Reassessment of the mitochondrial 12S-rRNA gene for DNA barcoding of museum specimens of shelled marine gastropods from Japan

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Abstract. DNA barcoding is an effective and powerful tool for taxonomic identification and thus very useful for biodiversity monitoring. This study investigated the usefulness of the mitochondrial 12S-rRNA gene for the DNA barcoding of shelled marine gastropods. To do so, we determined partial 12S-rRNA sequences of 75 vouchered museum specimens from 69 species of shelled gastropods from Japan. The specimens have been identified morphologically, and natural history data catalog. Sequence analyses through BLAST searches, maximum likelihood phylogenetic analysis, and species delimitation analysis suggested that the 12S-rRNA gene is helpful for barcoding shelled marine gastropods. They thus could be helpful to complement barcoding studies using other markers such as COI. The analyses successfully confirmed all samples' identity at higher taxonomy (subfamily and above), but much less so at the species level. Our result thus also underlines the lingering problem of DNA barcoding: The lack of comprehensive reference databases of sequences. However, since we provided sequences of properly curated, vouchered museum specimens in this study, our result reported here has thus also helped to give taxonomically reliable reference sequences for biodiversity monitoring and identifications of shelled gastropods which include many important fisheries species.

1 Introduction

With its ca. 35,000 of recorded species, Gastropoda, a class of shelled mollusks (Conchifera), is one of the most prominent invertebrate groups, in which, during its long evolutionary history, has radiated and occupied a diverse array of ecological niches in marine, freshwater, and biofouling/invasive organisms [2]. Many shelled members of this group are also greatly influenced by the recent ocean acidification event caused by global warming [1] [3-4]. As such, it is essential to monitor the diversity of this taxa constantly.

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Recent development in DNA sequencing and the building up of DNA sequence databases have allowed for the usage of DNA sequences for the quick and effective identifications and classifications of samples collected from the field with relatively high accuracy, a method called “DNA barcoding” [5]. Further development of DNA sequencing technology (e.g., Next Generation Sequencing) has allowed for the development of a non-invasive method of biodiversity monitoring using DNA sequences, called the eDNA (environmental DNA = eDNA) method, which is essential barcoding of DNA fragments shed off into the environment by living organisms [6-8]. For DNA barcoding and e-DNA to work, the availability of a robust, reliable, and exhaustive reference database of DNA sequences collected from target taxa, is essential. However, at present, biases in biodiversity studies might have caused such a reliable database to be not available for some taxa, including gastropods (e.g. [9]). In addition, Machida et al. [10] and Page [11] reported that currently, some data are not adequately curated. As a result, some taxa might become unidentifiable and thus become “dark taxa.” This becomes very problematic in monitoring studies, especially those conducted by people with inadequate taxonomical skills and resources, or if target taxa contain many possible undescribed or cryptic species, or when conducting eDNA-based monitoring for which morphological samples are simply unavailable.

On the technical side, the marker genes must have enough base substitutions to distinguish different species of the same genus [5], but not different enough to dramatically differentiate sympatric individuals of the same species. Several effective universal primers to amplify a region of the mitochondrial COI gene have been developed for metazoans, causing a tremendous amount of DNA barcoding studies to be conducted and thus empirically shown that the amplified COI segment has enough substitution rates to distinguish animals at the species level (e.g. [12-17]). However, in a previous study, we have suggested that doing DNA barcoding with only one genetic marker could be risky because of primarily technical problems (limited primer efficacies and the inability of a single quality to place samples properly at higher taxonomies) [16-17]. In that study [16-17], we also suggested that using multiple markers would help to alleviate the problems because it would allow for the collection of a more complete genetic data (primers of different genes might work on samples not amplifiable with those of one marker gene, and a combination of markers would allow for a more robust phylogeny), and thus allowing researchers to collect a more complete picture reflecting the actual biodiversity. Therefore, in that study, we evaluated and thus proposed the usefulness of the nuclear gene Histone-H3 (H3) as a marker for DNA barcoding of shelled gastropods, using previously developed primers [18].

In this study, to develop and assess the utility of another molecular marker for DNA barcoding-based studies of shelled gastropods, we investigated the usefulness of the mitochondrial gene 12S-rRNA using previously reported primers [19-20]. The 12S-rRNA gene has been used in previous molecular phylogenetics, phylogeography, and DNA barcoding of various metazoans, including gastropods. We sequenced the 12S-rRNA of different vouchered samples of shelled marine gastropods stored at the University Museum of The University of Tokyo, including some old museum samples (the oldest was sampled in 1999, and the latest was tested in 2015). Our result presented here, which was based on BLAST searches, phylogenetic analysis, and species delimitation analysis, has confirmed the usefulness of the 12S-rRNA as a genetic marker for DNA barcoding of shelled gastropods, which also include many fisheries species. The result also highlighted the lack of a robust and comprehensive reference database of this gene if it is to be used as a marker for gastropods. Meanwhile, because we used properly curated and vouchered museum specimens as samples, the natural history data of our representatives are reliable. Therefore, we have also contributed a set of reference sequence data from taxonomically reliable samples through this study, which is crucial for biodiversity monitoring using DNA barcoding and e-DNA.

2 Materials and methods

2.1 Sample collection

A total of 75 individuals of 69 species of shelled gastropods were used in this study. All samples used in this study were vouchered specimens stored in the University Museum, The University of Tokyo. These samples were initially collected from various locations in Japan, and then fixed and stored in 95% EtOH. Morphological identifications (based on [21]) of collected samples were conducted before or after fixation. Representatives were chosen at random, with one or two individuals per species. Most specimens are at least nine years old, with the most senior sample collected in 1999, and the latest in 2015. The list of samples is provided in Table 1.

2.2 DNA sequencing and sequence data acquisition

A piece of the muscle tissue from the mantle or the foot (about 0.25 mg) was cut out from each sample. Total genomic DNA was extracted using the standard CTABphenol-chloroform method. PCR was performed using a standard protocol but with an annealing temperature of 52°C. Three combinations of previously published three primers (Table 2) [19-20] were used to amplify the 12S-rRNA fragment of samples. Sanger sequencing of amplicons (using both the forward and reverse primers) was outsourced (FASMAC Co. Ltd., Kanagawa, Japan). For comparison, we also sequenced a fragment of the COI gene of all samples, using previously published primers [22-24]. The list of primers used in this study is shown in Table 2. Obtained sequences were then checked for contamination by BLASTn searches [25]. Sequence fidelity was confirmed and edited manually on the software MESQUITE ver. 3.61 [26-27], by also simultaneously checking the chromatograms by eye (visualized on ApE ver.2.0.61 [28]). After sequence editing, all forward and reverse sequences were assembled manually by eye.

2.3 Sequence identification (DNA barcoding) through BLASTn searches

In order to confirm if the obtained sequences were homologous to previously published arrangements, and thus to get taxonomic information of the organisms from which the lines were obtained, we performed BLASTn searches on the assembled sequences. We consider a sample as correctly identified if the morphological identification matches the BLASTn search result. We also confirmed at which taxonomic level a particular sequence was identified (species, genus, family, order) to check the availability of reference sequences on GenBank and the fidelity of the GenBank sequences. BLASTn searches were conducted for both the 12S-rRNA and COI gene sequences.

Table 1. List of analyzed OTU in this study, along with the BLASTn search results for DNA

Sample No.	Species	12S					
		Genebank ID	BLASTn result	Homology Level	E-value	Identity (%)	Gaps%
SS45	<i>Batillaria multiformis</i>	NC_047187.1	<i>Batillaria attramentaria</i>	Genus	0	98	0
B308	<i>Batillaria zonalis</i>	HQ833855.1	<i>Batillaria cumingi</i>	Genus	0	98	0
SS104	<i>Planaxis sulcatus</i>	HQ833854.1	<i>Planaxis sulcatus</i>	Species	0	99	0
B309	<i>Semisulcospira libertina</i>	NC_037771.1	<i>Semisulcospira coreana</i>	Genus	3.0E-160	93	0
B303	<i>Cerithidea cingulata</i>	AB535193.1	<i>Cerithidea djadjariensis</i>	Genus	8.0E-126	88	3
SS57	<i>Nerita albicilla</i>	LC215360.1	<i>Nerita albicilla</i>	Species	0	99	0
SS124	<i>Nerita helicinaoides</i>	MT161611.1	<i>Nerita chamaeleon</i>	Genus	6.0E-162	94	0
SS59	<i>Nerita japonica</i>	LC565707.1	<i>Nerita japonica</i>	Species	0	99	0
SS126	<i>homiodoris japonica</i>	MT161611.1	<i>Nerita chamaeleon</i>	Genus	1.0E-173	96	0
SS120	<i>Nerita striata</i>	KF728888.1	<i>Nerita fulgurans</i>	Genus	2.0E-162	94	0
SS103	<i>Cassidula mustelina</i>	KJ920319.1	<i>Cassidula nucleus</i>	Species	2.0E-171	96	0
SS141	<i>Erronea errone</i>	HQ833858.1	<i>Erronea errone</i>	Species	0	98	0
SS83	<i>Echinolittorina radiata</i>	AJ623151.1	<i>Echinolittorina radiata</i>	Species	0	99	0
SS142	<i>Notocochlis gualteriana</i>	MK507895.1	<i>Notocochlis sp.</i>	Genus	0	99	0
SS143	<i>Canarium mutabile</i>	MW244820.1	<i>Tridentarius dentatus</i>	Family	1.0E-163	94	0
SS110	<i>Conomurex luhuanus</i>	KY853669.1	<i>Conomurex luhuanus</i>	Species	0	99	0
B349	<i>Mitrella burchardi</i>	HQ833864.1	<i>Mitrella bicincta</i>	Genus	0	99	0
B366	<i>Fusinus ferrugineus</i>	NC_045906.1	<i>Fusinus longicaudus</i>	Genus	0	98	0
SS115	<i>Pleuroploca trapezium</i>	MN322355.1	<i>Turritatirus turritus</i>	Subfamily	2.0E-137	90	1
B355	<i>Nassarius conoidalis</i>	NC_041310.1	<i>Nassarius conoidalis</i>	Species	0	99	0
B342	<i>Nassarius fraterculus</i>	NC_037604.1	<i>Nassarius fraterculus</i>	Species	0	99	0
SS102	<i>Nassarius albescens</i>	KY489008.1	<i>Nassarius fenestratus</i>	Genus	0	99	0
SS144	<i>Nassarius coronatus</i>	KY488995.1	<i>Nassarius coronatus</i>	Species	0	99	0
SS44	<i>Cantharus mollis</i>	HQ833883.1	<i>Cantharus cecillei</i>	Genus	0	97	0
B344	<i>Erzincopsis menkeana</i>	FM999097.1	<i>Pisania striata</i>	Family	5.0E-158	93	0
SS140	<i>Pterygia dactylus</i>	KR087379.1	<i>Pterygia dactylus</i>	Species	0	100	0
SS123	<i>Coralliophila neritoidea</i>	AJ293679.1	<i>Coralliophila neritoidea</i>	Species	0	98	0
SS106	<i>Drupella cornus</i>	FR853980.1	<i>Drupella cornus</i>	Species	0	99	0
B356	<i>Oppomorus funiculatus</i>	HE583824.1	<i>Morula funiculata</i>	Species	0	99	0
B318	<i>Nucella lima</i>	KJ093800.1	<i>Nucella heysseana</i>	Genus	0	100	0
B336	<i>Ocenebra inornatus endermonis</i>	NC_046052.1	<i>Ocenebrellus falcatus</i>	Genus	0	98	0
SS81	<i>Mancinella echinata</i>	HE584089.1	<i>Mancinella echinata</i>	Species	0	99	0
SS70	<i>Mancinella siro</i>	HE584090.1	<i>Mancinella grossa</i>	Genus	0	99	0
SS86	<i>Menathais tuberosa</i>	KU747972.1	<i>Menathais tuberosa</i>	Species	0	99	0
B302	<i>Reishia bronni</i>	NC_039165.1	<i>Thais luteostoma</i>	Genus	0	98	0
SS75	<i>Reishia bronni</i>	HQ833878.1	<i>Thais luteostoma</i>	Genus	0	99	0
B293	<i>Thais clavigera</i>	HE584119.1	<i>Reishia clavigera</i>	Species	0	99	0
SS34	<i>Thais clavigera</i>	HE584119.1	<i>Reishia clavigera</i>	Species	0	99	0

Table 1. (Continued).

Sample No.	Species	12S					
		Genebank ID	BLASTn result	Homology Level	E-value	Identity (%)	Gaps%
SS116	<i>Vasum turbinellum</i>	HQ833909.1	<i>Vasum turbinellus</i>	Species	0	99	0
B363	<i>Homiodoris japonica</i>	KP635442.1	<i>Homiodoris japonica</i>	Species	0	99	0
B323	<i>Acmaea pallida</i>	MT370382.1	<i>Niveotectura pallida</i>	Species	5.0E-132	93	0
B253	<i>Collisella dorsuosa</i>	AB106454.1	<i>Lottia dorsuosa</i>	Species	1.0E-152	99	0
B334	<i>Tectura emydia</i>	MT370375.1	<i>Lottia instabilis</i>	Genus	0	99	0
B357	<i>Lepeta kuragiensis</i>	AB238235.1	<i>Cryptobranchia kuragiensis</i>	Species	1.0E-149	99	0
SS95	<i>Lottia luchuana</i>	AB106453.1	<i>Lottia luchuana</i>	Species	2.0E-172	99	0
B396	<i>Nipponacmea boninensis</i>	KU316594.1	<i>Nipponacmea radula</i>	Genus	0	99	0
SS14	<i>Nipponacmea radula</i>	KU316566.1	<i>Nipponacmea fuscoviridis</i>	Genus	0	100	0
S391	<i>Nipponacmea schrenckii</i>	KU316582.1	<i>Nipponacmea schrenckii</i>	Species	0	96	0
SS17	<i>Patelloida pygmaea</i>	AB106436.1	<i>Patelloida pygmaea</i>	Species	7.0E-161	99	0
SS91	<i>Patelloida ryukyuensis</i>	AB238280.1	<i>Patelloida ryukyuensis</i>	Species	2.0E-166	100	0
B256	<i>Patelloida saccharina lanx</i>	LC142820.1	<i>Nipponacmea boninensis</i>	Family	1.0E-163	99	0
SS51	<i>Patelloida saccharina lanx</i>	AB106439.1	<i>Patelloida saccharina lanx</i>	Species	8.0E-171	100	0
SS2	<i>Cellana grata</i>	GQ455887.1	<i>Cellana toreuina</i>	Genus	0	100	0
SS209	<i>Cellana grata</i>	AB106427.1	<i>Cellana grata</i>	Species	3.0E-134	99	0
SS18	<i>Cellana nigrolineata</i>	LC600801.1	<i>Cellana nigrolineata</i>	Species	2.0E-141	99	0
SS174	<i>Cellana radiata</i>	AB106430.1	<i>Cellana radiata orientalis</i>	Species	2.0E-152	99	0
SS84	<i>Cellana testudinaria</i>	AB106431.1	<i>Cellana testudinaria</i>	Species	1.0E-153	100	0
SS3	<i>Cellana toreuina</i>	GQ455887.1	<i>Cellana toreuina</i>	Species	0	99	0
SS4	<i>Cellana toreuina</i>	GQ455860.1	<i>Cellana mazatlandica</i>	Genus	0	99	0
B247	<i>Scutellastra flexuosa</i>	AF058183.1	<i>Scutellastra flexuosa</i>	Species	6.0E-117	92	1
B257	<i>Siphonaria sirius</i>	KF001136.1	<i>Siphonaria sp.</i>	Genus	1.0E-172	100	0
B359	<i>Siphonaria sp.</i>	KF001069.1	<i>Siphonaria sp.</i>	Genus	8.0E-81	84	3
SS131	<i>Tectus pyramis</i>	MF138911.1	<i>Tectus pyramis</i>	Species	0	97	0
B268	<i>Chlorostoma argyrostoma lischkei</i>	HE800683.1	<i>Chlorostoma lischkei</i>	Genus	5.0E-167	100	0
B285	<i>Chlorostoma lischkei</i>	HE800683.1	<i>Chlorostoma lischkei</i>	Species	0	99	0
SS72	<i>Chlorostoma turbinatum</i>	HE800683.1	<i>Chlorostoma lischkei</i>	Genus	0	99	0
SS73	<i>Tegula pfeifferi pfeifferi</i>	NC_056356.1	<i>Omphalius rusticus</i>	Genus	0	98	0
SS67	<i>Omphalius nigerrimus</i>	NC_031862.1	<i>Omphalius nigerrimus</i>	Species	0	99	0
SS69	<i>Omphalius rusticus</i>	AF080631.1	<i>Tegula rusticus</i>	Species	0	99	0
B254	<i>Cantharidus japonicus</i>	AB505369.1	<i>Cantharidus jessoensis</i>	Genus	0	93	1
B345	<i>Cantharidus jessoensis</i>	AB505369.1	<i>Cantharidus jessoensis</i>	Species	0	99	0
B332	<i>Lunella coreensis</i>	MN604179.1	<i>Lunella coreensis</i>	Species	0	99	0
SS54	<i>Lunella coreensis</i>	MN604179.1	<i>Lunella coreensis</i>	Species	0	99	0
SS101	<i>Lunella coronata</i>	KX298890.1	<i>Lunella granulata</i>	Genus	0	99	0
B316	<i>Turbo stenogyvus</i>	FR695555.1	<i>Turbo kenwilliamsi</i>	Genus	0	94	1

Table 1. (Continued).

Sample No.	Species	COI					
		Genebank ID	BLASTn result	Homology Level	E-value	Identity (%)	Gaps%
SS45	<i>Batillaria multiformis</i>	AB845820.1	<i>Batillaria multiformis</i>	Species	0	99	0
B308	<i>Batillaria zonalis</i>	MN389045.1	<i>Batillaria cumingii</i>	Genus	0	99	0
SS104	<i>Planaxis sulcatus</i>	MT620956.1	<i>Planaxis sulcatus</i>	Species	0	99	0
B309	<i>Semisulcospira libertina</i>	KM031760.1	<i>Semisulcospira libertina</i>	Species	0	98	0
B303	<i>Cerithidea cingulata</i>	HE680370.1	<i>Cerithideopsis cingulata</i>	Species	0	100	0
SS57	<i>Nerita albicilla</i>	EU253356.1	<i>Nerita albicilla</i>	Species	0	97	0
SS124	<i>Nerita helicinoides</i>	EU732252.1	<i>Nerita helicinoides</i>	Species	0	99	0
SS59	<i>Nerita japonica</i>	EU732262.1	<i>Nerita japonica</i>	Species	0	98	0
SS126	<i>homiodoris japonica</i>	MW277894.1	<i>Nerita polita</i>	Species	0	99	0
SS120	<i>Nerita striata</i>	EU732335.1	<i>Nerita undata</i>	Genus	0	100	0
SS103	<i>Cassidula mustelina</i>	MN389193.1	<i>Cassidula nucleus</i>	Species	0	99	0
SS141	<i>Erronea erronea</i>	MK507895.1	<i>Notocochlis sp.</i>	-	0	96	0
SS83	<i>Echinolittorina radiata</i>	HM560004.1	<i>Echinolittorina radiata</i>	Species	0	99	0
SS142	<i>Notocochlis gualteriana</i>	MK507895.1	<i>Notocochlis sp.</i>	Genus	0	95	0
SS143	<i>Canarium mutabile</i>	DQ525218.1	<i>Strombus mutabilis</i>	Species	0	96	0
SS110	<i>Conomurex luhuanus</i>	KY853669.1	<i>Conomurex luhuanus</i>	Species	0	96	2
B349	<i>Mitrella burchari</i>	JN052989.1	<i>Mitrella bicincta</i>	Genus	0	100	0
B366	<i>Fusinus ferrugineus</i>	HM180585.1	<i>Fusinus longicaudus</i>	Genus	0	100	0
SS115	<i>Pleuroploca trapezium</i>	KT753962.1	<i>Pleuroploca trapezium</i>	Species	0	97	0
B355	<i>Nassarius conoidalis</i>	KY783915.1	<i>Nassarius conoidalis</i>	Species	0	99	0
B342	<i>Nassarius fraterculus</i>	KX069666.1	<i>Nassarius fraterculus</i>	Species	0	100	0
SS102	<i>Nassarius albescens</i>	KY499727.1	<i>Nassarius albescens</i>	Species	0	99	0
SS144	<i>Nassarius coronatus</i>	KY451287.1	<i>Nassarius coronatus</i>	Species	0	97	0
SS44	<i>Cantharus mollis</i>	JN053007.1	<i>Cantharus cecillei</i>	Genus	3.0E-89	88	1
B344	<i>Enzinopsis menkeana</i>	KX519514.1	<i>Engina lanceolata</i>	Genus	0	93	0
SS140	<i>Pterygia dactylus</i>	KR087291.1	<i>Pterygia dactylus</i>	Species	0	96	0
SS123	<i>Coralliophila neritoidea</i>	MG917504.1	<i>Coralliophila violacea</i>	Species	0	99	0
SS106	<i>Drupella cornus</i>	FR853843.1	<i>Drupella cornus</i>	Species	0	99	0
B356	<i>Oppomorus funiculatus</i>	HE584045.1	<i>Morula funiculata</i>	Species	0	99	0
B318	<i>Nucella lima</i>	KJ093791.1	<i>Nucella heyseana</i>	Genus	0	99	0
B336	<i>Ocenebra inornatus endermonis</i>	HM180657.1	<i>Lunella corensis</i>	-	0	99	0
SS81	<i>Mancinella echinata</i>	KC466605.1	<i>Mancinella echinata</i>	Species	0	97	2
SS70	<i>Mancinella siro</i>	HE584344.1	<i>Mancinella siro</i>	Species	0	98	0
SS86	<i>Menathais tuberosa</i>	KU747972.1	<i>Menathais tuberosa</i>	Species	0	99	0
B302	<i>Reishia bronni</i>	HM180825.1	<i>Thais luteostoma</i>	Genus	0	99	0
SS75	<i>Reishia bronni</i>	NC_039165.1	<i>Thais luteostoma</i>	Genus	0	99	0
B293	<i>Thais clavigera</i>	HM180819.1	<i>Reishia clavigera</i>	Species	0	98	1
SS34	<i>Thais clavigera</i>	MH400316.1	<i>Reishia clavigera</i>	Species	0	99	0

Table 1. (Continued).

Sample No.	Species	COI					
		Genebank ID	BLASTn result	Homology Level	E-value	Identity (%)	Gaps%
SS116	<i>Vasum turbinellum</i>	HQ834084.1	<i>Vasum turbinellus</i>	Species	0	99	0
B363	<i>Homoiodoris japonica</i>	KP635442.1	<i>Homoiodoris japonica</i>	Species	0	99	0
B323	<i>Acmaea pallida</i>	LC416617.1	<i>Niveotectura pallida</i>	Species	0	97	0
B253	<i>Collisella dorsuosa</i>	KM221108.1	<i>Lottia dorsuosa</i>	Species	0	91	0
B334	<i>Tectura emydia</i>	MT814212.1	<i>Lottia instabilis</i>	Genus	0	99	0
B357	<i>Lepeta kuragiensis</i>	AB543974.1	<i>Cryptobranchia kuragiensis</i>	Species	0	99	0
SS95	<i>Lottia luchuana</i>	AB238471.1	<i>Lottia luchuana</i>	Species	0	99	0
B396	<i>Nipponacmea boninensis</i>	LC383956.1	<i>Japeuthria ferrea</i>	-	0	99	0
SS14	<i>Nipponacmea radula</i>	KC844158.1	<i>Nipponacmea fuscoviridis</i>	Genus	0	99	0
S391	<i>Nipponacmea schrenckii</i>	FR693994.1	<i>Lunella coreensis</i>	-	0	100	0
SS17	<i>Patelloida pygmaea</i>	AB238519.1	<i>Patelloida pygmaea</i>	Species	0	98	0
SS91	<i>Patelloida ryukyuensis</i>	AB238520.1	<i>Patelloida ryukyuensis</i>	Species	0	99	0
B256	<i>Patelloida saccharina lanx</i>	HM180776.1	<i>Patelloida saccharina lanx</i>	Species	0	99	0
SS51	<i>Patelloida saccharina lanx</i>	HM180776.1	<i>Patelloida saccharina lanx</i>	Species	0	99	0
SS2	<i>Cellana grata</i>	HM180722.1	<i>Notoacmea schrenckii</i>	-	0	100	0
SS209	<i>Cellana grata</i>	KM221072.1	<i>Cellana grata</i>	Species	0	98	0
SS18	<i>Cellana nigrolineata</i>	LC600801.1	<i>Cellana nigrolineata</i>	Species	0	100	0
SS174	<i>Cellana radiata</i>	AB238554.1	<i>Cellana radiata orientalis</i>	Species	0	99	0
SS84	<i>Cellana testudinaria</i>	AB238563.1	<i>Cellana testudinaria</i>	Species	0	99	0
SS3	<i>Cellana toreuma</i>	HM180724.1	<i>Notoacmea schrenckii</i>	-	0	100	0
SS4	<i>Cellana toreuma</i>	KM221072.1	<i>Cellana grata</i>	Genus	0	99	0
B247	<i>Scutellastra flexuosa</i>	KT149318.1	<i>Scutellastra flexuosa</i>	Species	0	87	0
B257	<i>Siphonaria sirius</i>	KF000832.1	<i>Siphonaria sp.</i>	Genus	0	99	0
B359	<i>Siphonaria sp.</i>	MF652008.1	<i>Siphonaria fuegiensis</i>	Genus	1.0E-175	84	0
SS131	<i>Tectus pyramis</i>	MN388983.1	<i>Tectus pyramis</i>	Species	6.0E-177	95	0
B268	<i>Chlorostoma argyrostoma lischkei</i>	EU530144.1	<i>Chlorostoma lischkei</i>	Genus	0	99	0
B285	<i>Chlorostoma lischkei</i>	EU530145.1	<i>Chlorostoma lischkei</i>	Species	0	99	0
SS72	<i>Chlorostoma turbinatum</i>	LC413975.1	<i>Chlorostoma turbinatum</i>	Species	0	99	0
SS73	<i>Tegula pfeifferi pfeifferi</i>	HM180731.1	<i>Omphalius pfeifferi carpenteri</i>	Species	0	99	0
SS67	<i>Omphalius nigerrimus</i>	HE800629.1	<i>Tegula aff. Argyrostoma</i>	Genus	0	98	0
SS69	<i>Omphalius rusticus</i>	NC_056356.1	<i>Omphalius rusticus</i>	Species	0	99	0
B254	<i>Cantharidus japonicus</i>	EU530120.1	<i>Cantharidus callichroa</i>	Genus	0	90	0
B345	<i>Cantharidus jessoensis</i>	AB505280.1	<i>Cantharidus jessoensis</i>	Species	0	100	0
B332	<i>Lunella coreensis</i>	MN604179.1	<i>Lunella coreensis</i>	Species	0	99	0
SS54	<i>Lunella coreensis</i>	MN604179.1	<i>Lunella coreensis</i>	Species	0	99	0
SS101	<i>Lunella coronata</i>	KX298890.1	<i>Lunella granulata</i>	Genus	0	99	0
B316	<i>Turbo stenogyrus</i>	AM403915.1	<i>Turbo stenogyrus</i>	Species	0	99	0

Table 2. List the 12S-rRNA and COI gene primers used in this study.

Gene	Primer	Sequence(5'-3')	Reference
12S	12Sma (F)	CTGGGATTAGATACCCTGTTAT	[19]
	12S97L (F)	AACYCAAAGRACTTGCGGT	[20]
	12Smb (R)	CAGAGAGTGACGGGCGATTTGT	[19]
COI	LCO1490 (F)	GGTCAACAAATCATAAAGATATTGG	[22]
	LCOmod (F)	TCTACTAATCATAAGGAYATYGGNAC	[24]
	HCO2198 (R)	TAAACTTCAGGGTGACCAAAAAATCA	[22]
	H7005 (R)	CCGGATCCACNACRTARTANGTRTCRTG	[23]
	HCOmod (R)	ACTTCTGGGTGTCCRAARAAYCARAA	[24]

2.4 Sequence editing, dataset preparation, and phylogenetic analysis

Confirmed, edited, and assembled sequences were aligned using MAFFT v7 [29]., the dataset containing aligned sequences was edited in Gblocks 0.91b (Online ver.) to exclude ambiguously aligned regions [30]. Furthermore, visualized in Mesquite ver. 3.61 [26-27]. Maximum likelihood (ML) phylogenetic inference was conducted in RAxML-GUI v.1.5 [31] under the GTR-GAMMA substitution model, partitioned per-gene, with 1000X bootstrap samplings carried out to assess the robustness of the obtained topology. Model selection before phylogenetic analysis was carried out in MEGA X [32]. Two Bivalves, *Pinctada fucata* (AB250258.1) and *Crassostrea gigas* (EF484878.1), were used as outgroups. Phylogenetic analysis was conducted only on the 12S-rRNA gene sequences.

2.5 Species delimitation analysis

Species delimitation analyses were conducted on the sequence datasets of COI and 12S-rRNA using the Automatic Barcode Gap Discovery (ABGD) software [33] to see if the 12S-rRNA fragment was used in our barcoding could differentiate species]. The analyses used the aligned sequence data of the 12S-rRNA (length = 280 bp) and COI (length = 632 bp). Prior intraspecific divergence range (Pmin to Pmax) was set to 0.001 – 0.1, and the X value for the minimum relative gap width was set to 0.99 under the K2P model with TS/TV = 2.0.

3 Results

3.1 Sequence data acquisition

For the 12S-rRNA gene marker, we successfully amplified and thus obtained ca. 450 bp for 12Sma / 12Smb primer pairs (60 samples) and ca. 430 bp of 12S97L / 12Smb primer pairs (15 samples), making us successfully obtain the 12S-rRNA sequences of all individuals used in this study. After sequence editing and alignments, the 12S-rRNA sequence lengths used for phylogenetic analysis were 280 bp. We also successfully amplified the COI gene marker for all samples in this study (ca. 650 bp for LCO1490 / HCO2198 primer pairs = 54 samples; ca. 1100 bp for LCO1490 / H7005 primer pairs = 6 samples; ca. 650 bp for LCOmod_Kano2008 / HCOmod_Kano2008 primer pairs = 15 samples). The sequence length of the COI marker after sequence editing and alignments was 632 bp.

3.2 BLASTn searches-based DNA barcoding

BLASTn searches of the gene 12S-rRNA matched 39 species of 41 individuals (Table 1), with the sequence identities of 92%–100% and e-values of 0.00 to 6.00 e-117. Meanwhile, 30 samples of 29 species were confirmed only at the genus level (22 genera). The rest of the samples (four individuals) matched Genbank sequences at higher taxonomy (i.e., subfamily, family, and order levels). Meanwhile, for the COI gene, 47 species (50 individuals; 67%) were confirmed at the species level, with 25 individuals matched at the taxonomic levels of genera and above. Detailed results of the BLASTn searches are presented in Table 1.

3.3 Obtained phylogeny and the taxonomic placement of the samples

The phylogenetic tree obtained from the maximum likelihood phylogenetic inference performed on the 12s-rRNA sequences is shown in Figure 1. Most samples were placed along with their morphologically identified species and genera levels with relatively reasonable support values (e.g., *Cellana*, BS = 72%; *Chlorostoma*, BS = 70%; *Nerita*, BS = 53%). However, *Nassarius* and *Patelloida* were not monophyletic, although both genera were placed in their proper higher taxonomies (*Nassarius*: Buccinoidea; *Patelloida*: Lottiidae). Placements at the higher taxonomy levels (subfamily and above) for other genera and species also placed most of them in their valid taxa, despite the lack of strong bootstrap support on many clades, and improper taxonomic placements of some samples (Figure 1), for example, members of Muricoidea, *Nucella lima*, and *Ocenebra inornatus endermonis*, were placed in Buccinoidea, while *Notocochlis gualteriana* (Naticoidea) was placed in Muricoidea; the Cypraeoid *Erronea erronea* was placed in Buccinoidea; some members of Littorinimorpha, *Erronea erronea*, *Notocochlis gualteriana*, *Canarium mutabile*, *Conomurex luhuanus*, were instead put inside Neogastropoda, and *Cassidula mustelina* (Ellobiida) was included in Neogastropoda.

3.4 Species delimitations of target samples

Species delimitations conducted in ABGD on the 12S-rRNA gene dataset resulted in the identification of 61 species, while the same analysis on the dataset of the COI gene identified 65 species (Table 3). Meanwhile, our samples (75 OTUs) were morphologically identified as 69 species. As shown in Table 3, the result of ABGD on both genes generally agrees with the outcome of morphological identification. However, some closely related congeneric species were not properly delimited and identified as the same species in one of the markers or both. For example, *Batillaria multiformis* and *Batillaria zonalis* were identified as one species by the 12s-rRNA, while *Cellana grata* and *Cellana toreuma* were identified as one species by both gene markers.

Fig. 1. Maximum likelihood phylogenetic tree of our samples based on 12S-rRNA sequences obtained in this study. Numbers on a branch denote bootstrap value of an adjacent node. Bootstrap values lower than 41% are not written, while fully supported nodes are denoted by ■.

4 Discussion

4.1 Sequence data acquisition success rate on preserved museum samples

In this study, we tested the 12S-rRNA primers on various museum samples across the whole Gastropods. The usefulness of the COI primers has been shown in multiple previous studies [13-14], including those of ours [16-17]. In general, our results here indicate that the 12S-rRNA primers tested in this study are helpful and can amplify the 12S-rRNA sequence fragments for DNA barcoding to complement results obtained using other markers such as COI. Meanwhile, we successfully got DNA sequences of both gene markers for all samples using standard PCR protocols, even though some of the samples were old museum samples, which were stored at conditions not ideal for molecular works (e.g., room temperature storage). Therefore, our result also suggests that these primers could probably be used for museums studies, such as museum samples barcoding and studies to obtain molecular data out of old museum samples [34].

4.2 The non-exhaustiveness of Genbank for the identification of gastropods

We performed BLASTn searches of the COI and 12S-rRNA sequences obtained from the samples to see if the sequences of our taxa are present on Genbank, besides checking if our morphological identification was consistent with the sequence data on Genbank. The result of our BLASTn searches suggested that most samples only 55% of our samples were correctly identified using the 12S-rRNA and only 67% even when using COI, which is the most commonly used DNA barcoding marker [35]. This result thus underlines the problem of taxonomic bias in biodiversity observation, causing the incompleteness and/or non-exhaustiveness of the data base [9]. This could be problematic for studies depending on identification based on DNA sequences only, such as eDNA and metagenomics [6].

4.3 The phylogeny is relatively well-resolved for a single marker

We also conducted a phylogenetic analysis of the 12S-rRNA sequences to see if the gene could adequately place the samples in their proper taxa. We found that while most samples were properly grouped with their conspecifics or congeners, some samples were not (Figure 1). At present, we are unable to pinpoint the cause of these misplacements, which might include sequence errors, homoplasies and long-branch attractions, and the possible lack of identifying substitutions of the 12S-rRNA fragment used in our phylogenetic analysis. Meanwhile, classification at higher taxonomy, in general, is congruent with the recently proposed gastropod systematics [36-37]. This result is generally also in agreement with the result of our preliminary study [38]. However, detailed interrelationships did not agree entirely, and the statistical supports were low in most nodes of higher taxonomy. It is expected since the interrelationships among higher taxa (above genus) cannot usually be resolved using only single-gene data [16-17,38-39].

4.4 The 12S-rRNA marker was able to delimit most species in this study

We also conducted species delimitation analyses on the COI and 12S-rRNA to confirm if both characteristics could correctly identify/delimit the species of the samples, as identified morphologically. There was 69 morphospecies (out of 75 individuals) in our samples, which were identified by professional taxonomists/curators, which were also co-authors, of this study. Interestingly, however, both markers were unable to completely delimit all morphospecies (COI = 65 species, but 12S = 71 species), apparently having difficulties differentiating closely related congeneric species (Table 3).

The differences in species delimitation could probably be attributed to differences in the substitution rates of each taxon, which might be related to their different biology. This was also suggested by the species delimitation results of both genes before the removal of ambiguously aligned regions by GBlocks. These results indicated that the removal of ambiguously aligned regions might affect the detection of sequence diversity due to the removal of possible informative areas [40]. However, all in all, our result also indicated that both markers could delimit the samples at least at the genus level.

4.5 General conclusion and future directions

Our present results of species delimitation analysis, phylogenetic analysis, and BLASTn searches suggest that the short fragment of the 12S-rRNA gene used in this study is useful and effective enough to delimit various gastropod species, and thus useful for DNA barcoding and metabarcoding (eDNA studies) of shelled marine gastropods. The marker could thus be used to complement other DNA barcoding markers such as COI and 18S-rRNA. DNA

barcoding using multiple markers would allow researchers to capture a complete snapshot of biodiversity and avoid the numerous possible pitfalls caused by using only a single marker [16-17, 41-44].

Moreover, because our study presented appropriately used curated museum samples, our sequence data would become an essential addition to the reference database for future studies. Therefore, in the future, we will register our sequence data to an adequately curated database such as Genbank or DDBJ. We will also continue our study by testing more prospective markers on more properly curated museum samples of shelled marine gastropods from Japan to provide a comprehensive reference sequence database for further studies involving DNA barcoding, metabarcoding, and e-DNA.

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Table 3. Results of analysis of 12S-rRNA and COI using ABGD.

#	Sample No.	Morphological	12S	COI
1	SS45	<i>Batillaria multiformis</i>	<i>12S_species 1</i>	<i>COI_species 1</i>
2	B308	<i>Batillaria zonalis</i>		<i>COI_species 2</i>
3	SS104	<i>Planaxis sulcatus</i>	<i>12S_species 2</i>	<i>COI_species 3</i>
4	B309	<i>Semisulcospira libertina</i>	<i>12S_species 3</i>	<i>COI_species 4</i>
5	B303	<i>Cerithidea cingulata</i>	<i>12S_species 4</i>	<i>COI_species 5</i>
6	SS57	<i>Nerita albicilla</i>	<i>12S_species 5</i>	<i>COI_species 6</i>
7	SS124	<i>Nerita helicinoides</i>	<i>12S_species 6</i>	<i>COI_species 7</i>
8	SS120	<i>Nerita striata</i>		<i>COI_species 8</i>
9	SS59	<i>Nerita japonica</i>	<i>12S_species 7</i>	<i>COI_species 9</i>
10	SS126	<i>Homiodoris japonica</i>	<i>12S_species 8</i>	<i>COI_species 10</i>
11	SS103	<i>Cassidula mustelina</i>	<i>12S_species 9</i>	<i>COI_species 11</i>
12	SS141	<i>Erronea erronea</i>	<i>12S_species 10</i>	<i>COI_species 12</i>
13	SS142	<i>Notocochlis gualteriana</i>	<i>12S_species 11</i>	
14	SS83	<i>Echinolittorina radiata</i>	<i>12S_species 12</i>	<i>COI_species 13</i>
15	SS143	<i>Canarium mutabile</i>	<i>12S_species 13</i>	<i>COI_species 14</i>
16	SS110	<i>Conomurex luhuanus</i>	<i>12S_species 14</i>	<i>COI_species 15</i>
17	B349	<i>Mitrella burchardi</i>	<i>12S_species 15</i>	<i>COI_species 16</i>
18	B366	<i>Fusinus ferrugineus</i>	<i>12S_species 16</i>	<i>COI_species 17</i>
19	SS115	<i>Pleuroploca trapezium</i>	<i>12S_species 17</i>	<i>COI_species 18</i>
20	B342	<i>Nassarius fraterculus</i>	<i>12S_species 18</i>	<i>COI_species 19</i>
21	B355	<i>Nassarius conoidalis</i>	<i>12S_species 19</i>	<i>COI_species 20</i>
22	SS102	<i>Nassarius albescens</i>		<i>COI_species 21</i>
23	SS144	<i>Nassarius coronatus</i>		<i>COI_species 22</i>
24	SS44	<i>Cantharus mollis</i>	<i>12S_species 20</i>	<i>COI_species 23</i>
25	B344	<i>Enzinopsis menkeana</i>	<i>12S_species 21</i>	<i>COI_species 24</i>
26	SS140	<i>Pterygia dactylus</i>	<i>12S_species 22</i>	<i>COI_species 25</i>
27	SS123	<i>Coralliophila neritoidea</i>	<i>12S_species 23</i>	<i>COI_species 26</i>
28	SS106	<i>Drupella cornus</i>	<i>12S_species 24</i>	<i>COI_species 27</i>
29	B356	<i>Oppomorus funiculatus</i>	<i>12S_species 25</i>	<i>COI_species 28</i>
30	B318	<i>Nucella lima</i>	<i>12S_species 26</i>	<i>COI_species 29</i>
31	B336	<i>Ocenebra inornatus endermonis</i>		<i>COI_species 30</i>
32	SS81	<i>Mancinella echinata</i>	<i>12S_species 27</i>	<i>COI_species 31</i>
33	SS70	<i>Mancinella siro</i>	<i>12S_species 28</i>	<i>COI_species 32</i>
34	SS86	<i>Menathais tuberosa</i>	<i>12S_species 29</i>	<i>COI_species 33</i>
35	B302	<i>Reishia bronni</i>	<i>12S_species 30</i>	<i>COI_species 34</i>
36	SS75			<i>COI_species 35</i>
37	B293	<i>Thais clavigera</i>		
38	SS34	<i>Vasum turbinellum</i>	<i>12S_species 31</i>	<i>COI_species 36</i>
39	SS116	<i>Homiodoris japonica</i>	<i>12S_species 32</i>	<i>COI_species 37</i>
40	B363	<i>Homiodoris japonica</i>	<i>12S_species 32</i>	<i>COI_species 37</i>

Table 3. (Continued).

#	Sample No.	Morphological	12S	COI
41	B323	<i>Acmaea pallida</i>	12S species 33	COI species 38
42	B253	<i>Collisella dorsuosa</i>	12S species 34	COI species 39
43	B334	<i>Tectura emydia</i>	12S species 35	COI species 40
44	B357	<i>Lepeta kuragiensis</i>	12S species 36	COI species 41
45	SS95	<i>Lottia luchuana</i>	12S species 37	COI species 42
46	B396	<i>Nipponacmea boninensis</i>	12S species 38	COI species 43
47	SS14	<i>Nipponacmea radula</i>	12S species 39	COI species 44
48	SS17	<i>Patelloida pygmaea</i>	12S species 40	COI species 45
49	SS91	<i>Patelloida ryukyuensis</i>	12S species 41	COI species 46
50	B256	<i>Patelloida saccharina lanx</i>	12S species 42	COI species 47
51	SS51		12S species 43	
52	SS2	<i>Cellana grata</i>	12S species 44	COI species 48
53	SS3	<i>Cellana toreuma</i>		
54	SS209	<i>Cellana grata</i>	12S species 45	COI species 49
55	SS4	<i>Cellana toreuma</i>		
56	SS18	<i>Cellana nigrolineata</i>		COI species 50
57	SS174	<i>Cellana radiata</i>	12S species 46	COI species 51
58	SS84	<i>Cellana testudinaria</i>	12S species 47	COI species 52
59	B247	<i>Scutellastra flexuosa</i>	12S species 48	COI species 53
60	B257	<i>Siphonaria sirius</i>	12S species 49	COI species 54
61	B359	<i>Siphonaria sp.</i>	12S species 50	COI species 55
62	SS131	<i>Tectus pyramis</i>	12S species 51	COI species 56
63	B268	<i>Chlorostoma argyrostoma lischkei</i>	12S species 52	COI species 57
64	SS72	<i>Chlorostoma turbinatum</i>		
65	B285	<i>Chlorostoma lischkei</i>	12S species 53	
66	SS73	<i>Tegula pfeifferi pfeifferi</i>	12S species 54	COI species 58
67	SS69	<i>Omphalius rusticus</i>		COI species 59
68	SS67	<i>Omphalius nigerrimus</i>	12S species 55	COI species 60
69	B254	<i>Cantharidus japonicus</i>	12S species 56	COI species 61
70	B345	<i>Cantharidus jessoensis</i>		COI species 62
71	S391	<i>Nipponacmea schrenkii</i>	12S species 57	COI species 63
72	B332		12S species 58	
73	SS54	<i>Lunella coreensis</i>	12S species 59	
74	SS101	<i>Lunella coronata</i>	12S species 60	COI species 64
75	B316	<i>Turbo stenogyrus</i>	12S species 61	COI species 65
		69	61	65

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