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Two partial mitochondrial gene sequences (COI and 12S-rRNA) suggested that *Ceramaster japonicus* (Sladen, 1889) and *Ceramaster patagonicus* (Sladen, 1889) (Asteroidea: Goniasteridae) from the Japanese waters are probably of the same species

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Abstract

Our recent morphological studies on the echinoderm collection of the National Museum of Nature and Science, Tokyo (NMST), indicated that the goniasterid starfishes *Ceramaster japonicus* (Sladen, 1889) and *Ceramaster patagonicus* (Sladen, 1889) are distributed in the Pacific Ocean side of Japan. In this study, we studied the NMST samples of *C. japonicus and C. patagonicus* from Japan, by using two mitochondrial DNA genes, the COI and 12S-rRNA, as markers to test relationships between these species in Japan. *C. patagonicus* sequences from GenBank were mined and included in the analyses. Results of phylogenetic and haplotype network analyses of both genes (final sequence lengths: COI = 317 bp, 12S = 477 bp) suggested that "*Ceramaster patagonicus*" and "*Ceramaster japonicus*" from Japanese waters are almost certainly synonymous without any population structure inside Japan.

Keywords: Ceramaster, molecular, species, classification, deep-sea, goniasterids

Introduction

Understanding taxonomic and phylogenetic relationships at the species level has become crucial for understanding biogeography, ecology, and evolutionary events (e.g. Foltz *et al*. 2008). In terms of species, Japanese biodiversity is rich, especially for echinoderms, a group displaying over 1052 described species (Fujikura *et al*. 2010). *Ceramaster japonicus* (Figure 1) is one of the most commonly encountered deep-sea asteroid species in Japan and occupies a dominant position in benthic communities (600–1200 m) in Japanese waters (Horikoshi *et al*., 1990). It is a widely occurring goniasterid, which also occurs in the Aleutians and east to the Pacific coast of North America. *C. japonicus* has become a significant biological indicator species in studies of Suruga Bay (Takahashi et al, 1997) and is one of the most abundant species present in the collections of the National Museum of Nature and Science, Tokyo (NMST), Japan. This species' widespread occurrence in Japanese waters, its relative abundance and its likely ecological importance suggest it would be an important model species for phylogeographic sampling. This would provide further understanding for its diversification throughout Japanese waters and eventually throughout its nominal range in the North Pacific.

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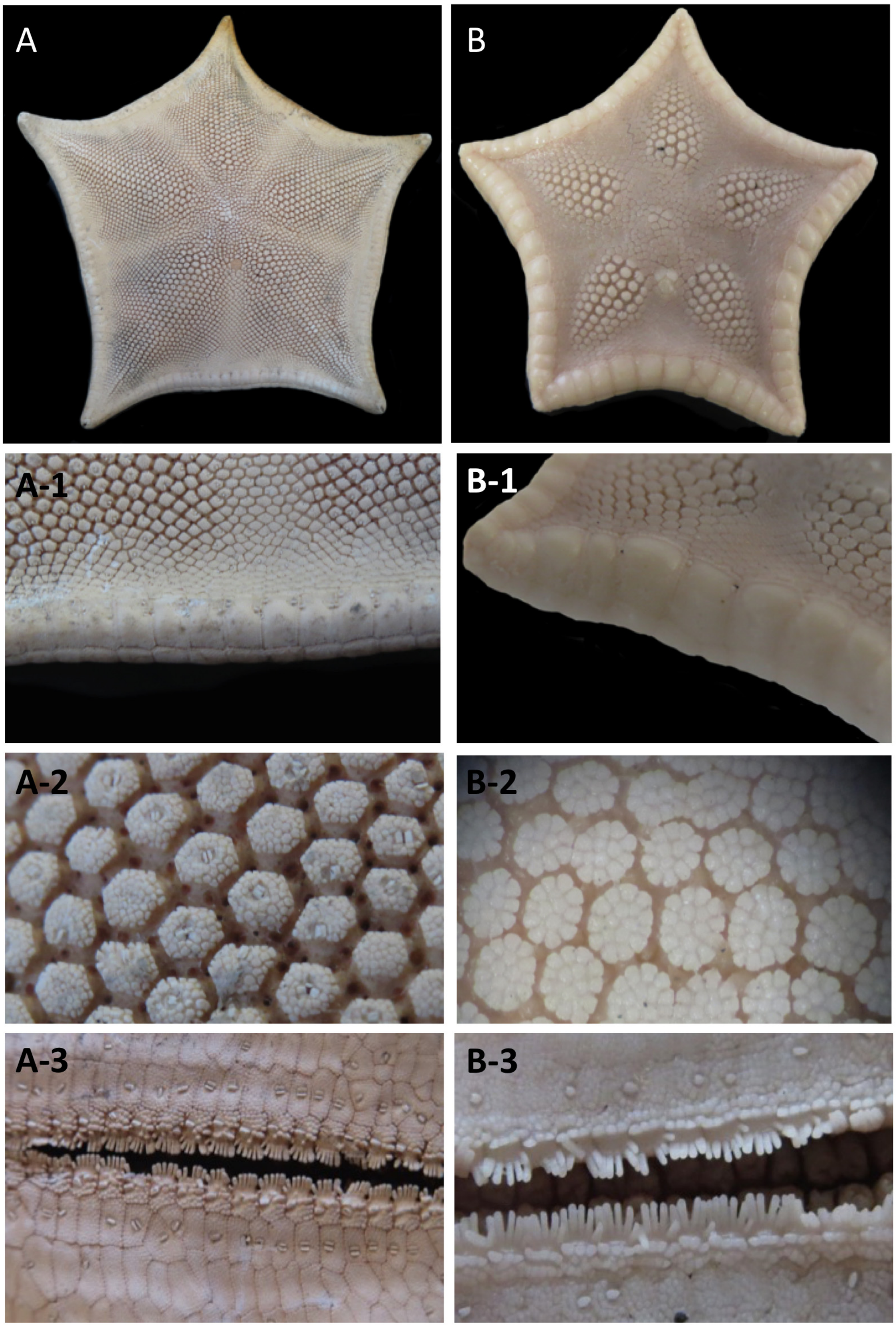

FIGURE 1. *Ceramaster* spp. showing relevant characters. Scale bar =15 mm for A and B. 2 mm for detail images. A. *Ceramaster japonicus* (CM-2041—unsequenced sample). R=73 mm, r=52 mm. A1. Superomarginal plates showing granular covering and pedicellariae. A2. Tabulae (abactinal surface) showing pedicellariae. A3. Adambulacral furrows showing spination. B. Specimen conforming to *Ceramaster patagonicus* (CM-2043—unsequenced sample). R=20 mm, r=15 mm. B1. Marginal plate surfaces showing large bald surfaces on plates. B2. Tabulae lacking pedicellariae. B3. Adambulacral furrows showing spination.

Ceramaster is a widely occurring genus in the family Goniasteridae, including approximately 20 species (Mah, 2018), which occur primarily at moderate to deep depths in cold to temperate water settings. Summary taxonomic accounts of *Ceramaster* in the North Pacific have been presented by Fisher (1911), Djakonov (1950/1968), and Lambert (2000). Of the approximately 20 species of *Ceramaster* currently recognized, approximately five of them occur along the coastal North Pacific region and phylogenetic relationships among have never been identified using quantitative tools.

Ceramaster patagonicus Sladen (1889) is a widely occurring species, and recorded from the Aleutian Islands and the North Pacific as well as New Zealand and further represented by subspecies in South Africa, Brazil and the Okhotsk Sea. Downey in Clark and Downey (1992) relegated *C. patagonicus* to a subspecies of *Ceramaster grenadensis* but this was not met with agreement by Clark (1993) or by Mah (2011) and is considered herein as a separate species complex in accord with O'Hara (1998) and Clark and McKnight (2001).

Our recent evaluations of the echinoderm collection of the National Museum of Nature and Science, Tokyo (NMST) have demonstrated that specimens of one additional nominal species, *C. patagonicus*, was also present in Japanese waters. Examination of these specimens showed morphological overlap suggesting close relationship between the two species. This prompted a phylogeographic evaluation of *C. japonicus* and Japanese specimens of *C. patagonicus* and their underlying taxonomic relationship.

In this paper, we report the result of our molecular taxonomy / phylogeography study of *Ceramaster patagonicus* and *Ceramaster japonicus* collected from the Japanese waters, in order to assess the genetic variation of the two Japanese *Ceramaster* species intra-specifically and inter-specifically in Japan. For this study, we extracted DNA from samples vouchered at the NMST, and sequenced two mitochondrial gene markers commonly used for DNA-Barcoding, molecular phylogenetics, and phylogeography studies, the COI and 12s-rRNA genes.

Materials and Methods

Morphological identification and taxon sampling

Vouchered museum samples of 39 *Ceramaster japonicus* and five *Ceramaster patagonicus* were collected from the Japanese waters on the Pacific Ocean side (Tosa-Bay, Ibaraki, Chiba, Shizuoka, Fukushima, and Hokkaido). Samples were collected from deep sea (depth: 548–3014 m) from year 1991–2012, and preserved in 70–90% EtOH. All samples were identified morphologically, based on the number of adambulacral furrow spines, the size of abactinal granules, and the differing marginal plate shapes. The specimens were then sorted and vouchered at National Museum of Nature and Science, Tokyo (NMST), Japan. Detailed sample list is shown in Table 1.

DNA extractions, PCR, and sequencings

Pieces of tube feet tissue (ca. 25 mg) were excised from the museum samples. Total genomic DNA was extracted and purified using DNeasy Blood and Tissue Kit (Qiagen), and was used as PCR templates. Previously reported and newly designed primers for both target mitochondrial genes (COI and 12S-rRNA) were used for amplification, in various combinations (Table 2). PCR reactions were conducted following standard protocols, with slight modifications on the annealing temperatures (42ºC–47ºC for COI, 50ºC for 12S-rRNA). Successfully amplified PCR products were purified using ExoProStar (GE Healthcare Life Sciences) following manufacturer's protocol. Sanger sequencings were outsourced (FASMAC Co. Ltd, Japan).

Sequence editing and datasets building

Obtained sequences were first aligned using the online version of MAFFT (MAFFT Server, Version 7, Katoh *et al* 2017) using the G-INS-i algorithm for COI, and the algorithm Q-INS-i for 12S to allow the consideration of secondary structures. Seven additional *C. patagonicus* sequences collected from GenBank were also included in the alignment. Aligned sequences were visualized in MEGA7 (Kumar *et al*, 2016), and edited manually. Obtained data sets of both genes (data sets 1) were then used for the subsequent Haplotype Network Analyses.

TABLE 1. List of samples analyzed in this study.

No .	Sample No.	NMST Voucher No.	Sample name	Sampling Year	Sampling Locations	Depth	Sequence	
							COI	12s
1	CM1396(1)	NSMT E-11883	*Ceramaster japonicus*	1993	S off Hokkaido (Erimo)	2977–3014 m	○	×
2	CM1396(2)	NSMT E-11884	*Ceramaster japonicus*	1993	S off Hokkaido (Erimo)	2977–3014 m	○	○
3	CM1396(3)	NSMT E-11885	*Ceramaster japonicus*	1993	S off Hokkaido (Erimo)	2977–3014 m	○	○
4	CM1396(4)	NSMT E-11886	*Ceramaster japonicus*	1993	S off Hokkaido (Erimo)	2977–3014 m	○	○
5	CM1396(5)	NSMT E-11887	*Ceramaster japonicus*	1993	S off Hokkaido (Erimo)	2977–3014 m	○	○
6	CM1396(6)	NSMT E-11888	*Ceramaster japonicus*	1993	S off Hokkaido (Erimo)	2977–3014 m	○	○
7	CM1396(7)	NSMT E-11889	*Ceramaster japonicus*	1993	S off Hokkaido (Erimo)	2977–3014 m	○	○
8	CM0248	NSMT E-11397	*Ceramaster japonicus*	2012	Ibaraki	2091–2079 m	×	○
9	CM0249	NSMT E-11398	*Ceramaster japonicus*	2012	Ibaraki	980–962 m	×	○
10	CM1394	NSMT E-11882	*Ceramaster japonicus*	1999	SE off Boso Peninsula (Kamogawa)	922–959 m	○	×
11	CM1237(2)	NSMT E-11864	*Ceramaster japonicus*	1999	Tosa Bay	763–803 m	×	○
12	CM1301(1)	NSMT E-11865	*Ceramaster japonicus*	2011	Tosa Bay	700 m	×	○
13	CM1301(2)	NSMT E-11866	*Ceramaster japonicus*	2011	Tosa Bay	700 m	×	○
14	CM1301(3)	NSMT E-11867	*Ceramaster japonicus*	2011	Tosa Bay	700 m	×	○
15	CM1301(4)	NSMT E-11868	*Ceramaster japonicus*	2011	Tosa Bay	700 m	×	○
16	CM1301(5)	NSMT E-11869	*Ceramaster japonicus*	2011	Tosa Bay	700 m	×	○
17	CM1301(6)	NSMT E-11870	*Ceramaster japonicus*	2011	Tosa Bay	700 m	×	○
18	CM1301(8)	NSMT E-11872	*Ceramaster japonicus*	2011	Tosa Bay	700 m	○	○
19	CM2003	NSMT E-11379	*Ceramaster japonicus*	1999	Tosa Bay	621–622 m	○	○
20	CM0250	NSMT E-11399	*Ceramaster japonicus*	1999	Tosa Bay	807–832 m	○	○
21	CM1238(1)	NSMT E-11879	*Ceramaster japonicus*	1999	Tosa Bay	621–622 m	×	○
22	CM1238(2)	NSMT E-11880	*Ceramaster japonicus*	1999	Tosa Bay	621–622 m	○	×
23	CM1239(1)	NSMT E-11861	*Ceramaster japonicus*	2008	Off of Miyako Bay	1586–1617 m	×	○

...Continued on next page

TABLE 1. (Continued)

No.	Sample No.	NMST Voucher No.	Sample name	Sampling Year	Sampling Locations	Depth	Sequence	
							COI	12s
24	CM0624(1)	NSMT E-11385A	*Ceramaster patagonicus*	2004	Chiba	730–742 m	○	○
25	CM0624(2)	NSMT E-11385B	*Ceramaster patagonicus*	2004	Chiba	730–742 m	○	○
26	CM0632	NSMT E-11304	*Ceramaster patagonicus*	1998	Tosa Bay	765–773 m	×	○
27	CM0633	NSMT E-11400	*Ceramaster patagonicus*	1998	Tosa Bay	654–686 m	×	○
28	CM0634	NSMT E-11386	*Ceramaster patagonicus*	1998	Tosa Bay	744–786 m	×	○
29	DSPEC003-07	-	*Ceramaster patagonicus*	2007	Canada, British Columbia, Howe Sound/Sunshine Coast		○	×
30	DSPEC034-07	-	*Ceramaster patagonicus*	2007	Canada, British Columbia, Howe Sound/Sunshine Coast		○	×
31	DSPEC601-08	-	*Ceramaster patagonicus*	2008	Canada, British Columbia, Nanaimo		○	×
32	DSPEC602-08	-	*Ceramaster patagonicus*	2008	Canada, British Columbia, Nanaimo		○	×
33	NZECA507-10	-	*Ceramaster patagonicus*	-	New Zealand		○	×
34	NZECA511-10	-	*Ceramaster patagonicus*	-	New Zealand		○	×
35	NZECA513-10	-	*Ceramaster patagonicus*	-	New Zealand		○	×
36	EU723035.1	-	*Ceramaster patagonicus*	-	-		×	○
37	EU723021.1	-	*Ceramaster arcticus*	-	-		×	○

Goniasterid outgroup sequences were mined from GenBank (Table 1) (*Neoferdina cumingi* and *Hippasteria imperialis* for COI; *N. cumingi* and *H. spinosa* for 12S-rRNA), and then added to the post-edited aligned datasets using the "--add" function in MAFFT, and then re-aligned using the G-INS-i algorithm for COI, and Q-INS-i for 12S-rRNA. Ambiguously aligned sequences were edited manually. The obtained data sets were then used for phylogenetic analyses.

Haplotype Network Analysis and Phylogenetic Analysis

Haplotype Network Analyses were conducted using PopArt ver. 1.7 (Leigh and Bryant, 2015), under the TCS algorithm (Templeton-Crandall-Sing algorithm, Templeton *et al*, 1992). The TCS algorithm has been used extensively to infer the probability of genealogical interrelationships among organisms spanning different divergence times, including among those with only one to several nucleotide substitutions, such as the phylogeographic history of conspecific populations (Clement *et al*, 2002). Maximum Likelihood (ML) phylogenetic tree for each gene was inferred using the program RAxML-GUI Ver 1.5-beta (Silvestro and Michalak, 2012), under the GTR+G algorithm, with 1000 bootstrap replications.

TABLE 2. List of primers used in this study.

Gene	Primers	Sequence	Reference
COI	COIceF	ACTGCCCACGCCTAGTAATGATATTTTTATGGTNATGCC	Hoareau and Boissin (2010)
	COIceR	TCGTGTGTCTACGTCCATTCCTACTGTRAACATRTG	Hoareau and Boissin (2010)
	CISBR1-F	CCCCTAATGATCGGATCCCCCGACATGGCC	This study
	CISBR1-R	CCTATTGATACTATGGCGTAGACC	This study
	CISBR2-F	CCCTCTTTTCTCCTACTCTTAGCCTCAGCG	This study
	CISBR2-R	CCTGGAAGTATTAGGATGTATACTTCAGGG	This study
12s	12SF-F	GGTHAATTATGTGCCAGCCACCG	Mah and Foltz (2010)
	12SA-R	GAGAGTGACGGGCGATGTGT	Smith *et al.* (1993)
	12SBR1-F	CCTCACACGGTAAAAAGGTGGCAAAG	This study
	12SBR1-R	GCGATGTGTGCGCATTTTAGAGCTGG	This study
	12SBR2-F	CCCTGAAAAAACAAACCCACTAAAGC	This study
	12SBR2-R	GGGTATTACTGCTGAATCCTGTTTCAGC	This study

Results and Discussion

Morphological comparison between C. patagonicus *and* C. japonicus

Several NMST individuals of *Ceramaster* display characters which set them apart from *C. japonicus,* but presented identifying characteristics for what appeared to be *C. patagonicus* (Fig. 1). Fisher (1911: 207) observed specimens he assumed were juveniles of *C. japonicus.* which he described as having the "same form" as Sladen's description of *C. patagonicus*. He briefly described variation among these forms but did not elaborate further on how he arrived at this conclusion. *Ceramaster japonicus* is characterized by the presence of a completely granule-covered superomarginal plate surface, the presence of pedicellariae on the abactinal plates (tabulae) and marginal plates and six or seven furrow spines (more variably three to six in North American individuals).

In contrast, other specimens collected from Japanese waters were consistent with the description of *Ceramaster patagonicus* and possessed large bald (i.e., granule-free) areas on the superomarginal plate surface, lacking pedicellariae on abactinal and marginal plates, and possessed only four or five furrows spines. These "*patagonicus*" forms are also more strongly arcuate in shape with more pointed arm tips and broader interradial arcs with a wider superomarginal boundary present around the periphery of the abactinal surface. Shape of the marginal plates in these individuals was also different with these "patagonicus" forms showing a more strongly convex superomarginal surface and better coordination between superomarginals and inferomarginals than the conventional *C. japonicus* forms.

DNA sequencing of old museum samples

We were successful in obtaining sequences of both the 12S-rRNA and COI genes for most samples (Table 1). For 12S, 33 samples of *C. japonicus* and five samples of *C. patagonicus* were amplified. For COI, 14 samples of *C. japonicus* and two samples of *C. patagonicus* were amplified. Sequence lengths before manual quality check, manual editing, and dataset-building involving alignment and additional editing were ca. 600 bp for COI, and 580 bp for 12S-rRNA. Sequence lengths were reduced after the manual quality check, sequence editing, and datasets building processes. Only 318 bp of COI and 477 bp of 12S-rRNA were useful for the actual analyses. The drastic reduction in sequence length was caused mainly because of the inclusion of short sequences from GenBank and BoLD.

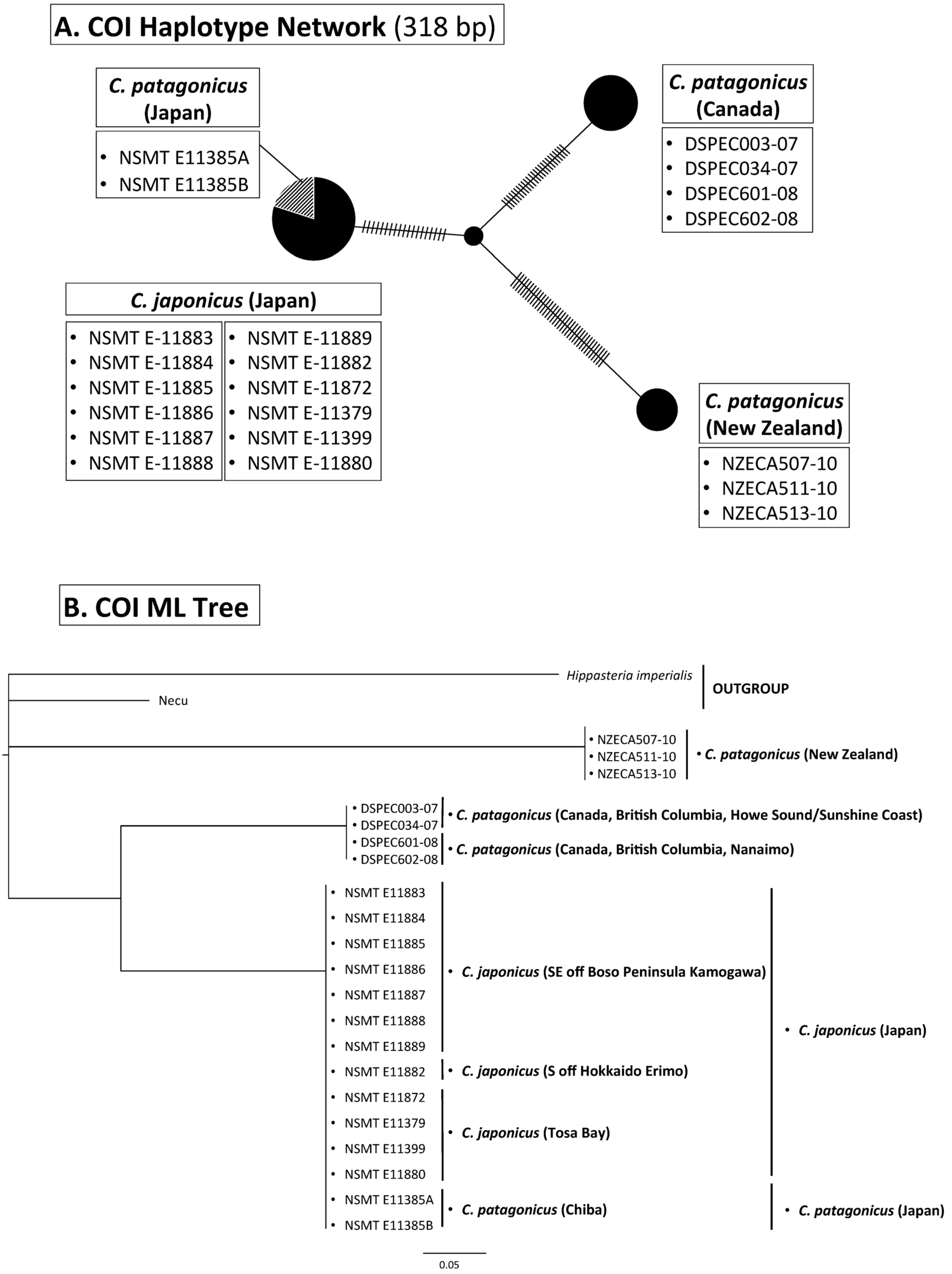

FIGURE 2. The lack of substitutions of the COI gene sequences indicates that *C. patagonicus* and *C. japonicus* from the Japanese waters are probably of the same species, while the relatively high number of substitutions among *C. patagonicus* of different geographical regions suggests that the species is probably not monophyletic. A. TCS Haplotype Network. Note that all Japanese samples, regardless of the species, are clustered into one haplotype group. Meanwhile, samples are clustered based on geographical area. B. The result is recapitulated in the Maximum Likelihood phylogenetic tree too.

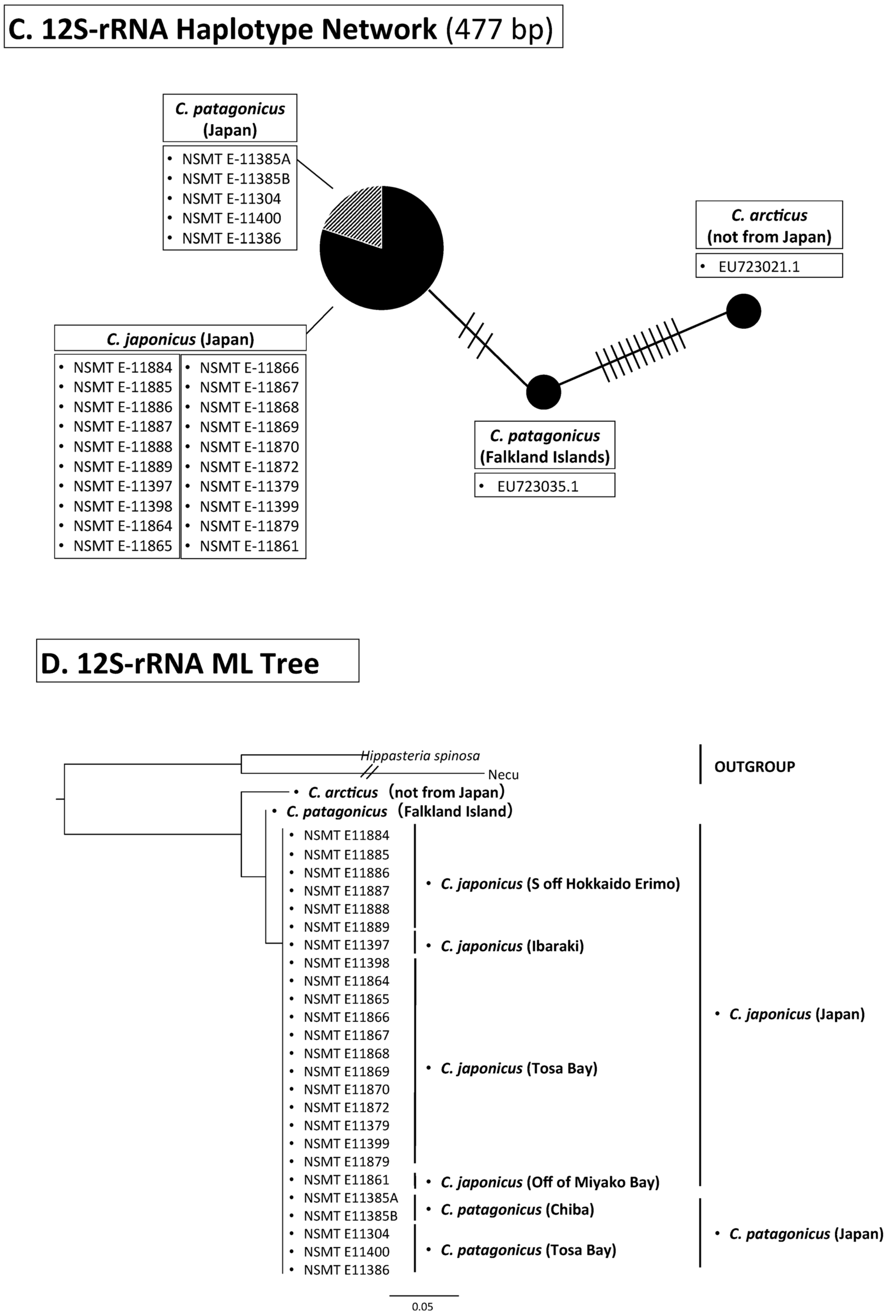

FIGURE 3. The lack of substitutions of the 12S-rRNA gene sequences also indicate that *C. patagonicus* and *C. japonicus* from the Japanese waters are probably of the same species. A. TCS Haplotype Network. B. Maximum Likelihood phylogenetic tree.

The oldest successfully amplified samples for both genes were collected in 1999, while the newest samples were collected in 2012. The oldest successfully sequenced sample for 12S-rRNA was collected in 1993. All of the samples mentioned above were vouchered *C. japonicus* samples at the National Museum of Science and Technology, Japan. The samples were preserved in 70%–80% EtOH and stored in the collection room at ambient room temperature.

Low genetic diversity

TCS haplotype network of both genes are congruent with each other (Figure 2A, 3A). The haplotype network of both genes showed that all *C. japonicus* and *C. patagonicus* from Japan to be clustered as one population, regardless of their sampling loci in Japan. Meanwhile, the topologies of our well-resolved maximum likelihood trees (Figure 2B, 3B) for both gene markers are not only congruent with each other, but also with the result of the TCS haplotype network analyses.

The TCS haplotype network including these samples showed that the *C. patagonicus* samples collected from different geographical area do not cluster together as one group, but are separated into different clusters with significant nucleotide substitutions among the different groups (Figure 2A, 3A). Meanwhile, there were only two *Ceramaster* 12S-rRNA sequence data available on GenBank (one of *C. patagonicus* from the Falkland Islands, one of *C. arcticus* of unrecorded locality). However, TCS haplotype network analysis of this gene also showed that *C.patagonicus* from Japan do not form a monophyletic species with its conspecific samples from the Falkland Islands, while *C. patagonicus* and *C. japonicus* from Japan form a monophyletic haplotype group with no nucleotide substitution. This result was also recapitulated in our phylogenetic trees. In our trees, *C. japonicus* and *C. patagonicus* samples from Japan were clustered together forming a monophyletic clade. However, the branches of the OTUs in this “Japanese clade" are not bifurcating and are polyphyletic instead. This is in congruence with our haplotype network, which indicates that both gene markers show no base substitutions among different individuals, making the OTUs to form one haplotype group.

Therefore, our results indicate that *Ceramaster patagonicus* from Japan were clustered with the allopatric *C. japonicus*, other *C. patagonicus* samples from areas outside of Japan (sequences obtained from Genbank) were clustered geographically based on their sampling localities (i.e. the New Zealand haplotype group and the Canadian haplotype group).

Interestingly, this remarkable lack of genetic diversity occurs not only across wide geographic area, but apparently, also across depth. Specimens of *Ceramaster japonicus* surveyed for our study were sampled primarily from Japanese waters, but across a wide bathymetric range, from 548 m to 3014 m. However, we found that these individuals showed remarkably low genetic diversity, regardless of the broad bathymetric occurrence of the surveyed specimens.

Taxonomic considerations of Japanese Ceramaster patagonicus *and* C. japonicus

Results from the molecular phylogeography support the Japanese “*Ceramaster patagonicus*” specimens within the *Ceramaster japonicus* cluster, running contrary to morphological characters which agree with the descriptions that identify *Ceramaster patagonicus*. Although this suggests that these two species could be synonymous, there as yet remains an incomplete survey of morphological variation and confirmation with type specimens. *Ceramaster patagonicus* is technically the senior name, pre-dating *C. japonicus* by 3 pages (Sladen 1889). The type locality for the *Ceramaster patagonicus* “concept” was from near the Atlantic entrance to the Strait of Magellan which was not sampled for this analysis. Further variation from throughout the range of *Ceramaster patagonicus* and its related species will need to be assessed.

Ceramaster japonicus’ full morphological range is incompletely documented. Clark (1993) lists the full bathymetric range of this species as 194-1410 m. Although Fisher (1911) has briefly covered the morphological range for this species, variation along its bathymetric range is poorly understood and characters such as pedicellariae and furrow spine number might be environmentally related phenotypic variation.

Comparisons with other widely occurring taxa

Hippasteria phrygiana, another widely occurring goniasterid, is present in cold and temperate water settings in both North and South Hemispheres, by several representative species and subspecies in South Africa, Brazil, New Zealand, and the Aleutians (Mah *et al.* 2014). However molecular phylogeography by Foltz *et al.*

(2008) showed very low genetic distances between populations, indicating that these were all one widely-occurring species, *Hippasteria phrygiana*, which showed a considerable amount of variation across its range. There are, in contrast, other examples of asteroid genera with species showing distinct species lineages in different areas (e.g. *Coscinasterias*, Waters and Roy 2003; *Astropecten*, Zulliger & Lessios 2010).

Concluding remarks and future directions

The result obtained from our phylogenetic and haplotype network analyses indicated that the two species collected from the Japanese waters are probably of the same species with almost no genetic difference, while conspecific samples of other regions would form a distinct population of the area, and not clustered with samples from Japan, with big genetic difference among the clusters (=populations), as indicated by the number of nucleotide substitutions among them. This further suggests the possibility that, the morphological differences used to differentiate the two species, such as their body size, skin colors, and size and shapes of particular body parts could be actually individual variations caused by developmental plasticity, heterochrony/heterokairy, age differences, and/or environmental acclimatization.

This result also underlines that molecular taxonomy and phylogeography might help to unravel the necessities of revisiting taxonomic descriptions of marine organisms (e.g. Shaffer *et al*, 2018) including deep-sea species, in order to understand its hidden diversity or lack thereof. Studies employing recent techniques such as metabarcoding (eDNA) might help to reveal the reality of such diversity (e.g. Arroyo *et al*, 2016). However, without a solid database containing sequences from properly identified specimens, metabarcoding are not effective (Bucklin *et al*, 2016; Machida *et al*, 2009). Re-analyzing museum samples, both morphologically, and when possible, molecularly, might allow the achievement of such aim (e.g. Bode *et al*, 2017; Smith-Vaniz and Johnson, 2016).

Our report here, however, should be considered as a pilot study for further research, since our results pose many additional questions. For example, since we only used mitochondrial genes, we cannot deny any possibility of introgression causing the lack of diversity on the female/mitochondrial lineage (e.g. Breusing *et al*, 2017; Mastrantonio *et al*, 2016; Petersen *et al*, 2010), or other sex-biased dispersal causing possible mitonuclear discordance (Toews and Brelsford, 2012). Accordingly, future studies including analyses using nuclear markers such as ATPS-1-alpha (Mah and Foltz, 2011) and/or ITS2 (Moore *et al*, 2018), or a genome-wide association studies on nuclear SNPs using methods such as MIG-Seq (Suyama and Matsuki, 2015) is warranted. Another issue to address by future molecular studies would be to include more samples of *Ceramaster japonicus*, *Ceramaster patagonicus*, and other congeners, collected from other parts of Japan and of the world, in order to look at their actual species delimitation.

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