq sequencing are available from the DDBJ Sequence Read Archive (DRA008193/DRR172676).

## Acknowledgements

TK was supported by JSPS KAKENHI Grant Number 16K07494 and MM was supported by JST CREST Grant Number JPMJCR13A2, Japan and by Environment Research and Technology Development Fund (4-1602) of the Ministry of the Environment, Japan. We deeply thank Shoji Tsuzuki and Ryuta Yoshida (Tateyama Station,

Ochanomizu University) for providing us with valuable specimens for this study, Takehiko Fukuchi for assisting in obtaining reference sequences of the 16S rRNA and two referees for reviewing the manuscript and for offering valuable comments for improvements. Shane T. Ah Yong (Australian Museum, Sydney), Peter K. L. Ng (Lee Kong Chian Natural History Museum, National University of Singapore) and Ling-Ming Tsang (National Taiwan Ocean University, Keelung) also kindly reviewed and edited the manuscript.

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