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DNA BARCODING STUDY OF SHELLED GASTROPODS IN THE INTERTIDAL ROCKY COASTS OF CENTRAL WAKAYAMA PREFECTURE, JAPAN, USING TWO GENE MARKERS

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ABSTRACT: The coasts of Wakayama Prefecture are known to be among the most biologically diverse coastal areas of Japan, and thus have a rich assemblage of shelled gastropods. In this paper, we report the result of our DNA Barcoding of shelled gastropods of the intertidal area of the Nada Coast in central Wakayama, using the mitochondrial genes COI and the nuclear gene Histone H3 as markers. In order to do so, we collected up to five individuals from 12 species of shelled gastropods from the intertidal rocky beach. Collected samples were first identified morphologically and then vouchered at the University Museum of the University of Tokyo. DNA sequence comparisons and phylogenetic analyses indicated that both genes have enough substitutions to differentiate species. We also found that the sequence data for most of our target species are not available on Genbank. Our result presented here indicated that we were not only successful in barcoding/identifying the target gastropod species in the area, but also contributed to the building of a set of reference DNA sequences for future DNA-based environmental and biodiversity monitoring, besides providing sequence data for future systematics studies of this group.

Keywords: DNA Barcoding, Gastropoda, Biodiversity Monitoring, Intertidal, DNA Database

1. INTRODUCTION

Recent development in sequencing technology has made DNA Barcoding a practical method for taxonomic identification of samples collected from the field, due to its relatively high accuracy [1]. Moreover, when coupled with Environmental DNA (eDNA), DNA Barcoding has been acknowledged as a powerful method for biodiversity monitoring due to its efficiency and minimal invasiveness [1–4].

In order for these methods to be effective, the availabilities of useful DNA markers and comprehensive reference databases, including those of DNA, are crucial. However, the lack of such reliable databases has been a problem. For example, Troudet et al [5] reported taxonomic representative bias in the Global Biodiversity Information Facility (GBIF) database. They found that while popular vertebrate taxa such as birds and mammals were over-represented, many major invertebrate lineages, including the Gastropods, were poorly represented. Page [6] and Machida et al [7] suggested that the lack of properly curated reliable data, including DNA data registered in Genbank, has hampered correct identifications of various "dark" taxa.

The highly diverse marine Gastropoda (ca. 32,000–40,000 extant species [2]) and other mollusks have been known to be sensitive to environmental changes [8–10]. The intertidal shelled members of this group are known for their abundance and wide distribution in various rocky coasts. The presence of the external calcified shell is useful for a quick but relatively accurate taxonomic identification. Because of these characteristics, the intertidal shelled marine Gastropods is relatively easy to monitor and survey, and thus a useful model taxon for the assessment of the impacts of environmental changes, at both the global and local scales.

The coasts of Wakayama Prefecture, including the Nada Coast in central Wakayama (Fig. 1), are known to be among the most biologically diverse coastal areas of Japan [11–12], and thus have a rich assemblage of shelled Gastropods. However, although many ecological and species observation studies have been reported (e.g. [13–14]), very little molecular-based biodiversity studies have been conducted in the area.

In this study, we report the result of our DNA Barcoding study on shelled Gastropods of the intertidal area of the Nada Coast in central Wakayama, using the mitochondrial gene cytochrome c oxidase I (COI) and the nuclear gene Histone H3 (H3). Our aims were thus: to identify the collected samples using molecular (DNA Barcoding) method in order to corroborate the

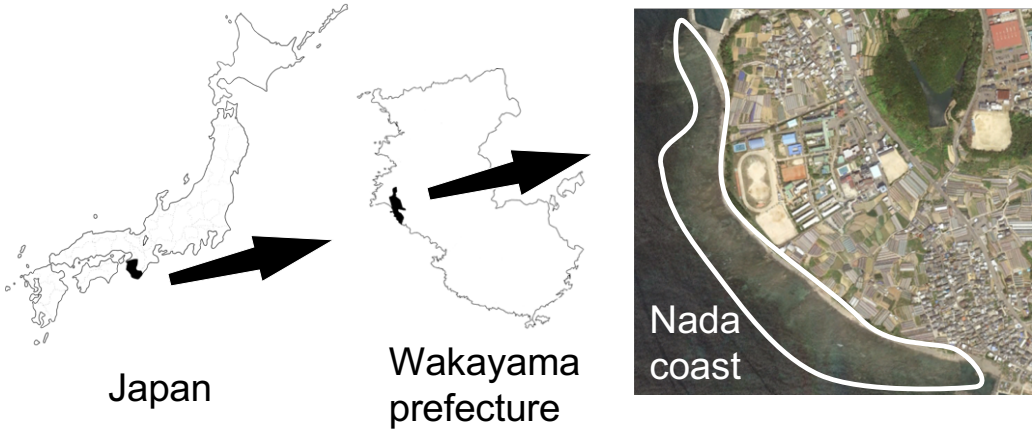

Figure 1. Sampling location of this study: The Nada Coast in Wakayama Prefecture, Japan.

results of morphological identifications, to confirm the usefulness of the two gene markers used in this study, and to provide a reliable sequence dataset linked to vouchered museum samples for future barcoding studies, DNA-based environmental and biodiversity monitoring, and systematics studies.

2. MATERIALS AND METHODS

2.1 Taxon Sampling

43 individuals of shelled gastropods from ten species were collected from the rocky intertidal area of the Nada Coast in central Wakayama. (Fig. 2, Table 1). Samples were identified morphologically based on current taxonomical classification [15] and then preserved in 95% ethanol and vouchered at the University Museum of the University of Tokyo.

2.2 DNA Sequence Data Obtainment

Pieces of muscle tissue (ca. 0.25 mg) were excised from samples. DNA was extracted using standard CTAB-phenol-chloroform method. The COI gene was amplified using LCO1490 and HCO2198 [16] and LCO mod_Kano2008 and HCO mod_Kano 2008 [17]. Primer pairs H3aF' and H3aR [18] were used for the H3 gene. PCR reactions were then conducted at the annealing temperatures of 45º and 50º, respectively. Sanger sequencings of successful PCR products were outsourced to FASMAC Ltd. (Japan).

2.3 Sequence Identification / Barcoding

BLASTn searches optimized for highly similar and somewhat similar sequences settings were conducted to identify sequences obtained from the samples. BLAST hits were then checked for their sequence similarities, percent identities, and e-values. We also checked whether a hit was of the

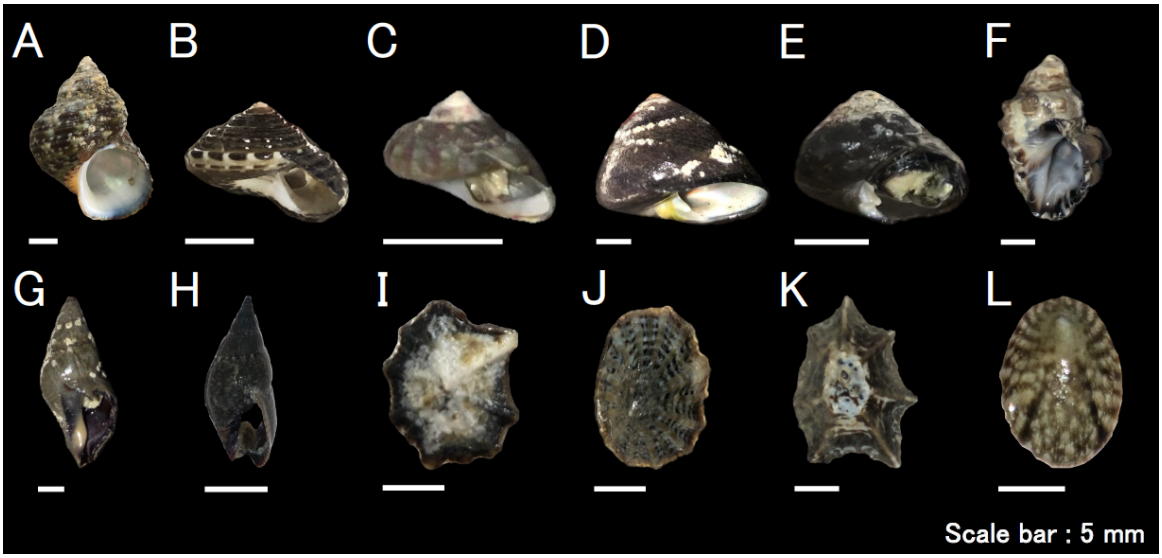

Figure 2. Target species of this study. A: *Turbo stenogyrus*, B: *Eurytrochus cognatus*, C: *Diloma suavis*, D: *Chlorostoma xanthostigma*, E: *Omphalius nigerrimus*, F: *Reishia clavigera*, G: *Japeuthria ferrea*, H: *Nassarius velatus*, I: *Siphonaria sirius,* J: *Siphonaria japonica*, K: *Patelloida saccharina lanx*, L: *Nipponacmea fuscoviridis*

Table 1. Sample list of this study, and the BLAST search results of sequences obtained from the samples

specinen ID	specimen	Vouchered No.	COI				H3			
			Accession No.	BLAST Hits	E-value	Percent Identity	Accession No.	BLAST Hits	E-value	Percent Identity
M-DV-013	*Japeuthria ferrea1*	33030	LC383955	*Japeuthria cingulata*	0	90%	LC384080	*Neptunea cumingi*	5.0E-118	96%
M-DV-014	*Japeuthria ferrea2*	33031	LC383956	*Japeuthria cingulata*	0	90%	LC384081	*Neptunea antiqua*	3.0E-119	97%
M-DV-137	*Japeuthria ferrea3*	33032	LC383957	*Japeuthria cingulata*	7.0E-173	91%	LC384082	*Neptunea cumingi*	3.0E-125	96%
M-DV-017	*Reishia clavigera1*	33037	LC383965	*Reishia clavigera*	0	99%	LC384090	*Thais luteostoma*	2.0E-141	99%
M-DV-133	*Reishia clavigera2*	33038	LC383966	*Reishia clavigera*	0	100%	LC384091	*Thais luteostoma*	2.0E-142	99%
M-DV-134	*Reishia clavigera3*	33039	LC383967	*Reishia clavigera*	0	100%	LC384092	*Thais luteostoma*	3.0E-140	100%
M-DV-094	*Nipponacmea fuscoviridis1*	33040	LC383961	*Nipponacmea fuscoviridis*	0	99%	LC384086	*Nipponacmea fuscoviridis*	4.0E-138	100%
M-DV-095	*Nipponacmea fuscoviridis2*	33041	LC383962	*Nipponacmea fuscoviridis*	0	94%	LC384087	*Nipponacmea fuscoviridis*	1.0E-144	100%
M-DV-096	*Nipponacmea fuscoviridis3*	33042	LC383963	*Nipponacmea fuscoviridis*	0	99%	LC384088	*Nipponacmea fuscoviridis*	4.0E-144	100%
M-DV-097	*Nipponacmea fuscoviridis4*	33043	LC383964	*Nipponacmea schrenckii*	0	100%	LC384089	*Nipponacmea fuscoviridis*	4.0E-133	99%
M-DV-007	*Patelloida lanx2*	33044	LC383960	*Patelloida saccharina lanx*	0	90%	LC384085	*Haplognathia ruberrima*	5.0E-143	99%
M-DV-092	*Patelloida lanx3*	33045	LC383958	*Patelloida saccharina lanx*	0	99%	LC384083	*Patelloida saccharina*	2.0E-147	99%
M-DV-093	*Patelloida lanx4*	33046	LC383959	*Patelloida saccharina lanx*	0	99%	LC384084	*Haplognathia ruberrima*	4.0E-139	99%
M-DV-029	*Siphonaria sirius 1*	33047	LC383968	*Siphonaria sp*	0	99%	LC384093	*Siphonaria denticulata*	1.0E-119	98%
M-DV-030	*Siphonaria sirius 2*	33048	LC383969	*Siphonaria sp*	0	99%	LC384094	*Siphonaria denticulata*	5.0E-118	98%
M-DV-067	*Siphonaria sirius 3*	33049	LC383970	*Siphonaria sp*	0	99%	LC384095	*Siphonaria denticulata*	3.0E-120	98%
M-DV-068	*Siphonaria sirius 4*	33050	LC383971	*Siphonaria sp*	0	99%	LC384096	*Siphonaria denticulata*	2.0E-116	98%
M-DV-069	*Siphonaria sirius 5*	33051	LC383972	*Siphonaria sp*	0	100%	LC384097	*Siphonaria denticulata*	3.0E-120	98%
M-DV-140	*Siphonaria japonica2*	RM33136	LC384342	*Siphonaria sp*	0	99%	LC384345	*Siphonaria denticulata*	1.0E-113	96%
M-DV-141	*Siphonaria japonica3*	RM33137	LC384343	*Siphonaria japonica*	0	99%	LC384346	*Siphonaria denticulata*	1.0E-113	96%
M-DV-158	*Siphonaria japonica5*	RM33138	LC384344	*Siphonaria sp*	0	99%	LC384347	*Siphonaria denticulata*	1.0E-119	98%
M-DV-024	*Omphalius nigerrimus1*	33052	LC383976	*Omphalius nigerrimus*	0	98%	LC384101	*Tegula eiseni*	1.0E-134	97%
M-DV-025	*Omphalius nigerrimus2*	33053	LC383977	*Omphalius nigerrimus*	0	97%	LC384102	*Tegula eiseni*	6.0E-132	96%
M-DV-026	*Omphalius nigerrimus3*	33054	LC383978	*Omphalius nigerrimus*	0	98%	LC384103	*Tegula eiseni*	2.0E-136	97%
M-DV-027	*Omphalius nigerrimus4*	33055	LC383979	*Omphalius nigerrimus*	0	98%	LC384104	*Tegula eiseni*	3.0E-125	96%
M-DV-129	*Eurytrochus cognatus 1*	33062	LC383973	*Eurytrochus cognatus*	0	99%	LC384098	*Gibbula zonata*	1.0E-139	97%
M-DV-130	*Eurytrochus cognatus 2*	33063	LC383974	*Eurytrochus cognatus*	0	99%	LC384099	*Gibbula turbinoides*	9.0E-120	97%
M-DV-131	*Eurytrochus cognatus 3*	33064	LC383975	*Eurytrochus cognatus*	0	99%	LC384100	*Gibbula zonata*	4.0E-139	97%
M-DV-132	*Eurytrochus cognatus 4*	33065	LC383980	*Eurytrochus cognatus*	0	99%	LC384105	*Gibbula zonata*	6.0E-137	97%
M-DV-009	*Chlorostoma xanthostigma1*	33056	LC383981	*Chlorostoma argyrostomum*	0	99%	LC384106	*Tegula eiseni*	4.0E-124	95%
M-DV-135	*Chlorostoma xanthostigma2*	33057	LC383982	*Chlorostoma argyrostomum*	0	99%	LC384107	*Tegula eiseni*	1.0E-134	96%
M-DV-136	*Chlorostoma xanthostigma3*	33058	LC383951	*Chlorostoma argyrostomum*	0	99%	LC384076	*Tegula eiseni*	4.0E-139	96%
M-DV-032	*Turbo stenogyrus1*	33059	LC383952	*Turbo stenogyrus*	0	97%	LC384077	*Turbo sp*	3.0E-124	99%
M-DV-033	*Turbo stenogyrus2*	33060	LC383953	*Turbo stenogyrus*	0	98%	LC384078	*Turbo sp*	5.0E-127	99%
M-DV-034	*Turbo stenogyrus3*	33061	LC383954	*Turbo stenogyrus*	0	98%	LC384079	*Turbo sp*	5.0E-127	99%
M-DV-201	*Nassarius velatus2*	33033	LC383947	*Nassarius siquijorensis*	0	94%	LC384072	*Nassarius conoidalis*	1.0E-154	99%
M-DV-202	*Nassarius velatus3*	33034	LC383948	*Nassarius idyllius*	0	94%	LC384073	*Nassarius hepaticus*	5.0E-153	99%
M-DV-203	*Nassarius velatus4*	33035	LC383949	*Nassarius idyllius*	0	94%	LC384074	*Nassarius conoidalis*	2.0E-152	99%
M-DV-204	*Nassarius velatus5*	33036	LC383950	*Nassarius idyllius*	0	94%	LC384075	*Nassarius hepaticus*	2.0E-152	99%
M-DV-150	*Pictodiloma suavis2*	RM33139	LC458676	*Pictodiloma suavis*	0	98%	LC458680	*Gibbula zonata*	7.0E-137	96%
M-DV-151	*Pictodiloma suavis3*	RM33140	LC458677	*Pictodiloma suavis*	0	98%	LC458681	*Gibbula zonata*	2.0E-136	96%
M-DV-152	*Pictodiloma suavis4*	RM33141	LC458678	*Pictodiloma suavis*	0	98%	LC458682	*Gibbula zonata*	4.0E-139	96%
M-DV-153	*Pictodiloma suavis5*	RM33142	LC458679	*Pictodiloma suavis*	0	98%	LC458683	*Gibbula zonata*	4.0E-139	96%

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same taxonomic grouping with the morphological classification of the particular sample at the species, genus, or family level.

2.4 Sequence Manipulations, Data Sets Building, and Phylogenetic Analyses

Confirmed sequences were aligned using MAFFT version 7 [19]. We used the online version of Gblocks 0.91b to remove ambiguously aligned positions with the least stringent settings [20]. Mesquite version 3.40 [21] was used for sequence visualizations. Outgroup bivalves were selected based on the availability of gene sequence data. Sequences of *Crassostrea gigas* (Acc. No.: AB904889) and *Pinctada fucata* (Acc. No.: GQ355871) for COI outgroups. The out groups for H3 were *C. gigas* (Acc. No.: HQ809488) and *P. fucata* (Acc. No.: HQ329300).

We built six datasets for phylogenetic analyses: Dataset A: COI; Dataset B: COI with the 3rd codon removed; Dataset C: H3; Dataset D: H3 with the 3rd codon removed, Dataset E: A+C concatenated, and Dataset F: B+C concatenated.

The outgroup sequences *C. gigas* and *P. fusca* were chimeric (collected from different individuals) on Datasets E and F.

All phylogenetic trees of all datasets (A–F) were constructed using two inference methods: the Maximum Likelihood (ML) analysis using RAxML-GUI v.1.5 on the GTR-GAMMMA substitution model, with 1000 bootstrap replications [22], and Neighbor-joining (NJ) analysis using MEGA v.7 [23] with the partial deletion option and the Maximum Composite Likelihood model, also with 1000 bootstrap replications.

3. RESULTS

3.1 Obtained Sequences

We succeeded in amplifying the target genes from all samples. We obtained 700 bases for COI, and 318 bases for H3. After sequence editing, alignment, and removals of ambiguously aligned regions, we obtained 628 positions for COI, and 295 positions for H3. 420 positions were left when the 3rd codon positions were omitted from the COI sequences, and 197 positions for H3 without the third codons.

3.2 DNA Barcoding-based Identification

All BLAST searches of the obtained COI, and Histone H3 sequences using BLASTn on the nr database hit gastropod sequences of the same families, and, if reference sequence data were available, genera and species (Table 1). For COI, reference sequences were available in Genbank for all samples at the family and genus levels. However, four species hit only their congeneric sequences (*Chlorostoma xanthostigma*, *Japeuthria ferrea, Nassarius velatus, Siphonaria sirius*).

For Histone H3, only two taxa were identified by BLAST at the species level (*Nipponacmea fuscoviridis* and *Patelloida lanx*) and six genera were not available on Genbank (*Japeuthria*, *Omphalius*, *Eurytrochus*, *Chlorostoma*, and *Pictodiloma*), and thus samples of these genera hit only at the family level (Table 1).

3.3 Phylogenies

Phylogenetic analyses on single gene datasets (data sets A–D) and concatenated datasets (datasets E–F) resulted in well-supported trees (Fig. 3, 4).

All species were monophyletic in the single gene trees, but only on datasets where the third codon is included in the analyses (Datasets A, C; Fig. 3). The topologies of the COI and H3 gene trees were different (Fig. 3). At the family level, the topologies of the single gene trees disagree with morphology-based systematics by Sasaki [21], except for the Histone H3 tree in which the 3rd codon was included in the analysis (Datasets C). For example, *Siphonaria* (Pulmonata) was not included in Neogastropoda, in the gene trees inferred from Datasets A, B, and D.

On the other hand, ML and NJ analyses on the concatenated datasets resulted in trees with well-resolved topologies, regardless of the inclusion of the 3rd codons of COI (Fig. 4). Monophylies at the species, genus, order, subfamily, and family levels in both inference methods, for both Datasets E (COI 3rd codon included) and F (COI 3rd codon excluded) were observed (Fig. 4).

4. DISCUSSION

Phylogenetic analyses on concatenated datasets showed that removing the 3rd codons of the COI gene improves tree topology and taxonomic placement of samples, as indicated by the increased bootstrap supports. Taxonomic groupings also agreed with the morphology-based classification by Sasaki [24]. Mitochondrial genomes are known to have a relatively fast evolutionary rates, and thus the 3rd codons of the mitochondrial gene COI were probably saturated [25], causing homoplasies and thus incorrect phylogenetic inference caused by long branch attractions.

The nuclear gene Histone H3 is known to have a low substitution rate, and was considered useful for phylogenetic analyses at the higher taxonomic levels [26]. However, our result suggested that this

Figure 3. Phylogenetic trees of our samples based on single gene sequences. Trees were inferred using Maximum-likelihood (ML) and Neighbor-joining (NJ) methods. The tree on the left side was analyzed using COI sequences, and the right one using H3 sequences. Numbers above the nodes represent bootstrap values (1000 replicates). Four bootstrap values on each node were obtained from the inferences of the four Datasets (A–D, please read the main text for details) using both ML and NJ. Only the topologies of ML trees inferred from Dataset A (for COI) and Dataset C (for Histone H3) are shown. The four bootstrap values of the COI tree are shown as follow: Dataset A (ML) / Dataset A (NJ) / Dataset B (ML) / Dataset B (NJ), while those of the Histone H3 tree are shown as follow: Dataset C (ML) / Dataset C (NJ) / Dataset D (ML) / Dataset D (NJ). Scale bar indicates branch length.

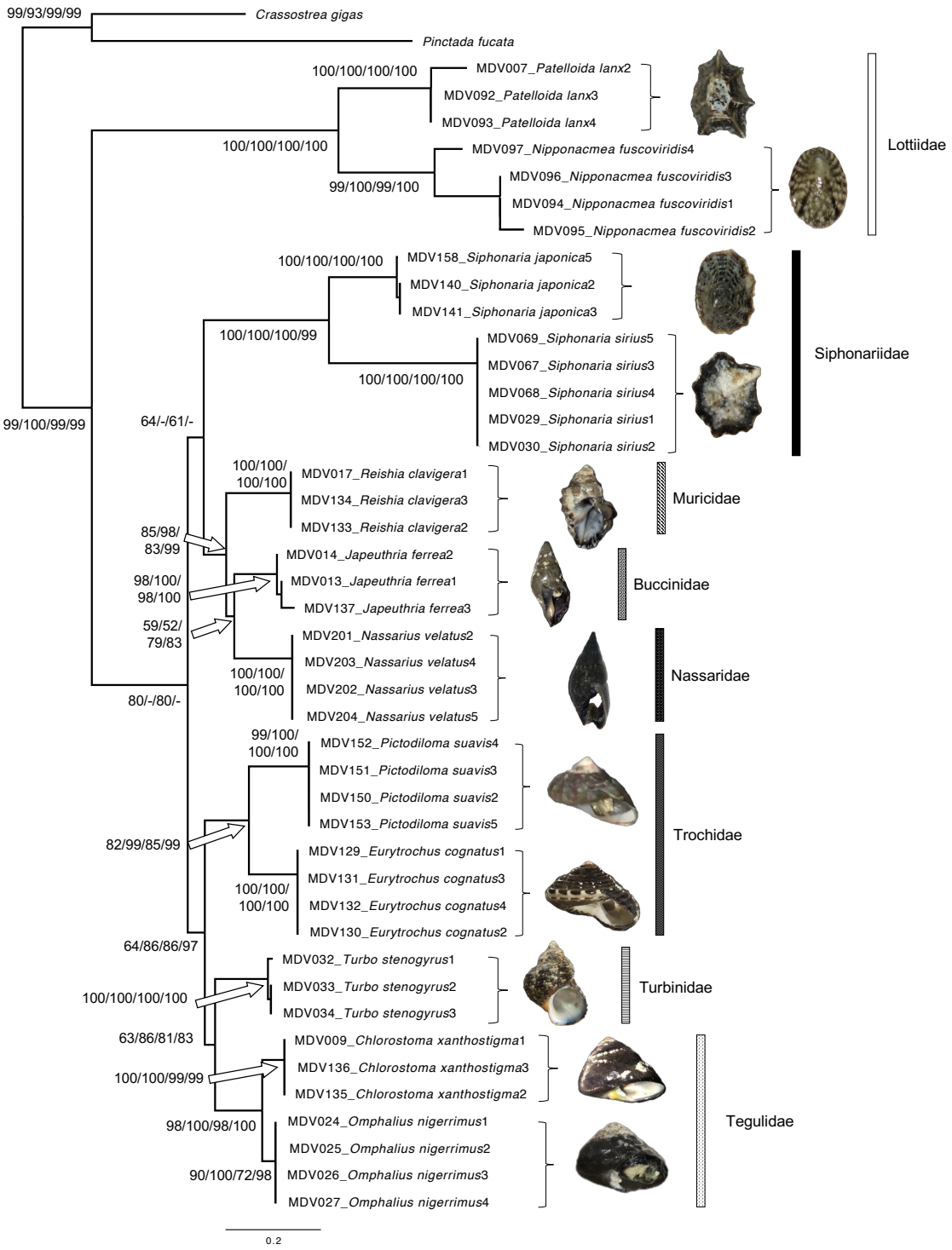

Figure 4. The phylogenetic trees of our samples based on concatenated sequences of the genes COI and H3. The topology shown is the tree inferred using Maximum Likelihood (ML) method, on Dataset E (COI and Histone H3 sequences with their 3rd codons left intact, and Dataset F (COI without the 3rd codons, and Histone H3 sequences with their 3rd codons left intact. Please read the main text for details). The four bootstrap values on each node are shown as follow: Dataset E (ML) / Dataset E (NJ) / Dataset F (ML) / Dataset F (NJ). Scale bar indicates branch length.

gene could also be useful for species-identification, provided that the 3rd codons are included in the analysis. This is probably because the exclusion of the 3rd codon positions of this gene causes the lack of enough nucleotide variations, and thus detrimental to lower taxonomy phylogenetic inference. This result is also indicated by the result of Dataset D (only H3, with the 3rd codon excluded): the topology of Dataset D tree was not well resolved at both the species and higher taxonomic levels (Fig. 3).

Despite the lack of resolution at the higher taxonomic level when used singularly, both genes were successful in placing conspecific samples in a single monophyletic group with high bootstrap supports regardless of the inference method (NJ or ML), provided that the 3rd codons are included in the analysis. The ability of both genes to identify conspesific samples was also indicated with the samples of *Siphonaria sirius* and *S. japonica*. Both genes, when the third codon was included, have enough substitutions place samples of both species, properly. Meanwhile, DNA Barcoding using both gene markers is useful to differentiate samples which form / morphological features, to non-taxonomists, might look similar and thus difficult to classify, such as the two trochid snails (Fig. 4). The two species of *Siphonaria* also showed that even species showing drastically different morphological features could actually be of the same genus (Fig. 4). This result underlines the handiness and practicality of DNA Barcoding for environmental monitoring.

All results considered, we suggest that both the mitochondrial COI and nuclear Histone H3 genes are useful to differentiate species for barcoding purposes. We also suggest that, utilizing two gene markers or more is useful as a fail-safe in DNA Barcoding and eDNA studies: one marker gene might pick sequences of organisms uncollected using another marker gene, while, as our study here suggested, when information of a gene is not available on the reference database such as Genbank, the data of other markers might be available, and thus using multiple markers could help to avoid misidentification or the lack of identification.

5. CONCLUSION

We succeeded in providing novel sequence data of the mitochondrial COI and nuclear Histone H3 genes for 43 individuals of 12 intertidal shelled gastropod species collected from Nada Coast in central Wakayama. We showed that both marker genes are useful for species identifications. Since our samples are vouchered as museum collections (Table. 1), the sequences we provided here will be useful as references for future DNA Barcoding and eDNA studies. Moreover, the sequences could also be useful for future molecular phylogenetic studies of Gastropods.

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