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Morphological and Genetic Diversity of *Briareum* (Anthozoa: Octocorallia) from the Ryukyu Archipelago, Japan

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The primary problem hindering the study of octocorals is the disordered situation regarding their taxonomy, chiefly caused by insufficient knowledge of valid morphological taxonomic characters. *Briareum* is an octocoral genus found in the Atlantic and Pacific in shallow tropical and subtropical waters, and occurs in both encrusting and branching colony forms. Their simple morphology and morphological plasticity have hindered taxonomic understanding of this genus. In this study three morphologically distinct types (= type-1, -2, and -3) of *Briareum* from the Ryukyu Archipelago and their genetic diversity were examined. Colony, anthostele morphology, and sclerite length were examined for each type. Four molecular markers (mitochondrial cytochrome *c* oxidase subunit 1, mitochondrial mismatch repair gene, nuclear 18S ribosomal DNA, internal transcribed spacer 2 (ITS2)) were used to evaluate molecular phylogenetic status of these variations. Although one morphological type ("deep" small colonies, = type-3) showed small differences in nuclear ITS2 sequences compared to the other two types, the remaining types had identical sequences for all molecular markers examined. The results suggest extremely low genetic diversity despite highly variable morphology of *Briareum* species in Okinawa. Nevertheless, considering the distribution patterns and discontinuous morphology of type-3 compared to the other two morphotypes, genetic isolation of type-3 is plausible. In *Briareum*, small variances in nuclear ITS2 sequences of type-3 may have much more importance than in molecular phylogenies of other octocorals. Further phylogenetic investigations and comparison with *Briareum* specimens from other regions are necessary to conclusively taxonomically identify the three types.

Key words: octocoral, molecular phylogeny, ITS-rDNA, *Briareum*, taxonomy

INTRODUCTION

Octocorals are sessile benthic marine invertebrates, widely distributed from shallow to deep areas (Bayer, 1961; Fabricius and Alderslade, 2001). In coral reefs, species and individuals are abundant, and octocorals are also abundant in extratropical and polar areas where hermatypic corals are lacking. Many species distributed in tropical and subtropical areas are zooxanthellate (Sánchez et al., 2003a). Octocorals are therefore thought to be important in diverse marine ecosystems, although there are few studies to confirm this. Additionally, octocorals are noted as sources of novel natural chemicals, and a large number of studies for these chemicals have been conducted (e.g. Rodríguez et al., 1995; Chen et al., 2006).

Octocorals are a morphologically well-defined group, characterized by having eight tentacles and eight septa. The monophyly of Octocorallia is also strongly supported by many molecular phylogenetic studies (e.g., France et al., 1996; Berntson et al., 1999). In contrast, species-level taxonomy of many octocorals is much more confusing (Bayer, 1981). The overall colony shape of many octocorals is not stable unlike as in some other colonial marine invertebrates. Additionally, most species of octocorals have quite simple morphologies and often lack easily examined, taxonomically valid diagnostic characters except for sclerites. Although sclerites are the traditionally used and principal diagnostic characters for many octocoral species, the wide and complex variety of sclerites hinders formulation of a sclerite-based morphotaxonomy system. Furthermore, in several species environmental plasticity of the morphology of sclerites has been reported (West et al., 1993).

Due to these problems, the formal taxonomy of many octocoral species has been confused for many years. Many synonymous species are likely to exist, while many other species remain to be described because of the difficulty in proper description. Thus, the current situation of octocoral taxonomy hinders further progress of their diversity-related research, and therefore examination and reorganization of the taxonomy of Octocorallia is urgently needed.

Recently, many molecular phylogenetic studies have been conducted to examine presumed relationships in Octocorallia and these studies have successfully revealed systematic positions of some families and genera (e.g., Berntson et al., 2001; Sánchez et al., 2003b; McFadden et al., 2006b; Aguilar et al., 2012; Reijnen et al., 2013). On the other hand, reexaminations of species-level taxonomy in

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Octocorallia by molecular phylogenetic methods have only just begun for most groups of octocorals (McFadden et al., 2009). The systematic relationships of octocoral taxa inferred by molecular phylogenetic methods often do not fit with traditional morphological taxonomy, and therefore both morphological and phylogenetic investigations are necessary to reconstruct valid and practical taxonomy/classifications.

Briareum (Octocorallia: Alcyonacea) is a genus of octocorals widely distributed in coral reefs of the Caribbean, Red Sea, Indo-West Pacific, and West Africa. *Briareum* has a quite simple morphology among octocorals, with few diagnostic taxonomical characters. Colonies are encrusting, matlike, or digitate in form, with finger-like projections. Polyps are monomorphic, and can retract into the coenenchyme, which consists of a cortex and medullar layer. The color of the surface of colonies varies from purple to pale pink, cream, gray, or white. The colors of polyps are generally brownish, pale pink, or fluorescent green and the polyps contain zooxanthellae. Verseveldt (1940) gave details regarding the morphology and anatomy of Briareidae and *Briareum*. Some recent molecular phylogenetic studies have shown that the genera *Briareum* and *Erythropodium* may be the most ancestral groups in the octocorallian radiation (Berntson et al., 2001; McFadden et al., 2006a).

Briareum is known to show morphological plasticity in colony shape and sclerite length (West et al., 1993). These problems make *Briareum* an especially difficult genus when considering species identification. As valid distinguishing characters for species are not known, the actual number of species of *Briareum* is unknown, although Chen et al. (2006) mention that there are four valid species in genus *Briareum*. Due to these identification problems, *Briareum* was not examined by phylogenetic methods until Bilewitch et al. (2010) studied two forms of Atlantic *Briareum* (encrusting vs. digitate; once divided as *B. polyanthes* and *B. asbestinum*) using both morphological and molecular phylogenetic techniques, and concluded that these two forms are conspecific.

From Japan, *B. violaceum* (*Clavularia violacea*; *Pachyclavularia violacea*; *Pachyclavularia erecta*) and *B. excavatum* (*Solenopodium excavatum*) have been recorded (Utinomi, 1956; Benayahu, 2002). From Taiwan, *B. marquesarum* (*Erythropodium marquesarum*; *Solenopodium marquesarum*) has also been recorded (Utinomi, 1959). In the Ryukyu Archipelago in southern Japan, there are three morphological types of *Briareum*; two are morphologically distinct in colony color (purple = type-1, pale or pink = type-2), and another type (= type-3) is distinguished by its small colony size and fluorescent tentacles. All three types are encrusting and there are no records of digitate *Briareum* spp. from Japan. In this study, these three types of *Briareum* were morphologically analyzed (sclerite shape, size, type composition, distribution, arrangement; colony shape, color), and phylogenetic relationships were inferred by using four molecular markers.

Mitochondrial cytochrome *c* oxidase subunit 1 (COI) and mismatch repair protein (msh1), and nuclear 18S ribosomal DNA (18S rDNA) and internal transcribed spacer 2 of ribosomal DNA (ITS2) were used to infer the phylogenetic relationships of the three types of *Briareum*. COI is a molecular marker that has been used to deduce phylogenetic relationships of Cnidaria. Although Reimer et al. (2004) and Sinniger et al. (2008) have shown that some species of the hexacorallian order Zoantharia can be distinguished by analyses of COI, generally for octocorals, COI sequences have low variability among species and genera, and this marker is considered more suitable for investigating higher-level (genus and higher) phylogenies. Msh1 is a mitochondrial region specific to octocorals and is considered to be a homolog of the prokaryote MutS gene (France et al., 1996; Berntson et al., 1999). *Msh1* shows much more variability than COI, and is appropriate for more detailed phylogenetic investigations in many groups of octocorals (Sánchez et al., 2003b; Wirshing et al., 2005; McFadden et al., 2006b). 18S rDNA generally evolves slowly, and has utility in examining affinity in broad taxa, while ITS2 is the most variable among molecular markers used for octocoral molecular phylogenetic analyses, and is even effective for detecting hybridization (McFadden and Hutchinson, 2004). However, ITS2 sequences often have high levels of intragenomic variation (McFadden and Hutchinson, 2004; Bilewitch et al., 2010).

In this study, using these four molecular markers, the phylogenetic relationships of specimens of the three morphological types of *Briareum* were examined and compared with morphological differences.

MATERIALS AND METHODS

Sampling

Specimens of *Briareum* were collected from several locations around Okinawa Island (Sesoko Island, Manzamo, Shin-Yona Tunnel Beach, Miyagi-kaigan, Cape Maeda), from other islands in the Ryukyu Archipelago (Miyako Island, Ishigaki Island, Iriomote Island, Kohana Island, Tanegashima Island), and from Chichijima Island in the Ogasawara Islands at depths between 1.5 m to 26 m (Table 1) from November 2008 to August 2009. Sampled colonies were preserved in 99.5% ethanol. Digital images were also taken in situ by digital camera with a waterproof case to assist in distinguishing types of *Briareum* and to record approximate colony size.

Morphological analyses

Digital images were examined to check colony size, colony color, and anthostele form type (Fig. 1). Sclerites were isolated from anthosteles and from the surface of coenenchyme, the inner part of the coenenchyme, and the medulla by dissolving small pieces of tissues with household bleach containing sodium hypochlorite, followed by rinsing with distilled water and air-drying. Sclerite specimens were stuck to aluminum specimen mounts using carbon double-faced tape, and then examined and pictured with a scanning electron microscope (SEM) (Keyence VE-8800). For sclerites from anthosteles and surface of coenenchyme, length and width were measured and recorded using observational software (VE-H2A Ver. 1.0, Osaka, Japan).

DNA extraction and PCR amplification

DNA was extracted from tentacle tissue of ethanol-preserved samples by guanidine extraction protocol following Sinniger et al. (2009). PCR amplifications were performed using HotStarTaq DNA polymerase (Qiagen, Tokyo, Japan) following the manufacturer's instructions. Mitochondrial *msh1* was amplified by the primers ND42599F: 5′-GCCATTATGGTTAACTATTAC-3′ (France and Hoover, 2002) and Mut-3458R: 5′-TSGAGCAAAAGCCACTCC-3′ (Sánchez et al., 2003b) under conditions as follows: 15 min at 94°C; 35 cycles of 1.5 min at 94°C, 1.5 min at 58°C, and 1 min at 72°C; and 5 min at 72°C. COI was amplified by the primers COII-8068: 5′-

Table 1. *Briareum* specimens examined in this study and their collection information.

Sample number	Locality	Latitude	Longitude	Date	Collector	Depth (m)	Type*
B001	Cape Maeda, Onna Vil., Okinawa Is.	26°45′N	127°77′E	2008	J. Reimer	–	
B002	Sesoko Is, Motobu Town	26°63′N	127°86′E	2007.11	Y. Miyazaki	< 2	1
B003	Sesoko Is, Motobu Town	26°63′N	127°86′E	2007.11	Y. Miyazaki	< 2	1
B004	Sesoko Is, Motobu Town	26°63′N	127°86′E	2007.11	Y. Miyazaki	< 2	1
B005	Sesoko Is, Motobu Town	26°63′N	127°86′E	2007.11	Y. Miyazaki	< 2	1
B006	Sesoko Is, Motobu Town	26°63′N	127°86′E	2007.11	Y. Miyazaki	< 2	1
B007	Sesoko Is, Motobu Town	26°63′N	127°86′E	2007.11	Y. Miyazaki	< 2	2
B008	Sesoko Is, Motobu Town	26°63′N	127°86′E	2007.11	Y. Miyazaki	< 2	2
B009	Sesoko Is, Motobu Town	26°63′N	127°86′E	2007.11	Y. Miyazaki	< 2	2
B010	Sesoko Is, Motobu Town	26°63′N	127°86′E	2007.11	Y. Miyazaki	< 2	2
B011	Sesoko Is, Motobu Town	26°63′N	127°86′E	2007.11	Y. Miyazaki	< 2	2
B012	Sesoko Is, Motobu Town	26°63′N	127°86′E	2009.3.19	Y. Miyazaki	< 2	1
B013	Sesoko Is, Motobu Town	26°63′N	127°86′E	2009.3.19	Y. Miyazaki	< 2	1
B014	Sesoko Is, Motobu Town	26°63′N	127°86′E	2009.3.19	Y. Miyazaki	< 2	1
B015	Sesoko Is, Motobu Town	26°63′N	127°86′E	2009.3.19	Y. Miyazaki	< 2	2
B016	Sesoko Is, Motobu Town	26°63′N	127°86′E	2009.3.19	Y. Miyazaki	< 2	1
B017	Sesoko Is, Motobu Town	26°63′N	127°86′E	2009.3.19	Y. Miyazaki	< 2	1
B018	Sesoko Is, Motobu Town	26°63′N	127°86′E	2009.3.19	Y. Miyazaki	< 2	2
B019	Sesoko Is, Motobu Town	26°63′N	127°86′E	2008.12.28	Y. Miyazaki	< 2	1
B020	Sesoko Is, Motobu Town	26°63′N	127°86′E	2008.12.28	Y. Miyazaki	< 2	1
B021	Manzamo, Onna Vil, Okinawa Is.	26°50′N	127°85′E	2008.10.21	Y. Miyazaki	–	1
B022	Manzamo, Onna Vil, Okinawa Is.	26°50′N	127°85′E	2008.10.21	Y. Miyazaki	–	1
B023	Funauki, Iriomote Is. Takekomi Town	24°33′N	123°74′E	2009.3.17	M. Obuchi	26	1
B024	Sesoko Is, Motobu Town	26°63′N	127°86′E	2009.4.4	Y. Miyazaki	< 2	1
B025	Manzamo, Onna Vil, Okinawa Is.	26°50′N	127°85′E	2009.4.14	Y. Miyazaki	19.3	1
B026	Manzamo, Onna Vil, Okinawa Is.	26°50′N	127°85′E	2009.4.14	Y. Miyazaki	17.6	3
B027	Manzamo, Onna Vil, Okinawa Is.	26°50′N	127°85′E	2009.4.14	Y. Miyazaki	17.6	3
B028	Manzamo, Onna Vil, Okinawa Is.	26°50′N	127°85′E	2009.4.14	J. Reimer	14.9	3
B029	Manzamo, Onna Vil, Okinawa Is.	26°50′N	127°85′E	2009.4.14	J. Reimer	16.3	3
B030	Manzamo, Onna Vil, Okinawa Is.	26°50′N	127°85′E	2009.4.14	Y. Miyazaki	15.4	3
B031	Yona, Kunigami Vil, Okinawa Is.	26°77′N	128°19′E	2008.9.25	Y. Miyazaki	< 2	1
B032	Yona, Kunigami Vil, Okinawa Is.	26°77′N	128°19′E	2008.9.25	Y. Miyazaki	< 2	1
B033	Yona, Kunigami Vil, Okinawa Is.	26°77′N	128°19′E	2008.9.25	Y. Miyazaki	< 2	1
B034	Tanegashima Is.	–	–	2008.7.17	F. Iwase	25	1
B035	Ohama Is, Takekomi Town	24°35′N	123°96′E	2008.5.7	J. Reimer	–	2
B036	Beach of Hora Port, Miyako Is.	24°73′N	125°43′E	2008.5.7	Y. Irei	< 2	1
B037	Beach of Hora Port, Miyako Is.	24°73′N	125°43′E	2008.5.7	Y. Irei	< 2	2
B038	Beach of Hora Port, Miyako Is.	24°73′N	125°43′E	2008.5.7	Y. Irei	< 2	2
B039	Miyagi beach, Chatan, Town, Okinawa Is.	26°33′N	127°75′E	2009.4.21	Y. Miyazaki	1.5	1
B040	Miyagi beach, Chatan, Town, Okinawa Is.	26°33′N	127°75′E	2009.5.19	Y. Miyazaki	4.3	1
B041	Marugu, Ishigaki Is.	24°28′N	124°03′E	2009.4.29	J. Reimer	2.9	1
B042	Yonara Channel, Ishigaki Is.	24°33′N	123°94′E	2009.4.30	J. Reimer	9.0	2
B043	Urabishi, Ishigaki Is.	24°27′N	124°03′E	2009.4.29	J. Reimer	7.0	1
B044	Yonara Channel, Ishigaki Is.	24°33′N	123°94′E	2009.4.30	J. Reimer	16.9	2
B045	Chichijima Is, Ogasawara Vil.	27°10′N	142°19′E	2009.5	F. Sinniger	–	1
B046	Chichijima Is, Ogasawara Vil.	27°10′N	142°19′E	2009.5	F. Sinniger	–	1
B047	Miyanohama, Chichijima Is, Ogasawara Vil.	27°10'N	142°19′E	2009.5.9	J. Reimer	1.0	1
B048	Tatsumi Bay, Chichijima Is, Ogasawara Vil.	27°05′N	142°23′E	2009.5.10	J. Reimer	1.0	1
B049	Miyanohama, Chichijima Is, Ogasawara Vil.	27°10'N	142°19′E	2009.5.9	J. Reimer	1.0	1
B050	Manzamo, Onna Vil, Okinawa Is.	26°51′N	127°85′E	2009.8.4	Y. Miyazaki	14.7	3
B051	Manzamo, Onna Vil, Okinawa Is.	26°51′N	127°85′E	2009.8.4	Y. Miyazaki	4.1	1
B052	Manzamo, Onna Vil, Okinawa Is.	26°51′N	127°85′E	2009.8.4	Y. Miyazaki	5.6	1
B053	Manzamo, Onna Vil, Okinawa Is.	26°51′N	127°85′E	2009.8.4	Y. Miyazaki	16.7	3
B054	Manzamo, Onna Vil, Okinawa Is.	26°51′N	127°85′E	2009.8.4	Y. Miyazaki	17.3	3
B055	Manzamo, Onna Vil, Okinawa Is.	26°51′N	127°85′E	2009.8.4	Y. Miyazaki	15.4	3
B056	Manzamo, Onna Vil, Okinawa Is.	26°51′N	127°85′E	2009.8.4	Y. Miyazaki	15.1	3
B057	Manzamo, Onna Vil, Okinawa Is.	26°51′N	127°85′E	2009.8.4	Y. Miyazaki	16.4	3
B058	Manzamo, Onna Vil, Okinawa Is.	26°51′N	127°85′E	2009.8.4	Y. Miyazaki	5.8	1

*for type definitions, see Materials and Methods.

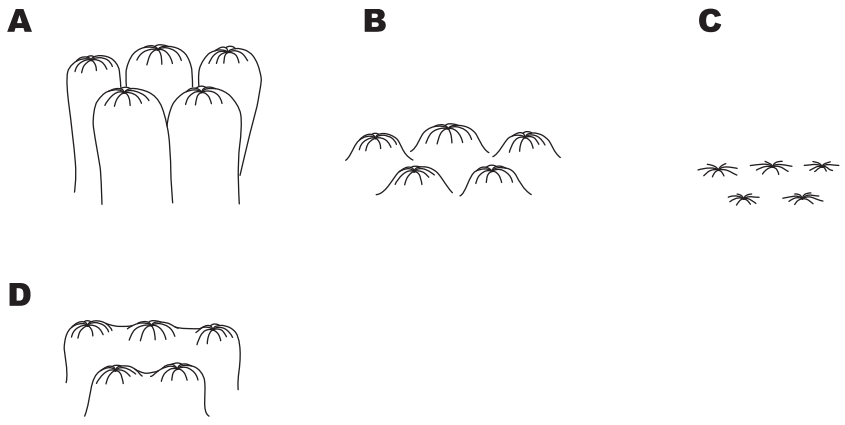

Fig. 1. Anthostele types of *Briareum*. **(A)** Colony with high calyces (height > width). **(B)** Colony with medium calyces (height < width). **(C)** Colony with flat anthosteles. **(D)** Calyces merged in rows.

Table 2. Octocoral GenBank sequences used as outgroups in constructing molecular phylogenetic trees in this study.

	Species	Accession numbers	Source paper
mitochondrial COI			
1	*Briareum asbestinum*	DQ640649	Medina et al. (2006a)
2	*Briareum asbestinum*	GQ342405	Parrin et al. (unpub.)
3	*Briareum hamrum*	GU355975	McFadden et al. (2011)
4	*Ellisella* sp.	FJ642932	Thoma et al. (unpub.)
5	*Titanideum frauenfeldii*	FJ264916	France and Thoma (unpub.)
6	*Telestula* cf. *spiculicola*	FJ264917	France and Thoma (unpub.)
7	*Cryogorgia koolsae*	FJ264910	France and Thoma (unpub.)
8	*Swiftia pallida*	FJ264905	France and Thoma (unpub.)
9	*Cryogorgia koolsae*	FJ264910	France and Thoma (unpub.)
10	*Swiftia pallida*	FJ264905	France and Thoma (unpub.)
11	*Anthothela nuttingi*	FJ264908	France and Thoma (unpub.)
12	*Acanthogorgia granulata*	FJ264903	France and Thoma (unpub.)
13	*Iridogorgia magnispiralis*	FJ268639	France and Pante (unpub.)
14	*Homophyton verrucosum*	GQ342403	Parrin et al. (unpub.)
15	*Ideogorgia capensis*	GQ342428	Parrin et al. (unpub.)
mitochondrial msh1			
16	*Briareum asbestinum*	FJ434352	Bilewitch et al. (unpub.)
17	*Briareum asbestinum*	AY533653	Sanchez and Cairns (2004)
18	*Briareum asbestinum*	DQ640649	Medina et al. (2006)
19	*Briareum asbestinum*	GQ342484	Parrin et al. (unpub.)
20	*Briareum asbestinum*	GQ342405	Parrin et al. (unpub.)
21	*Briareum hamrum*	GU355975	McFadden et al. (2011)
22	*Plexaura flexuosa*	EF659598	Prada et al. (2008)
23	*Chrysogorgia* sp.	EU268056	Brugler and France (2008)
24	*Radicipes gracilis*	DQ297424	McFadden et al. (2006a)
25	*Eleutherobia* sp.	DQ302809	McFadden et al. (2006a)
26	*Chrysogorgia chryseis*	DQ297421	McFadden et al. (2006a)
27	*Eleutherobia rotifera*	GQ342472	Parrin et al. (unpub.)
28	*Alcyonium valdiviae*	GQ342469	Parrin et al. (unpub.)
29	*Ideogorgia capensis*	GQ342502	Parrin et al. (unpub.)
30	*Titanideum frauenfeldii*	GU563314	McFadden et al. (2011)
31	*Homophyton verrucosum*	GQ342482	Parrin et al. (unpub.)
Nuclear ITS2			
32	*Plexaura kuna*	EF090737	Aguilar and Sanchez (2007)
33	*Alaskagorgia aleutiana*	EF090733	Aguilar and Sanchez (2007)
34	*Alcyonium digitatum*	EF090736	Aguilar and Sanchez (2007)
35	*Muricea muricata*	EF090734	Aguilar and Sanchez (2007)
36	*Pachyclavularia* sp.	AB055936	Fujiwara (unpub.)
37	*Briareum asbestinum*	FJ357419	Bilewitch et al. (unpub.)

CCATAACAGGACTAGCAGCATC-3′ (McFadden et al., 2004) and COI-OCTr: 5′-ATCATAGCATAGACCATACC-3′ (France and Hoover, 2002) under the following conditions: 5 min at 95°C; 35 cycles of 1 min at 94°C, 1 min at 40°C, and 1.5 min at 72°C; and 7 min at 72°C. Nuclear 18S rDNA was amplified by the primers 18SA: 5′-GAGGGAGCCTGAGAAATGG-3′ (Beebe et al., 2000) and 18SB: 5′-CCGTCAATTCCTTTAAGTTT-3′ (Beebe et al., 2000) under the following conditions: 15 min at 95°C; 40 cycles of 0.5 min at 95°C, 0.5 min at 58°C, and 2 min at 72°C; and 5 min at 72°C. Nuclear ITS2 was amplified by the primers 5.8S-436: 5′-AGCATGTCTGTCTGAGTGTTGG-3′ (Aguilar and Sánchez, 2007) and 28S-663: 5′-GGGTAATCTTGCCTGATCTGAG-3′ (Aguilar and Sánchez, 2007) under the following conditions: 15 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 50°C, and 2 min at 72°C; and10 min at 72°C.

Amplified products were visualized with 1.0% agarose gel electrophoresis. Positive PCR products were cleaned up by Exonuclease I and Shrimp Alkaline Phosphatase (Takara) before sequencing

Sequence analysis

Cycle sequencing was performed in both directions using the forward and reverse primers separately with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) under reaction conditions following the manufacturer's instructions. Reaction products were analyzed on an ABI PRISM 3700 DNA Analyzer (Applied Biosystems). The sequences were analyzed by 4Peaks Version 1.7 software (mekentosj.com, Amsterdam, Netherlands).

By using Se-AL v2.0a11 software (Rambaut, 2002), the nucleotide sequences of *msh1*, COI, 18S, and ITS2 from *Briareum* obtained in the present study were separately aligned with sequences of *Briareum* and other octocoral species retrieved from GenBank (Table 2). The alignments were checked by eye and manually edited to remove any ambiguous sites (e.g. double peaks) before phylogenetic analyses. For each alignment, none or only one to two base pairs were edited in this manner. Four aligned data sets were generated: 1) 752 sites of 54 sequences (*msh1*), 2) 602 sites of 43 sequences (COI), 3) 736 sites of 17 sequences (18S), and 4) 331 sites of 33 sequences (ITS2), respectively. The alignment data are available on request from the corresponding author.

Phylogenetic analyses

Maximum-likelihood (ML) analyses with PhyML (Guindon and Gascuel, 2003) of these datasets were independently performed using input trees generated by BIONJ (Gascuel, 1997) with the general time reversible (GTR) model. PhyML bootstrap trees (1000 replicates) were constructed using the same parameters as the individual ML trees.

Bayesian trees were reconstructed by using the program MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). One cold and three heated

Markov chain Monte Carlo (MCMC) chains with default-chain temperatures were run for 1,000,000 generations, sampling log-likelihoods (lnLs), and trees at 100-generation intervals (10,000 lnLs and trees were saved during MCMC). The first 30% (COI), 20% (*msh1*), and 10% (18S rDNA, ITS2) of all runs were discarded as "burn-in" for all datasets. The likelihood plots for all three datasets also showed that MCMC reached the stationary phase by these times. Thus, the remaining 7000 (COI), 8000 (*msh1*), or 9000 (18S rDNA, ITS2) trees were used to obtain posterior probabilities and branch-length estimates, respectively.

Neighbor-joining (NJ) trees were also reconstructed by using CLC Free Workbench 4 software (CLCbio.com, Aarhus North, Denmark) (500 replicates).

RESULTS

Morphological analyses

Three types of morphologically distinct *Briareum* (type-1, -2, and -3) were sampled from shallow waters of the Ryukyu Archipelago and the Japanese mainland.

Type-1 formed large colonies growing up to 1 m^2 in area (Figs. 2A, 2B). Anthostele shapes varied from A-type (anthostele projecting from coenenchyme and forming high calyces; Figs. 1, 2D) to B-type (projecting anthostele, but calyx height not exceeding width; Figs. 1, 2F). Some specimens showed calyces merged in rows and forming platform-like structures (Figs. 1, 2G). Surface colors of type-1 colonies were deep purple. The lengths of the polyp tentacles showed broad variation, and some specimens had long (> 20 mm) tentacles (Fig. 2C). Fully expanded polyps were completely open with extended tentacles and the oral disc having a palm-like shape (Fig. 2C, E). Colonies consisted of two layers: cortex and medulla. The surfaces of the cortex and anthosteles were covered with deep purple spindles. On the top of the anthosteles, there were flat spindles with prominent warts arranged in a horizontal direction (three sclerites on left in Fig. 3A). Long and pointed spindles with elliptic cross sections were arranged in longitudinal direction on the sides of anthosteles (four sclerites on right in Fig. 3A). Beneath these, colorless thick spindles were present (Fig. 3B). In the medulla, deep magenta colored spindles and irregularly branched spindles with high warts were found (Fig. 3C).

Type-2 also formed large colonies (reaching 1 m^2) (Fig. 4A, B). The majority of anthosteles were C-type (calyces not projecting from coenenchyme; Figs. 1, 4D), but some spec-

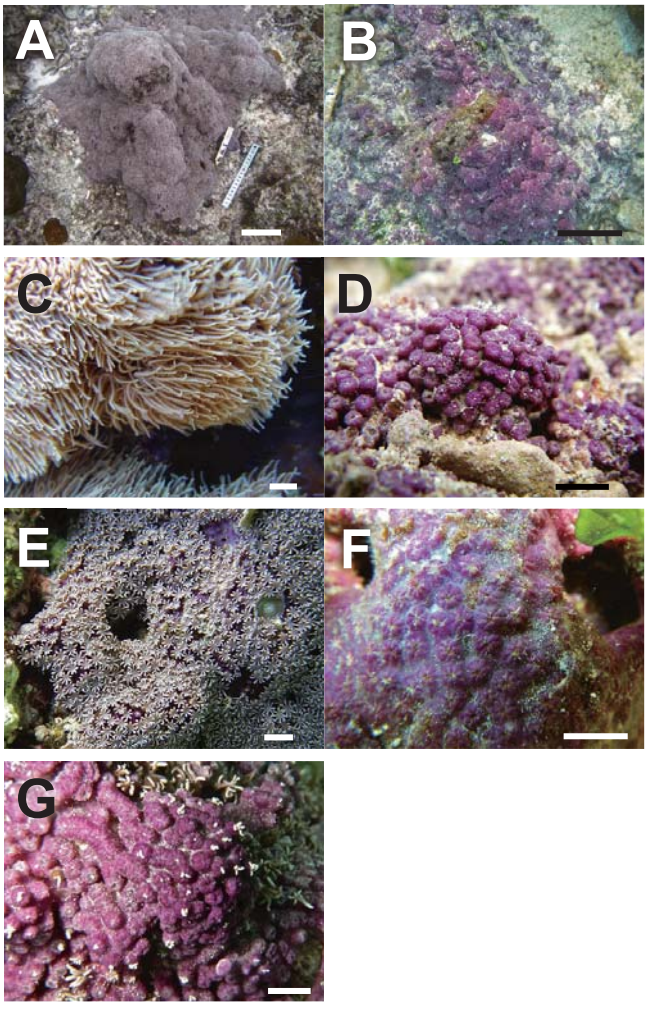

Fig. 2. Underwater photographs of type-1 *Briareum* (Sesoko Is, Japan). **(A, B)** Whole colonies (bars = 10 cm); **(C, E)** expanded polyps; **(D, F)** anthosteles; **(G)** calyces merged in rows (see Results) (bars = 10 mm). **(A, B, C, D, E)** = specimen B012; E = B013; F = 014; G = B017. Image taken March 19, 2009.

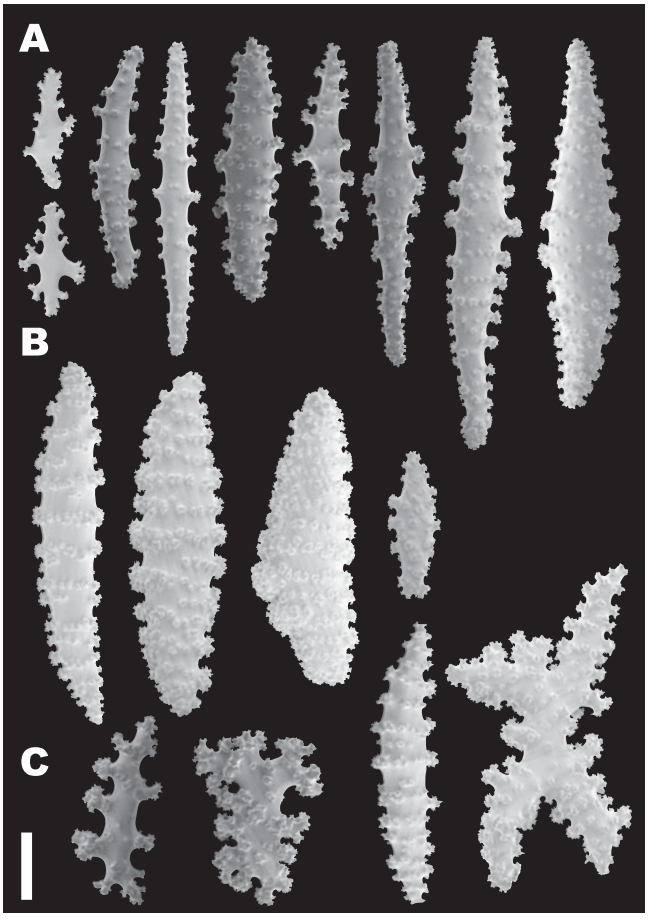

Fig. 3. Scanning electron microscopic images of sclerites from *Briareum* colonies type-1. **(A)** Sclerites from surface of cortex; **(B)** sclerites from innner cortex; **(C)** sclerites from medulla. Bar = 0.1 mm.

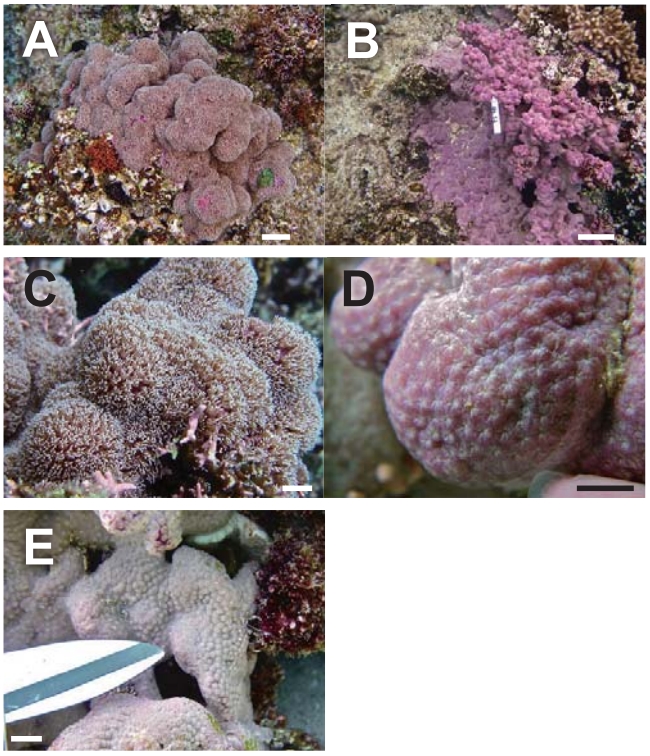

Fig. 4. Underwater photographs of type-2 *Briareum* (Sesoko Is, Japan). **(A, B)** Whole colonies (bars = 10 cm); **(C)** expanded polyps; **(D)** anthosteles; **(E)** white, shaded part of colony (see Results) (bars = 10 mm) **(A, B, C, D, E)** = specimen B015, image taken March 19, 2009.

imens had some B-type anthosteles. No specimens had merged calyces. Surfaces of type-2 colonies were pale purple to pink, but shaded parts of colonies were regularly white or ivory. The lengths of the polyp tentacles did not exceed 15 mm (Fig. 4C). Fully expanded polyps did not stretch tentacles laterally and looked "shriveled" (Fig. 4C). Colonies consisted of cortex and medulla. The surfaces of the cortex and anthosteles were covered with colorless and light purple sclerites (spindles). On the tops of anthosteles there were flat spindles with high warts arranged in a horizontal direction (three sclerites on left in Fig. 5A). Short spindles with elliptic cross sections were arranged in longitudinal direction on the side of anthosteles (right four of Fig. 5A). Beneath these, colorless thick spindles were present (Fig. 5B). In the medulla, deep magenta colored spindles and irregularly branched spindles with prominent warts were found (Fig. 5C).

Type-3 did not form large colonies (< 0.2 m^2 in coverage area) (Fig. 6A). Anthosteles were A-type or B-type (Figs. 1, 6B), but no specimens had merged calyces as seen in type-1. The surface color of type-3 colonies was deep purple. Lengths of the polyp tentacles showed variation although they never exceeded 20 mm. Fully expanded polyps stretched tentacles laterally (Fig. 6C, D). Colonies consisted of cortex and medulla. The surfaces of the cortex and anthosteles were covered with deep purple spindles On the tops of anthosteles, there were flat spindles with prominent warts arranged in a horizontal direction (left three of Fig. 7A). Long and pointed spindles with elliptic cross sections

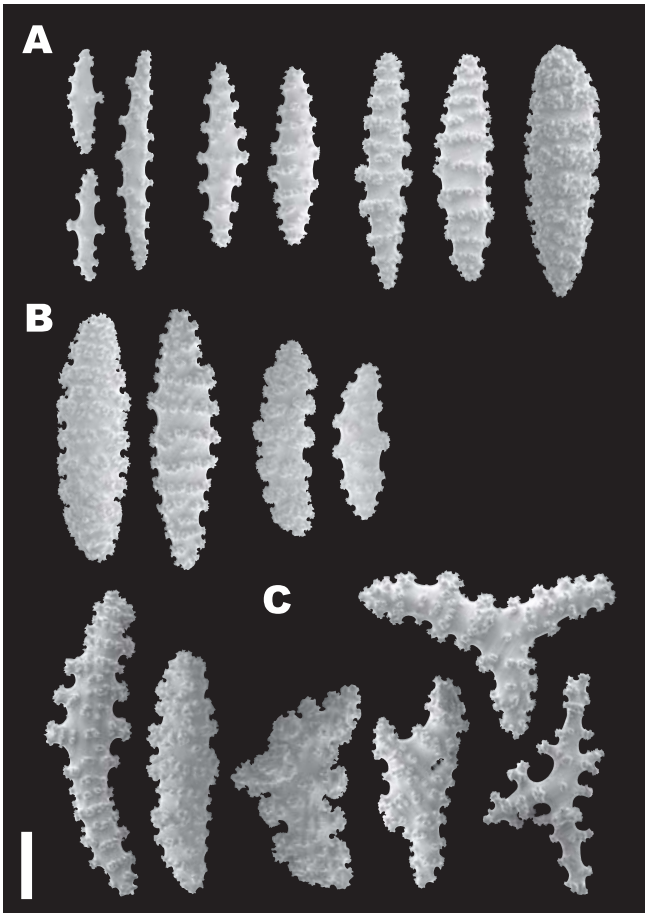

Fig. 5. Scanning electron microscopic images of sclerites from *Briareum* colonies type-2. **(A)** sclerites from surface of cortex; **(B)** sclerites from innner cortex; **(C)** sclerites from medulla. Bar = 0.1 mm.

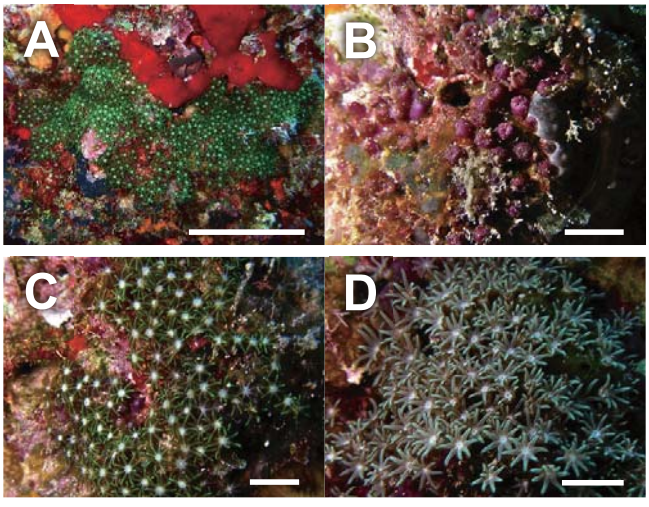

Fig. 6. Underwater photographs of type-3 *Briareum* (Manzamo, Japan). **(A, B)** Whole colonies **(A)** bar = 10 cm, **(B)** bar = 10 mm); **(C, D)** expanded polyps (bars = 10 mm). A = specimen B053, B = B057, C = B056, D = B054; E = B013; F = 014; G = B017. Images taken August 4, 2009.

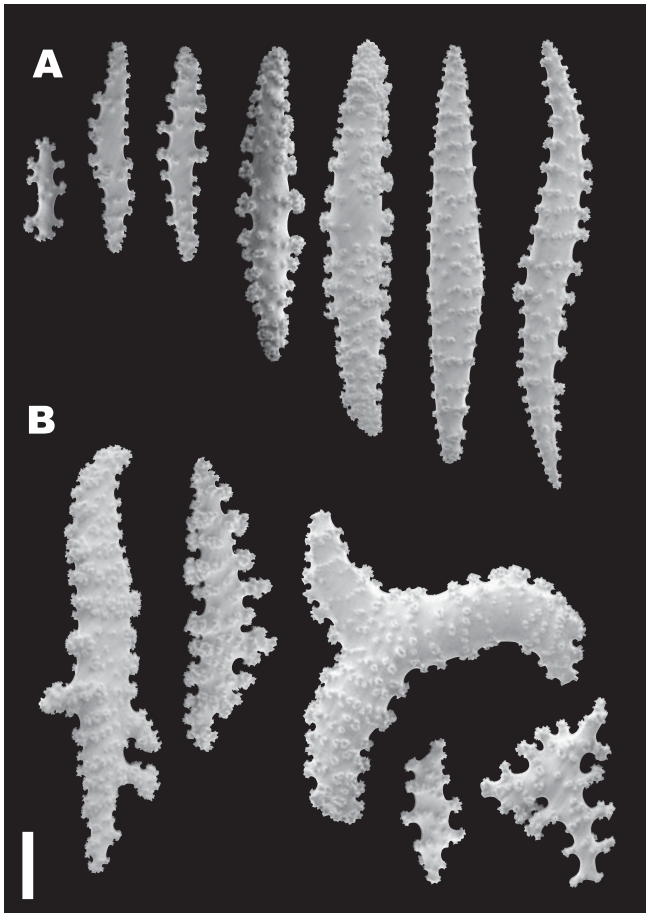

Fig. 7. Scanning electron microscopic images of sclerites from *Briareum* colonies type-3. **(A)** Sclerites from surface of cortex; **(B)** sclerites from medulla. Cortex of type-3 colonies was very thin and thick spindles as observed in the inner cortex of type-1 and -2 colonies were not present. Bar = 0.1 mm.

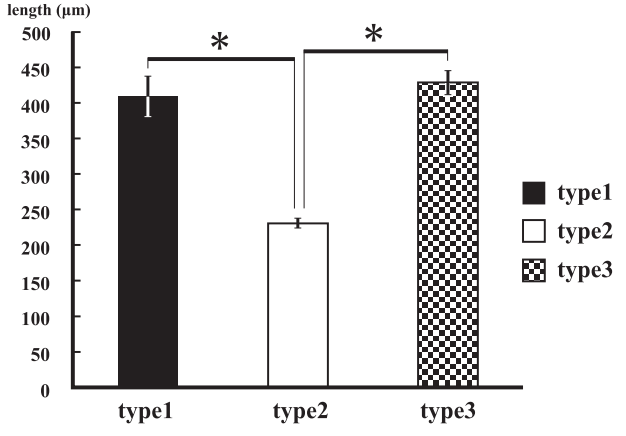

Fig. 8. Spindle length and width of three *Briareum* types. Each plot represents an individual specimen (specimen n = 11 for each type; sclerites n = 60 for each specimen). Error bars are SD for spindle length of each specimen.

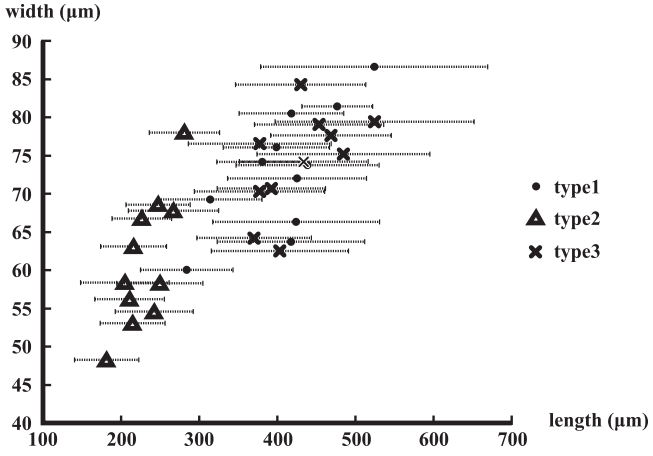

Fig. 9. Variation in spindle length among three types of *Briareum*. Error bars shows SD. *P < 0.01 (Steel-Dwass' test; specimen n = 11 for each type; sclerites n = 60 for each specimen).

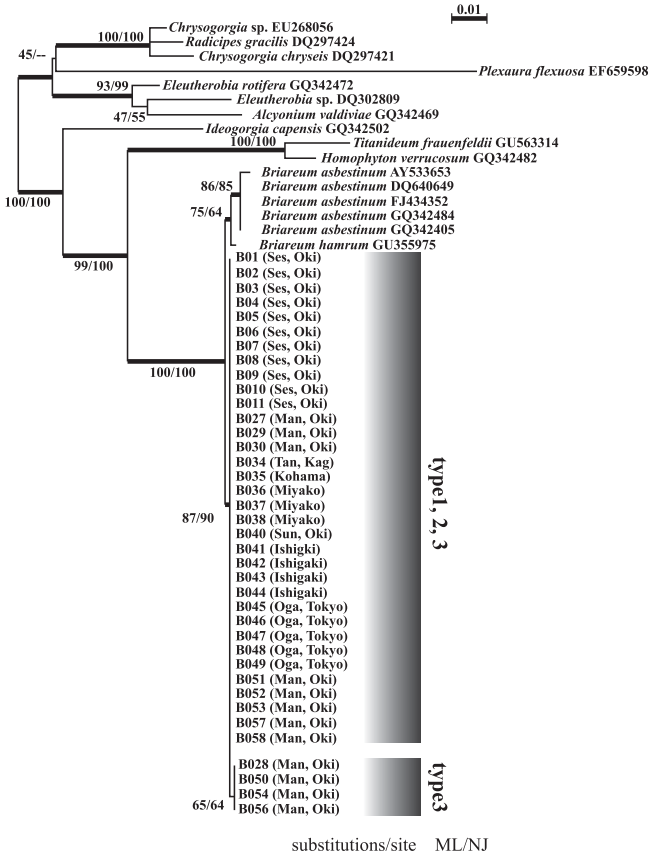

Fig. 10. Maximum likelihood tree for mitochondrial msh1 sequences. Values at branches represent ML and NJ bootstrap probabilities, respectively (> 50%). Sequences without accession numbers were newly obtained in this study. Bold lines represent branches with very high support in Bayesian analyses (> 0.95).

were arranged in longitudinal direction on the side of anthosteles (four sclerites on right in Fig. 7A). A layer with colorless sclerites (thick spindles) was absent. In the medulla, deep magenta-colored spindles and irregularly branched spindles with high warts were found (Fig. 7B).

The lengths of sclerites from anthosteles and surface of coenenchyme were significantly shorter in type-2 specimens

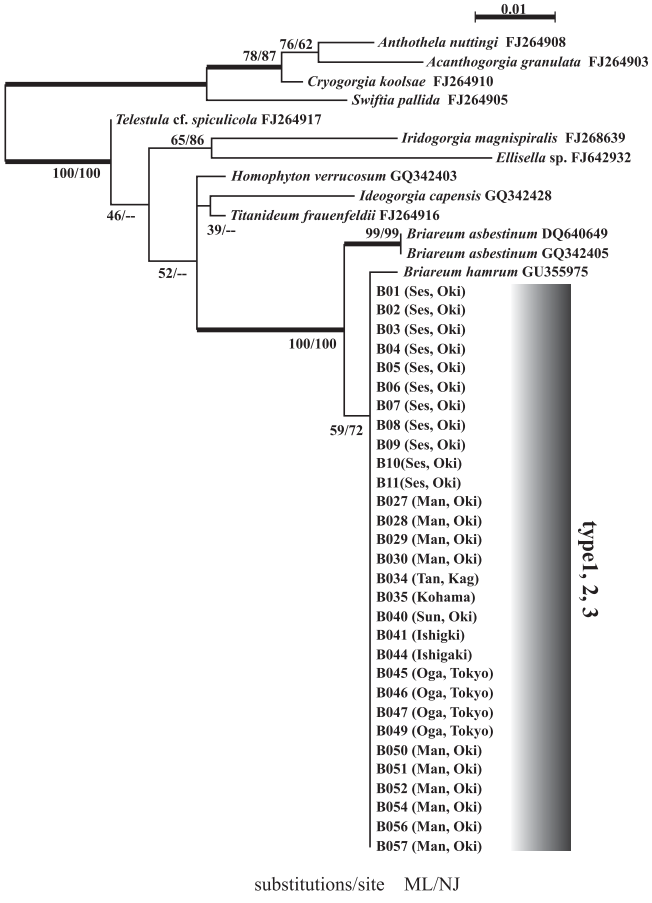

Fig. 11. Maximum likelihood tree for mitochondrial cytochrome oxidase subunit I (COI) sequences. Values at branches represent ML and NJ bootstrap probabilities, respectively (> 50%). Sequences without accession numbers were newly obtained in this study. Bold lines represent branches with very high support in Bayesian analyses (> 0.95).

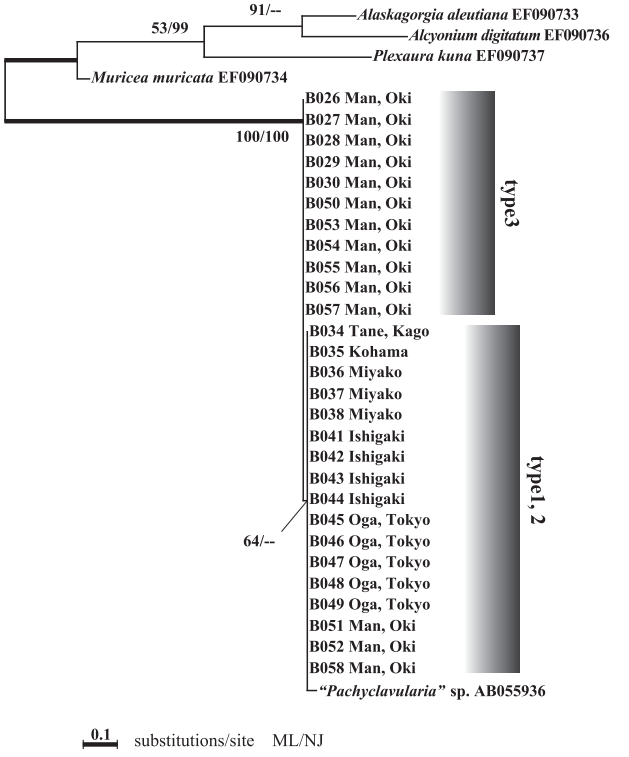

Fig. 12. Maximum likelihood tree for nuclear ITS2 sequences. Values at branches represent ML and NJ bootstrap probabilities, respectively (> 50%). Sequences without accession numbers were newly obtained in this study. Bold lines represent branches with very high support in Bayesian analyses (> 0.95).

than in type-1 and type-3 ($P < 0.05$), but no significant difference was observed between type-1 and type-3 sclerite lengths (Steel-Dwass' test; specimen $n = 11$ for each type; sclerites $n = 60$ for each specimen) (Figs. 8, 9).

Molecular analyses

Newly acquired sequences were deposited in GenBank under Accession Numbers AB763395–AB763399.

The resulting ML trees for *msh1* (Fig. 10) and mt COI (Fig. 11) alignments showed similar topologies. All *Briareum* sequences (sequences for samples of this study + sequences from previous studies in GenBank) made a strongly supported clade (ML = 100%: Bayes = 1.00, NJ = 100% in *msh1*, COI) within but clearly separate from other octocoral genera. *Briareum* sequences obtained in this study made one moderately well supported subclade separate from previously obtained *B. asbestinum* sequences from the Atlantic Ocean (ML = 87%; Bayes = 0.95, NJ = 90% in *msh1*). In the *msh1* alignment, four type-3 specimens had one unique nucleotide substitution (at sites 125–127) and formed a weakly supported subclade (ML = 65%; Bayes = 0.78, NJ = 64%). Every other specimen in this study (containing type-1, -2, and -3 specimen sequences) had identical *msh1* sequences. In COI and 18S rDNA alignments, all specimens of all types examined had identical sequences.

The resulting ML tree for nuclear ITS2 sequences is shown in Fig. 12. Although type-1 and -2 specimens had completely identical ITS2 sequences, type-3 ITS2 had two nucleotide differences over 192 nucleotides for this region (= 1.0% variation). Type-1 and -2 *Briareum* formed a weakly supported clade with GenBank sequences of "*Pachyclavularia violacea*" (AB055936) (note: *Pachyclavularia* is a junior synonym of *Briareum*) (ML = 64%, Bayes = 0.75, NJ < 50%). The ITS2 sequence of *B. asbestinum* from the Atlantic Ocean had 17 nucleotide differences from type-1, -2, and -3 sequences over 115 nucleotides for this region (= 14.8% variation). These *B. asbestinum* sequences were not included in molecular analyses due to their short lengths.

DISCUSSION

Discrimination of three types of *Briareum* specimens

Despite morphological distinction in surface color of the colony, anthostele shape, polyp opening form, and sclerite size, type-1 and type-2 of *Briareum* showed no variance in sequences examined in this study. Two hypotheses can be made from these results: 1) the two types are conspecific, and are simply environmental variations of the same species; or 2) type-1 and type-2 are distinct species that are reproductively isolated, but the molecular markers in this study failed to show variation between them. In contrast, all specimens of *Briareum* type-3—morphologically distinguish-

able by colony size, sclerites forms, and fluorescence in tentacles—had slightly variant ITS2 sequences from type-1 and type-2 specimens. While the status of type-1 and type-2 remain somewhat ambiguous, we propose that *Briareum* type-3 is a distinct species from type-1 and type-2.

Candidate species for the three types of *Briareum* in this study

Morphological characters (colony color/size, layer composition and thickness, shape of anthostele, sclerites morphology) of type-1 *Briareum* coincide well with descriptions for *Briareum violaceum* (*Pachyclavularia violacea*) by Utinomi (1956) and Verseveldt (1960). Although in Benayahu (2002) there is no formal diagnosis of *B. excavatum*, underwater images of *B. excavatum* within this paper are very similar to type-2 *Briareum*. There are no records of any *Briareum* species in the past literature with very small colonies, fluorescent tentacles, and living in comparatively deep habitats, as seen in type-3 in this study.

Relationship of type-1 and type-2

Octocorals are known to exhibit morphological plasticity in response to environmental factors, and this includes characters such as colony growth form, and the shape and the size of sclerites, all of which are traditionally used in taxonomic descriptions (e.g., Bayer, 1961; West et al., 1993; Prada et al., 2008). For *Briareum* there have been some studies investigating morphological plasticity of sclerite lengths and colony shapes. West et al. (1993) reported changes in colony branch shape and sclerite length affected by depth, by conducting transplant experiments on the Caribbean species *Briareum asbestinum*. West (1998) also concluded that morphological plasticity in *B. asbestinum* is an adaptation to exposure to strong waves in shallow habitats, and to predation by snails in deeper habitats. Fabricius and Alderslade (2001) suggested that variation in anthostele shape in an unidentified species of *Briareum* was an adaptation to silty habitats.

Considering the identical sequences for type-1 and type-2 for all molecular markers examined in this study, including ITS2, at least type-1 and -2 are extremely closely related congeners. Furthermore, the morphological characters investigated in this study are presumed to be susceptible to environmental factors based on the previous research mentioned above. Recently, a similar case has been reported in the Caribbean species *B. asbestinum*, which has two different colony shapes (branching vs. encrusting). In Bilewitch et al. (2010), these two forms of *B. asbestinum* were compared morphologically (sclerite size) and molecularly (mitochondrial *msh1* and 18S–ITS1–5.8S–ITS2–28S rDNA). From the lack of significant morphological differences excepting gross colony shape, and no specific variation in sequences from both forms, they concluded that these two forms of *Briareum* are conspecific.

Nonetheless, based on traditional morphological methods, it is somewhat difficult to regard type-1 and type-2 in this study as conspecific. Although variation in a single morphological character is usually inadequate to judge octocoral relationships, the combination of variant characters observed in this study (surface color of the colony, anthostele shape, and polyp expansion form) give these two types of *Briareum* distinctive appearances. Moreover, no colony exhibiting an intermediate morphology between the two types was observed, although there were variations in anthostele shape and sclerite length within each type. Above all, type-1 and type-2 colonies are sympatric, and the two types may even be found adjacent to each other (Fig. 13). It is very unlikely that any single environmental factor can therefore account for all the morphological dissimilarity between such closely located colonies. It may be that type-1 and -2 are currently undergoing rapid radiation, which would account for the observed lack of genetic variation between them. Thus, for now, as molecular evidence indicates the two types are conspecific, we consider these two types to be different morphotypes of the same species.

Fig. 13. Sympatric type-1 and type-2 *Briareum* colonies (Sesoko Isl., Japan) at a depth of 2 m.

Interestingly, while *Briareum* corresponding with type-1 morphology has been reported from Tanegashima Island (this study), the Ogasawara Islands (Utinomi, 1956; this study) and the Ryukyu Archipelago, type-2 *Briareum* has not been reported in Japan outside of the Ryukyu Archipelago. Further investigation into the distribution of type-2 *Briareum* and studies adopting molecular methods more responsive to detecting very close relationships (e.g., microsatellites) may reveal more accurately the status of *Briareum* type-1 and type-2.

Taxonomic status of type-3

While all type-1 and type-2 specimens had completely identical sequences of all molecular markers examined, type-3 sequences had a two-nucleotide difference in ITS2 sequences. The mitochondrial COI region, which is a good species-level marker in many groups of animals, is known to have a low molecular evolutionary rate in Octocorallia (Berntson et al., 1999, 2001; France and Hoover, 2002; McFadden et al., 2011). Sequences from the *msh1* gene shows more variation compared to COI, and in some groups of octocorals this region exhibits enough variability to separate congeneric species (France and Hoover, 2002; Sánchez et al., 2003b; Wirshing et al., 2005; McFadden et al., 2006b), but the variability is not sufficient to distinguish different but conspecific populations (France and Hoover,

2002), or closely related species in some genera (McFadden et al., 2006b). On the other hand, nuclear ITS2, the intergeneric region between 5.8S rDNA and 28S rDNA, is known to be a region of promising diagnostic utility for some lower eukaryotes (including some octocorals) (Aguilar and Sánchez, 2007).

Compared with variation in the ITS region observed between *Briareum* specimens in the present study and from the Atlantic, and for other octocoral genera, a two nucleotide difference over 192 nucleotides in type-3 sequences does not initially seem considerable. However, considering all other *Briareum* specimens in this study obtained from a broad area (northern and southern parts of the Ryukyu Archipelago, Tanegashima Island, Ogasawara Islands) shared identical ITS2 sequences and that all type-3 specimens (also distinguishable by consistent morphological characters) shared the same ITS2 variation implies some extent of differentiation corresponding to the species or subspecies level. More samples from other DNA regions and additional molecular analyses including RNA secondary structure (e.g., Aguilar and Sánchez, 2007) may more accurately reveal the phylogenetic relationships of *Briareum* type-3 with type-1 and -2.

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