Taxonomic status of the oreasterid sea star Bothriaster primigenius Döderlein, 1916 (Echinodermata, Asteroidea)

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Taxonomic status of the oreasterid sea star *Bothriaster primigenius* Döderlein, 1916 (Echinodermata, Asteroidea)

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Abstract: One of the oreasterid sea stars, *Bothriaster primigenius*, is a rarely encountered species in the tropical Indo-West Pacific. The taxonomic position of this species, and more specifically, whether to recognize this sea star as a species, remains unresolved. To resolve the question, we assessed the specific validity of this sea star based on morphological observations and molecular phylogenetic analysis. The results revealed that this species is a young individual of another oreasterid sea star, *Choriaster granulatus*, and is placed as a junior synonym for the same.

Key words: starfish, *Bothriaster primigenius*, *Choriaster granulatus*, species validity, COI, taxonomy

Introduction

Bothriaster is a monotypic genus found in the tropical Indo-western Pacific region. The only species, *Bothriaster primigenius* Döderlein, 1916, was first described in Indonesian waters based on a small specimen with R/r (the ratio of arm length R to disk radius r) = 27.5/13 mm (Döderlein, 1916; 1935). The distribution of this species in the Maldives, Philippine Islands, Guam, Papua New Guinea, Solomon Islands, and Queensland, Australia, was confirmed by Yamaguchi (1975), Rowe & Gates (1995), and Marsh & Fromont (2020). In addition to the distribution records, Marsh & Fromont (2020) noted that the maximum body size of this species was R/r = 46/20 mm.

The small body size of this sea star raises doubts about the validity of the species. Yamaguchi (1975) suspected that this species represents a juvenile form of *Choriaster granulatus* Lütken, 1869. Clark (1993) pointed out that the validity of the genus *Bothriaster* and the species *B. primigenius* was doubtful because the diagnostic characteristics were attributable to the immaturity of the lost holotype specimen. Liao & Clark (1995) reported that young individuals of *C. granulatus* have been described under other generic names, implying *C. granulatus* and *B. primigenius* are conspecific.

Recently, we found a series of sea star specimens morphologically connecting *B. primigenius* to *C. granulatus*, sampled from Okinawa Island, southern Japan. The purpose of this study was to elucidate the taxonomic status of *B. primigenius* using external features and genetic information of the examined specimens, including other oreasterid sea stars that inhabit Japanese waters.

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Materials and Methods

Four starfish specimens of various body sizes (R ranging from 13 to 55 mm) obtained from the coast of Okinawa Island were used for morphological observations. The specimens were tentatively named from the smallest to largest individuals as follows: *Bothriaster primigenius*, intermediate type 1, intermediate type 2, and *Choriaster granulatus* (Table 1, Fig. 1A). To closely examine the skeletal structure, a portion of the abactinal skin was removed using sodium hypochlorite. The skeletal structure was observed using a stereomicroscope.

In addition to the morphological examination, the mitochondrial cytochrome oxidase subunit I (COI) gene region was used for molecular phylogenetic analysis.

The sea star species for the analysis were composed of the smallest individual of *B. primigenius* used for the morphological observations mentioned above, and other oreasterid species including two specimens of fully grown *C. granulatus* which were collected from the southern Japanese waters (Table 1, Fig. 1).

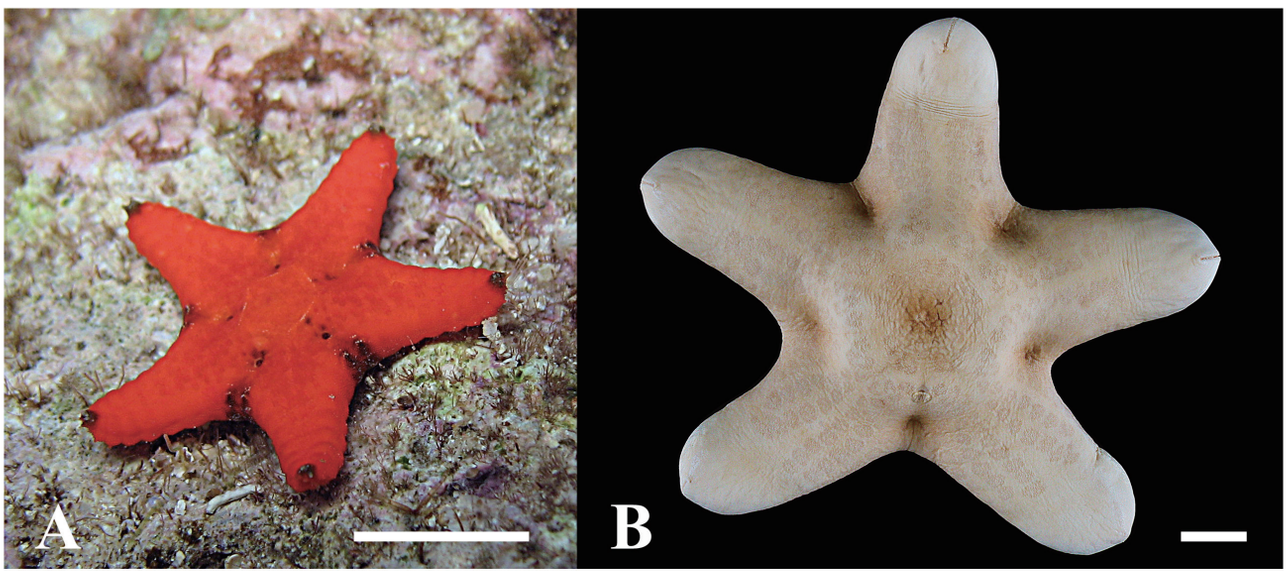

Fig. 1. Oreasterid sea stars used in this study. A: living specimen of *Bothriaster primigenius*, OMNH-Iv-10644; B: alcohol-preserved specimen of *Choriaster granulatus* s1, OMNH-Iv-10646. Scale bars: A = 10 mm, B = 20 mm.

Table 1. Oreasterid sea stars used in this study.

Species	Body size	Locality	Repository**	Accession No.
**Astrosarkus idipi*	R/r = 204/110 mm	Okinawa Is., Okinawa Pref.	OCA	LC771234
**Bothriaster primigenius*	R/r = 13/7 mm	Okinawa Is., Okinawa Pref.	OMNH-Iv-10644	LC771235
intermediate type 1	R/r = 19/9 mm	Sesoko-jima, Okinawa Pref.	RUMF-ZE-01113	
intermediate type 2	R/r = 33/16 mm	Okinawa Is., Okinawa Pref.	OMNH-Iv-10645	
Choriaster granulatus	R/r = 55/22 mm	Okinawa Is., Okinawa Pref.	RUMF-ZE-01123	
**Choriaster granulatus* s1	R/r = 96/45 mm	Okinawa Is., Okinawa Pref.	OMNH-Iv-10646	LC771236
**Choriaster granulatus* s2	R/r = 95/42 mm	Okinawa Is., Okinawa Pref.	OCA	LC771237
**Culcita novaeguineae*	R/r = 103/91 mm	Okinawa Is., Okinawa Pref.	OCA	LC771238
**Halityle regularis*	R/r = 98/72 mm	Kume-jima, Okinawa Pref.	RUMF-ZE-00036	LC771239
**Pentaceraster alveolatus*	R/r = 105/42 mm	Ojika-jima, Nagasaki Pref.	OMNH-Iv-10647	LC771240
**Protoreaster nodosus*	R/r = 164/66 mm	Okinawa Is., Okinawa Pref.	OCA	LC771241

*Specimen used for molecular phylogenetic analysis. **OCA: living specimen kept in the Okinawa Churaumi Aquarium, OMNH: Osaka Museum of Natural History, RUMF: Ryukyu University Museum (Fujukan).

Genomic DNA was extracted from one or two pieces of tube feet from each specimen using the NucleoSpin Plant II kit (Macherey-Nagel), following the manufacturer's protocol. Buffer PL1 was used for tissue lysis. The COI fragment was amplified using the COI ast F1:5'-ATGCAACTWAGACGATGAYT-3' and COI ast R1:5'-GGCCATTCAKCTRAATACCTT-3' primers. The thermocyclic conditions for PCR included the initial denaturation at 94°C for 2 min, 35–45 cycles of denaturation at 94°C for 15 sec, annealing at 52°C for 30 sec, extension at 72°C for 1 min, and a final extension at 72°C for 1 min. Successful PCR products were purified with a mixture of Exonuclease I (New England Biolabs) and rAPid Alkaline Phosphatase (Roche) and sequenced using an ABI 3130 Genetic Analyzer. The accession numbers of the sequences are listed in Table 1.

Multiple alignments of the COI sequences were performed using MAFFT v7.520 (Katoh & Standley, 2013) using the L-INS-i strategy. The COI sequence of the crown-of-thorns starfish *Acanthaster planci* (Linnaeus, 1758) (GenBank accession No. AB116377.2), which is phylogenetically closely related to oreasterid sea stars (Mah & Foltz, 2011), was included in the alignment and further phylogenetic analyses as an outgroup. The maximum likelihood (ML) tree was constructed using RAxML GUI 2.0.10 (Edler *et al*. 2021) with a GTR+I+G model. The reliability of the tree topology was evaluated using bootstrap analysis with 1000 replicates.

Results

Comparison of external morphology. The typical specimen of *Bothriaster primigenius* was characterized by a

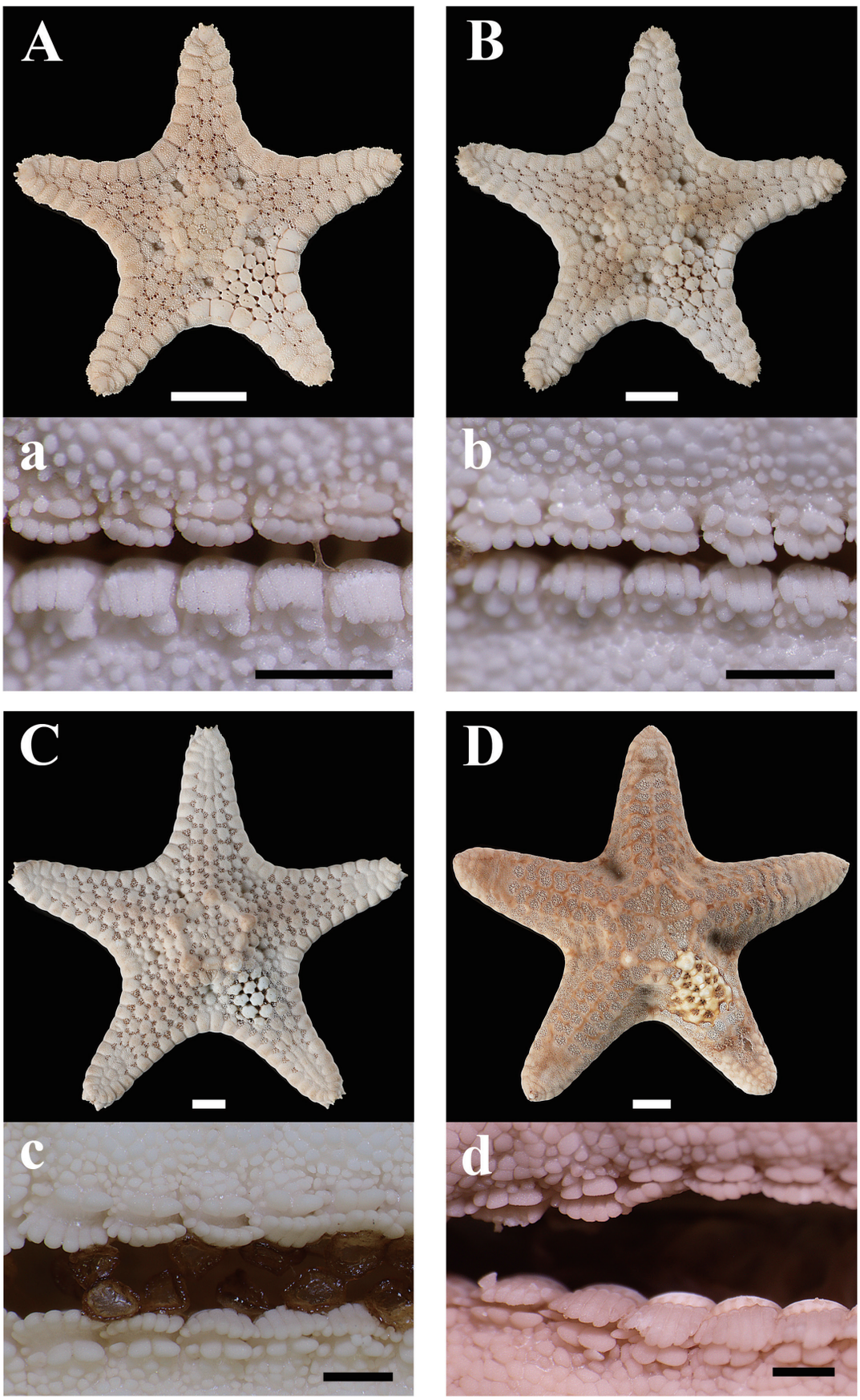

Fig. 2. Sea stars used for morphological observations in this study. *Bothriaster primigenius* (A) and its adambulacral spines (a), OMNH-Iv-10644; intermediate type 1 (B) and its adambulacral spines (b), RUMF-ZE-01113; intermediate type 2 (C) and its adambulacral spines (c), OMNH-Iv-10645; *Choriaster granulatus* (D) and its adambulacral spines (d), RUMF-ZE-01123. Scale bars: A–C = 5 mm. D = 10 mm, a-d = 1 mm.

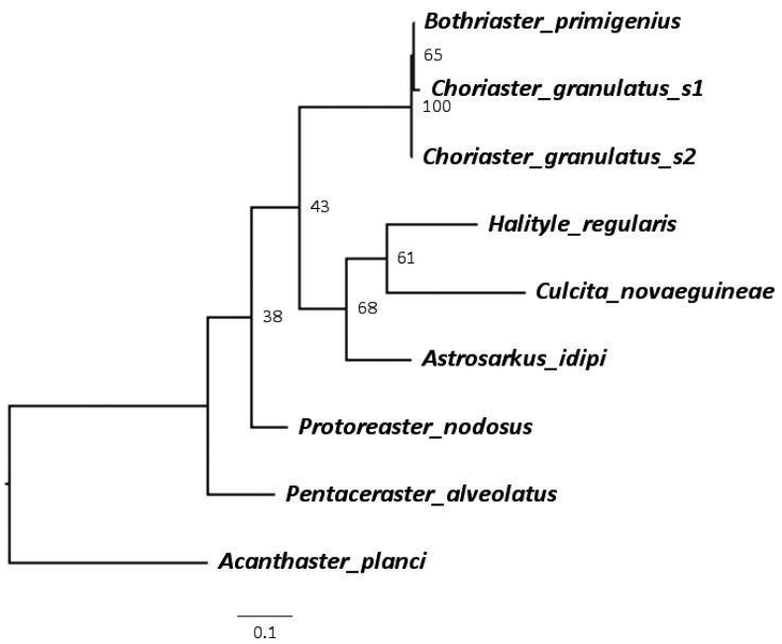

Fig. 3. Maximum likelihood (ML) tree for oreasterid sea stars based on the mitochondrial cytochrome oxidase subunit I (COI) sequences. Scale bar shows branch length in substitutions per site. *Acanthaster planci* is an outgroup.

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B. primigenius      1 aaatctacaaagtaatagtaaccgcccacgccctagtaatgatattcttcatggtaatgccaataatgatcggcggattcggaaactgactcatcccactaatgattggagccccagaca 120
C. granulatus s1    1 ....................................................................t..................................... 120
C. granulatus s2    1 ....................................................................t..................................... 120

B. primigenius    121 tggcgttcccccgaatgaataaaatgagattctgactagtccccccatcattccttctcctcgtagcctcagccggggtggagagaggggccggaacaggctgaacaatatacccccccac 240
C. granulatus s1  121 ..................c.....................................................................g..................... 240
C. granulatus s2  121 ....................................................................................................... 240

B. primigenius    241 tatcaagtggcctagctcacgcaggggatccgttgacctggccatattctcgcttcacttagcaggcgcctcatcaatccttgcatccattaaatttattactacagtaataaaaatgc 360
C. granulatus s1  241 ....................................................................................................... 360
C. granulatus s2  241 ....................................................................................................... 360

B. primigenius    361 gaacccctggaatttcgtttgaccgcctcccgctattcgtgtgatctgtattcgtaacggcattcctactcctcctttcactcccagttctagccggagcaataacaatgctactaactg 480
C. granulatus s1  361 ....................................................a.......................................................... 480
C. granulatus s2  361 ..............................a.....................................................................  480

B. primigenius    481 accgaaaagtaaaaaccacattctttgaccccgcaggaggggggacccaatactattccaacacttattctgattcttcggtcaccctgaagtttatattctcattttaccagggttcg 600
C. granulatus s1  481 ....................................................................................g.....t. 600
C. granulatus s2  481 ....................................................................................................... 600

B. primigenius    601 gcatgatctcccacgtcatagcccattactcggggaagagagaacctttcgggtatttgggaatggtcta                                          670
C. granulatus s1  601 ..........................c.....................................                                          670
C. granulatus s2  601 ....................................................................                                          670
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Fig. 4. Alignment of the COI sequences of *Bothriaster primigenius* and *Choriaster granulatus*. Periods in the sequences of two *C. granulatus* specimens mean that the base of the position is same as in the sequence of *B. primigenius.*

stellated body shape with R/r = 13/7 mm and a pentagonal skeletal ridge consisting of 10 raised abactinal plates in the center of the disc (Fig. 1A, Fig. 2A). The abactinal plates were polygonal in shape with rounded corners, and their surfaces were covered with coarse granules. The papulae emerged between the abactinal plates. Eight superomarginal plates were present. The arm tip, including the terminal plate, has conical projections. The actinal and inferomarginal plates were covered with granules, similar to those on the abactinal plates. The furrow spines were digitiform in shape, numbered 5–6 per adambulacral plate (Fig. 2a). Robust subambulacral spines, numbered 3–5, were placed directly at the back of the furrow series.

The specimens of intermediate type 1 with R/r = 19/9 mm (Fig. 2B) and intermediate type 2 with R/r = 33/16 mm (Fig. 2C) exhibited the same body shape, granulation, and

arrangement of adambulacral spines (Figs. 2b, c) as typical *B. primigenius* specimen. The major difference between typical and intermediate specimens was the number of papulae. The intermediate type 1 had more papulae around the abactinal plate, whereas type 2 had numerous papulae that formed a mottled papular area.

As for the external features of the *Choriaster granulatus* specimen with R/r = 55/22 mm (Fig. 2D), thick skin developed all over the body, concealing the skeletal structure; however, the pentagonal skeletal ridge in the center of the disc was observed by denuding the skin. Supplemental plates inserted between the polygonal abactinal plates formed a reticulated structure carrying numerous papulae. The conical projections at the arm tip, which are characteristic of smaller specimens, completely disappeared. The number, shape, and arrangement of the adambulacral spines were similar to those of the smaller specimens (Fig. 2d).

Molecular phylogenetic analysis. In the present study, we sequenced 670–878 bp of mitochondrial COI genes from eight specimens of seven oreasterid sea star species. Although the bootstrap values of each branch were low, our ML tree showed that two spine-poor species with long arms, namely *Choriaster granulatus* and *Bothriaster primigenius*, formed a monophyletic clade (Fig. 3). Three other spine-poor species with short arms, namely *Culcita novaeguineae* (Müller & Troschel, 1842), *Halityle regularis* (Fisher, 1913), and *Astrosarkus idipi* (Mah, 2003), also formed a monophyletic clade, which is a sister group to the spine-poor species with long arms, suggesting the monophyly of all spine-poor species. The remaining spiny species, *Pentaceraster alveolatus* (Perrier, 1875) and *Protoreaster nodosus* (Linnaeus, 1758), did not form monophyletic clades.

One of the most notable findings of the phylogenetic analysis was the close relationship between *B. primigenius* and *C. granulatus* (Fig. 4). The difference in the COI nucleotide sequence between the two species was only two or seven out of 670 bp (0.30% and 1.04%, respectively), which was almost identical between the two *C. granulatus* species (five out of 670 bp, 0.75%). In comparison, the COI sequences of other oreasterids showed greater differences. For example, the difference between the COI sequences of *C. novaeguineae* and *H. regularis*, close relatives in our phylogenetic tree (Fig. 3), was 106 of 708 bp (14.97%).

Discussion

Our observations revealed that the external features of the examined specimens continuously changed. As the body grew, the thickness of the skin gradually increased and the spines at the arm tip disappeared. Notable morphological changes were observed in the number and distribution patterns of the papulae. The papulae, which had been growing in isolation and were located around the abactinal plates of the small specimens, increased in number, eventually aggregated, and were housed in the reticulated skeletal interspaces with growth.

In contrast to the significant morphological changes mentioned above, the number and arrangement of the adambulacral spines were almost the same in all individuals. In particular, the number and shape of the furrow spines were very similar between the small and large specimens.

From a morphological perspective, all specimens examined in this study were positioned as the same species, with a growth pattern accompanied by gradual changes in body structure. The similarity of the structure of the adambulacral spines, which is an important criterion for the identification of sea stars (Southward & Campbell, 2006), supported the identity of all specimens.

In addition to the morphological analysis, a molecular phylogenetic approach revealed that a series of specimens composed of *B. primigenius*, and *C. granulatus* could be attributed to the same species. Although the phylogenetic relationship of each oreasterid species was considered tentative because of the low bootstrap values at every node in the present analysis, the high COI sequence similarity between *B. primigenius* and *C. granulatus* showed that they were not only morphologically but also genetically close to each other.

Our study indicated that *B. primigenius* is a young individual of *C. granulatus*. Ryanskiy (2020) stated that clusters of papulae can help distinguish young individuals of *C. granulatus* from *B. primigenius*; however, the gradual change in the number and distribution pattern of papulae shown in this study suggests that this identification criterion is invalid. Similar significant morphological changes, including the characteristic growth pattern of papulae, have been observed in other oreasterid species. A famous example is the growth of *Culcita novaeguineae*, whose young individual, showing a goniasterid-like body shape, was described as a completely different species (Domantay & Roxas, 1938).

The present study showed that it is appropriate to place *B. primigenius* as a junior synonym for *C. granulatus*. Our samples for the molecular phylogenetic analysis were limited because the so-called "*B. primigenius*" sea stars are rarely encountered species and it was difficult to extract the necessary DNA from fixed specimens preserved for long periods in alcohol. Further analysis of additional specimens and gene regions will support our results and make them more robust and reliable.

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