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Identification of species of teatfish (Holothuroidea: Holothuriida) in Japan based on mitochondrial cytochrome oxidase subunit I (COI) sequences, morphology, and ossicles

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Abstract: Holothurians referred to as "teatfish" contain three species, *Holothuria* (*Microthele*) *nobilis*, *H.* (*M.*) *whitmaei* and *H.* (*M.*) *fuscogilva*, that have recently been included in CITES Appendix II for sustainable catch and trade. Taxonomic key traits of these species are primarily genetic sequences and there are few morphological differences among them, except for color patterns of the body. Although located in the northern limit of their distribution, there have been no reports identifying species of teatfish in Japan based on both morphology and genetic traits. This study investigated sequences of mitochondrial cytochrome oxidase subunit I (COI) and morphology including ossicles of black and white teatfish collected from Okinawa Island and Yaeyama Islands, Japan. The entirely black specimens and white specimens having black mottles on the dorsal side were identified as *Holothuria* (*M.*) *whitmaei* and *H.* (*M.*) *fuscogilva*, respectively, based on the agreement of genetic and morphological traits and with published data. There is no clear difference in COI sequences between specimens from Japan and other Pacific regions for both species. The only inconsistent finding with previous reports was that one specimen of *H.* (*M.*) *whitmaei* lacked Cuvierian tubules, which are usually present in this species, implying plasticity of this trait. Mean diameter of the disc and height of the pillars of table ossicles were shorter and longer, respectively, in *H.* (*M.*) *fuscogilva* than in *H.* (*M.*) *whitmaei* ($p<0.05$, *t*-test).

Key words: genetic, molecular, sea cucumbers, spicules

Introduction

Tropical holothurians are traded as luxury Chinese foods ("bêche-de-mer" or "trepang") and fished all around the world, together with temperate species such as *Apostichopus japonicus* (Selenka, 1867). Particularly in developing countries of the Indo-Pacific, bêche-de-mer fisheries have been important industries for the local economy. Among the more than 58 species of edible holothurians in the world (Purcell et al. 2012), "teatfish" are the most commercially valuable group in tropical areas (Purcell 2014, Purcell et al. 2018). Teatfish are composed of three species as *Holothuria* (*Microthele*) *nobilis* (Selenka, 1867), *H.* (*M.*) *whitmaei* Bell, 1887, and *H.* (*M.*) *fuscogilva* Cherbonnier, 1980, and also include an unknown species type "pentard" (Uthicke et al. 2004, Asha 2013). These three species of teatfish in addition to elephant trunkfish *H.* (*M.*) *fuscopunctata* Jaeger, 1833 compose subgenus *Holothuria* (*Microthele*). Because of their high commercial value, teatfish have tended to be overexploited from an earlier period than other holothurian species (Eriksson & Byrne 2015), resulting in worldwide degradation of the resources (CITES 2019, FAO 2019). Based on this situation, the three species *H. nobilis*, *H. whitmaei* and *H. fuscogilva* were included in CITES Appendix II in 2019 (Simone et al. 2020), which was the second listing of holothurians after *Isostichopus fuscus* in Equador was included in CITES Appendix III in 2003 (Toral-Granda 2008). Hereafter, species-based resource management will be required when exporting teatfish (i.e. issuing a non-detrimental finding: NDF).

This CITES listing of teatfish was enabled by the recent

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resolution (Uthicke et al. 2004) of taxonomic confusion (Selenka 1867, Cherbonnier 1980, Massin 1999). According to the latest taxonomy (Uthicke et al. 2004), white teatfish *H. fuscogilva* is present throughout the Indo-Pacific, while the distribution of the two species of black teatfish is separated without overlap as *H. whitmaei* and *H. nobilis* in the Pacific and the Indian Oceans, respectively.

However, *H. nobilis* has been recently reported in Japan (Minami 2011, Minami & Tamaki 2013, Yamana et al. 2020), which is contrary to the present understanding about their distribution ranges described above. Therefore, it is necessary to confirm which species Japanese teatfish correspond to, and to elucidate the genetic relationships between the Japanese and the known species of teatfish, in terms of local fishery management and understanding about the diversity of this group.

As the three species of teatfish are phylogenetically closely-related to each other, there are few morphological traits available to differentiate one from the others (Uthicke et al. 2004). The only clear morphological difference among the three species was color patterns of the body; black teatfish *H. whitmaei* is entirely black (Bell 1887), while *H. nobilis* is also black-colored but has white blotches around "teats", that is, protrusions on the lateral body (Uthicke et al. 2004). White teatfish *H. fuscogilva* is white- or beige-colored but in most cases has irregular-shaped black mottles on the top of the dorsal body (Cherbonnier 1980). But their color patterns are highly variable, both individually and ontogenically; particularly, their young animals show quite different color patterns from the typical ones as described above (Uthicke et al. 2004, Purcell & Tekanene 2006). There are still other morphological differences, which are, however, too variable within species to be used as taxonomic keys; Cuvierian tubules are thick and numerous in black teatfish *H. whitmaei* and *H. nobilis*, while often absent in white teatfish *H. fuscogilva* (Cherbonnier 1980, Uthicke et al. 2004). However, *H. fuscogilva* sometimes has this organ, though it is rudimentary (Massin 1999, Uthicke et al. 2004). Another morphological difference is that ossicles (spicules) referred to as "simple buttons" are present in the ventral body wall of *H. fuscogilva* (Cherbonnier 1980), which are usually absent in *H. nobilis* and *H. whitmaei*. But this is not always true as simple buttons are occasionally lacking in *H. fuscogilva* (Massin 1999), and sometimes appear also in *H. whitmaei* (Uthicke et al. 2004). Therefore, DNA sequences were used as important evidence to taxonomically differentiate the three species (Uthicke et al. 2004). This study aims to provide the first description of teatfish species from Japan based on both morphological and genetic traits.

Materials and Methods

Two and three individuals of black and white teatfish, respectively, were collected by diving or purchased from fishermen in Yaeyama Islands, and two individuals of each black and white teatfish were purchased from fishermen

Table 1. A list of collected specimens of teatfish.

Specimen	Date of collection	Place	Sediment	
			Depth	Type
White teatfish (Okinawa 1) (RUMF-ZE-04001)	30th Jun 2020	Off Naha Port (Shinko Breakwater No. 1); N26°14.4′, E127°38.6′	20–30 m	Sand
White teatfish (Okinawa 2) (RUMF-ZE-04002)	30th Jun 2020	Off Naha Port (Shinko Breakwater No. 1); N26°14.4′, E127°38.6′	20–30 m	Sand
Black teatfish (Okinawa 3)	29th Jun 2020	Off Naha Port (Urasoe Breakwater No. 1); N26°15.6′, E127°39.3′	15–20 m	Sand-rubble, bare bedrock
Black teatfish (Okinawa 4) (RUMF-ZE-04003)	4th Jul 2020	Off Naha Port (Urasoe Breakwater No. 1); N26°15.6′, E127°39.3′	15–20 m	Sand-rubble, bare bedrock
White teatfish (Yaeyama 1) (RUMF-ZE-04004)	6th May 2020	Yonara Strait, Yaeyama Isls.	7–8 m	Sand
White teatfish (Yaeyama 2) (RUMF-ZE-04005)	14th May 2020	Yonara Strait, Yaeyama Isls.	10–20 m	—
White teatfish (Yaeyama 3) (RUMF-ZE-04006)	20th May 2020	South of Kohama Isl., Yaeyama Isls.	12–18 m	Sand-rubble
Black teatfish (Yaeyama 4) (RUMF-ZE-04007)	15th May 2020	Yonara Strait, Yaeyama Isls.	5–10 m	—
Black teatfish (Yaeyama 5) (RUMF-ZE-04008)	15th May 2020	Yonara Strait, Yaeyama Isls.	5–10 m	—
Black teatfish (Yaeyama 6)	May 2020	Yaeyama Isls.	—	—
Black teatfish (Yaeyama 7)	May 2020	Yaeyama Isls.	—	—
Black teatfish (Yaeyama 8)	—	Yaeyama Isls.	—	—
Black teatfish (Yaeyama 9)	—	Yaeyama Isls.	—	—

through Urasoe-Ginowan fishery cooperative association (FCA) in Okinawa Island (Table 1). Black and white teatfish were collected from 5–20 m and 7–30 m depths, respectively (Table 1). These specimens were fixed in 70% ethanol, after anesthetization in seawater or tapwater in which magnesium chloride was dissolved (only for live animals from Yaeyama). The fixed specimens were stored in Ryukyu University Museum (Fujukan).

The number of tentacles, presence of Cuvierian tubules, and shapes of radial and interradial elements of calcareous rings were observed by dissecting the specimens. Calcareous rings were observed for each three specimens of black and white teatfish (Okinawa 1, 2, and 4 and Yaeyama 2–5 in Table 1).

To observe ossicles, small pieces of tissues were sampled from the dorsal and ventral body wall, tube feet, papillae and tentacles from four and five specimens of black and white teatfish, respectively (Okinawa 1–4 and Yaeyama 1–5 in Table 1). Diluted commercial bleach was used to remove the tissue to isolate ossicles, then the samples were rinsed with pure water by decantation for several times and dried at 105°C overnight. The dried ossicles were observed under a scanning electron microscope (SEM) (NeoScope JCM-6000, JEOL) or a light microscope (BX51, Olympus).

For DNA sequence analysis, a piece of body wall was removed from each specimen preserved in 70% ethanol, epidermis of which was scraped off using a razor to minimize contamination (e.g. bacteria). In addition to the preserved specimens, DNA samples were also collected from four live animals of black teatfish collected in Yaeyama Islands (Yaeyama 6–9 in Table 1), which had been kept in seawater-flowing tanks in the laboratory. DNA from the tissue was extracted using DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol. Nucleotide of 674 bp in the mitochondrial cytochrome oxidase subunit I (COI) was amplified using primers COIeF (5′-ATA ATG ATA GGA GGR TTT GG-3′) and COIeR (5′-GCT CGT GTR TCT ACR TCC AT-3′) (Arndt et al. 1996, Uthicke et al. 2004) and Taq polymerase. Thermal cycles of the PCR were the same as in Uthicke & Benzie (2003). In the initial trials, DNA was not amplified by PCR, but this problem could be avoided by decreasing the amount of tissue (3–10 mg) used in extraction or/and by diluting template in PCR up to ca. 10^4 times. The PCR products were purified and sequenced in Macrogen (Kyoto, Japan). Sequencing reactions were performed in a BioRad DNA Engine Dyad PTC-220 Peltier Thermal Cycler using an ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template in both directions using the primers described above. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems). The sequences were deposited in DNA Data Bank of Japan (DDBJ) (Table 2).

The obtained sequences were aligned using the program ClustalW (Thompson et al. 1994) in MEGA X (Kumar et al. 2018) together with sequences deposited in GenBank and listed in Uthicke et al. (2004) and Uthicke et al. (2010) (Table 2), and cut into 529 bp. Among these sequences, AY700231.1 and AY700232.1 were eliminated from the analysis, because these were not located in the clade of their species in a phylogenetic tree in a pre-trial. These sequences may be mixed up in the GenBank deposition (Dr. S. Uthicke pers. comm.). Then, a neighbor-joining tree (Saitou & Nei 1987) was drawn based on genetic distance calculated by Kimura two parameter (Kimura 1980) using MEGA X. TCS networks (Clement et al. 2002) were drawn using PopART software (http://popart.otago.ac.nz).

Results

Description of black teatfish specimens

Macroscopic morphology

The specimens of black teatfish were entirely black on the dorsal surface (Fig. 1a, b), and black to dark brown on the ventral side (Fig. 1f, g). Papillae were present over the whole dorsal area. The ventral three ambulacra were integrated, and gray- to brown-colored tube feet were densely distributed. The mouth was on the ventral side, which had 20 gray-colored tentacles. The anus was at the posterior end of the body, and the five anal papillae were calcified to be "teeth". Teats, longitudinally aligned protrusions, were on both sides of the lateral body. Cuvierian tubules were present in 3 specimens, but absent in one specimen. The one specimen lacking Cuvierian tubules has no other features different from other specimens.

Ossicles and calcareous rings

Ossicles were tables and ellipsoid buttons that occurred in the dorsal (Fig. 2a, b) and ventral body wall (Fig. 2c, d). Dorsal papillae possessed perforated rods and perforated plates (Fig. 2e) in addition to buttons and tables. Ossicles in the ventral tube feet were mainly perforated plates (Fig. 2f) (length: 126±35, 47.1–236 μm, width: 95.9±24.8, 43.7–149 μm as mean±standard deviation and range, n=86 from 4 specimens) and ellipsoid buttons. Ossicles in the tentacles were mainly tables (Fig. 2g) and various sizes of rods (length: 118±115, 21.1–970 μm, width: 14.0±9.7, 2.75–50.2 μm, n=99) (Fig. 2h). The tables in the tentacles (Fig. 2g) were less developed than those in other parts; the pillars and the crown of the table were often incomplete or lacking, and the diameter of the disc was small (48–66 μm). Tables in other tissues; diameter of the disc was 72.8±11.1 (44.0–108) μm (n=228), height of the pillars was 42.3±9.4 (24.6–64.3) μm (n=79). Buttons in the dorsal body wall (Fig. 2b) and papillae were round and complete ellipsoid-shaped, while buttons in the ventral body wall (Fig. 2d) and tube

Table 2. A list of sequences used in the phylogenetic analysis.

Species	Location/Label	GenBank/DDBJ accession	Reference
Holothuria (*Microthele*) *fuscogilva*	GBR 1	AY700763.1	Uthicke et al. (2004)
	GBR 2	AY700764.1	Uthicke et al. (2004)
	GBR 3	AY700765.1	Uthicke et al. (2004)
	GBR 4	AY700766.1	Uthicke et al. (2004)
	GBR 5	AY700767.1	Uthicke et al. (2004)
	Fiji 1	AY700768.1	Uthicke et al. (2004)
	Fiji 2	AY700769.1	Uthicke et al. (2004)
	PNG 1	EU848237.1	Uthicke et al. (2010)
	Samoa 1	EU848240.1	Uthicke et al. (2010)
	Seychelles 1	EU848241.1	Uthicke et al. (2010)
	Samoa 2	EU848252.1	Uthicke et al. (2010)
	Samoa 3	EU848253.1	Uthicke et al. (2010)
	Cook Island 1	EU848254.1	Uthicke et al. (2010)
	Okinawa 1	LC593266	This study
	Okinawa 2	LC593261	This study
	Yaeyama 1	LC593267	This study
	Yaeyama 2	LC593268	This study
	Yaeyama 3	LC593260	This study
H. (*M.*) *whitmaei*	Fiji 3	AY176777.1	Uthicke and Benzie (2003)
	Fiji 4	AY176778.1	Uthicke and Benzie (2003)
	PNG 2	AY176780.1	Uthicke and Benzie (2003)
	PNG 3	AY176781.1	Uthicke and Benzie (2003)
	Australia 1	AY176810.1	Uthicke and Benzie (2003)
	Australia 2	AY176811.1	Uthicke and Benzie (2003)
	GBR 6	AY700760.1	Uthicke et al. (2004)
	GBR 7	AY700761.1	Uthicke et al. (2004)
	GBR 8	AY700762.1	Uthicke et al. (2004)
	Cook Island 2	EU848239.1	Uthicke et al. (2010)
	Indonesia	EU848242.1	Uthicke et al. (2010)
	Palau	EU848245.1	Uthicke et al. (2010)
	New Caledonia	EU848290.1	Uthicke et al. (2010)
	Okinawa 3	LC593262	This study
	Okinawa 4	LC593263	This study
	Yaeyama 4	LC593264	This study
	Yaeyama 5	LC593265	This study
	Yaeyama 6	LC593269	This study
	Yaeyama 7	LC593270	This study
	Yaeyama 8	LC593271	This study
	Yaeyama 9	LC593272	This study
H. (*M.*) *nobilis*	La Réunion 1	AY176775.1	Uthicke and Benzie (2003)
	La Réunion 2	AY176.776.1	Uthicke and Benzie (2003)
	Seychelles 2	EU848246.1	Uthicke et al. (2010)
H. (*M.*) *fuscopunctata*	New Caledonia	EU848238.1	Uthicke et al. (2010)
	GBR	EU848272.1	Uthicke et al. (2010)
	GBR	EU848273.1	Uthicke et al. (2010)
	GBR	EU848274.1	Uthicke et al. (2010)
	PNG	EU848288.1	Uthicke et al. (2010)
	GBR	EU848289.1	Uthicke et al. (2010)
	GBR	EU848291.1	Uthicke et al. (2010)
H. (*Halodeima*) *atra*	GBR	AY700770.1	Uthicke et al. (2004)
Actinopyga miliaris	GBR	AY700773.1	Uthicke et al. (2004)
Bohadschia vitiensis	GBR	AY700772.1	Uthicke et al. (2004)
Apostichopus californicus	Pacific USA	U32198.1	Arndt et al. (1996)
Thelenota ananas	GBR	AY700771.1	Uthicke et al. (2004)

Abbreviations: Great Barrier Reef (GBR), Papua New Guinea (PNG)

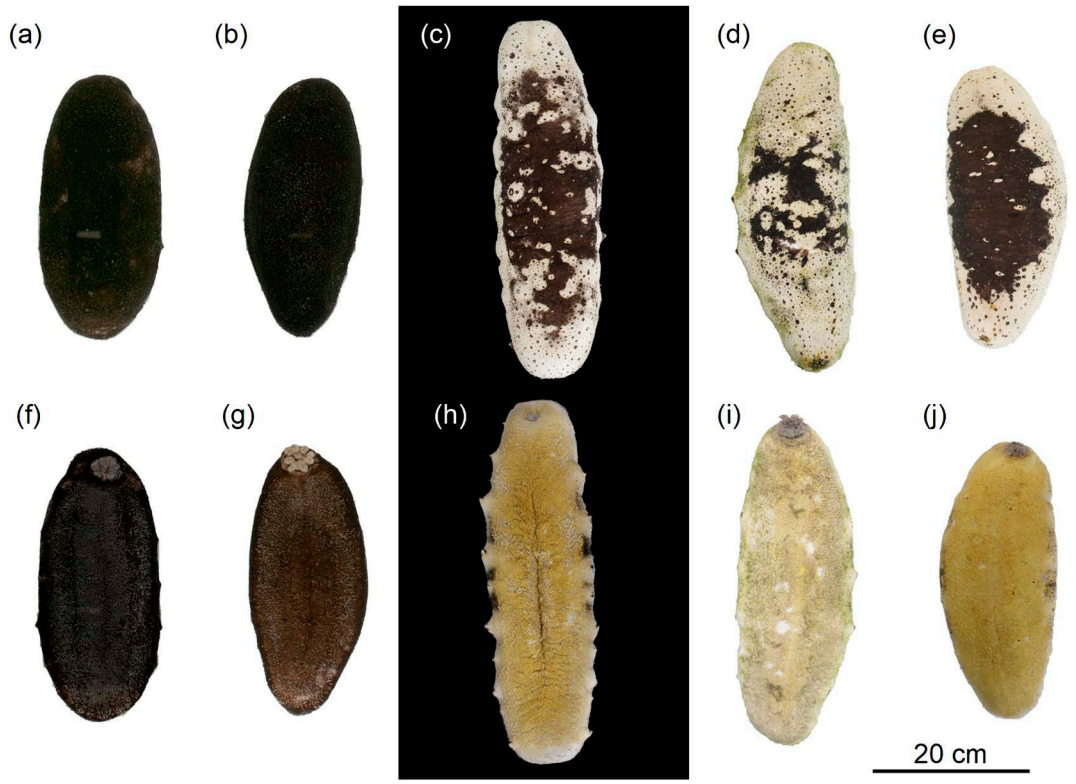

Fig. 1. Photographs of dorsal (a–e) and ventral (f–j) sides of black teatfish *Holothuria* (*Microthele*) *whitmaei* Bell, 1887 (a, b, f and g) and white teatfish *H.* (*M.*) *fuscogilva* Cherbonnier, 1980 (c, d, e, h, i and j) specimens before fixation. Specimens: Yaeyama 4 (a and f), Yaeyama 5 (b and g), Yaeyama 1 (c and h), Yaeyama 2 (d and i), Yaeyama 3 (g and j).

feet were flatter and somewhat similar to simple buttons, but completely flat-shaped (simple) buttons were not present. The buttons were 65.4±11.2 (35.7–125) μm long and 33.5±4.8 (19.8–55.9) μm wide (n=209). Radial elements of the calcareous ring had four projections on the upper side and a round depression on the lower side, while interradial elements had one projection on the upper side (Fig. 4a).

Description of the white teatfish specimens

Macroscopic morphology

The body color was ivory, and also there were black to sepia, irregular-shaped mottles of various sizes on the central area of the dorsal side (Fig. 3c, d, e). Dorsal papillae were ivory- or goldenrod-colored, and located in the mottle or small dark spots. Gray-colored papillae were distributed over the whole dorsal area. The mouth was on the ventral side, and there were 20 tentacles, which were pale, gray-colored. The anus was at the posterior end of the body, surrounded by five calcified teeth (papillae). The ventral color was ivory to goldenrod (Fig. 1h, i, j). The ventral three ambulacra were integrated, in which tube feet were densely distributed. Teats were present along both sides of the lateral body. The top of the teats was whitish, around which an incomplete black ring was present. Cuvierian tubules were small (2–3 mm thick, 2–3 cm long) in 2 specimens and absent in one specimen.

Ossicles and calcareous rings

Ossicles in the dorsal body wall were comprised of tables (Fig. 3a), ellipsoid buttons (Fig. 3b), and a few perforated plates. The ventral body wall contained numerous simple buttons (Fig. 3d), clearly flat and discernible from ellipsoid buttons, and a few tables (Fig. 3c) and perforated plates. The ventral tube feet possessed abundant simple buttons (Fig. 3e) and a few tables and perforated plates (Fig. 3f) (length: 128±40, 42.9–269 μm, width: 63.2±22.6, 28.1–129 μm, n=87 from 5 specimens), while ellipsoid buttons were rare. Ossicles of the tentacles were mainly rods of variable sizes (length: 157±114, 15.9–612 μm, width: 15.9±11.5, 3.2–49.3 μm, n=108) (Fig. 3g), and some buttons, tables and small perforated plates (Fig. 3h). The simple buttons were 66.9±18.2 (30.5–111) μm long and 37.0±13.5 (3.9–74.7) μm wide (n=89), while the ellipsoid buttons stayed slightly shorter as 65.4±9.1 (46.1–90.6) μm long and 36.5±4.8 (23.6–54.7) μm wide (n=140), comparable to that of the ellipsoid buttons in the black teatfish specimens. The diameter of the disc of tables was 63.8±12.4 (30.7–90.8) μm (n=305), the average of which was significantly shorter than that of black teatfish (72.8±11.1 μm, n=228) ($p<0.05$, Welch's two sample *t*-test). The height of the pillars of tables was 53.1±14.2 (29.9–115) μm (n=174), the average of which was significantly longer than that of black teatfish (42.3±9.4 μm, n=79) ($p<0.05$, Welch's two sample *t*-test). Shape of the calcareous ring (Fig. 4b) showed generally the same structure as that in

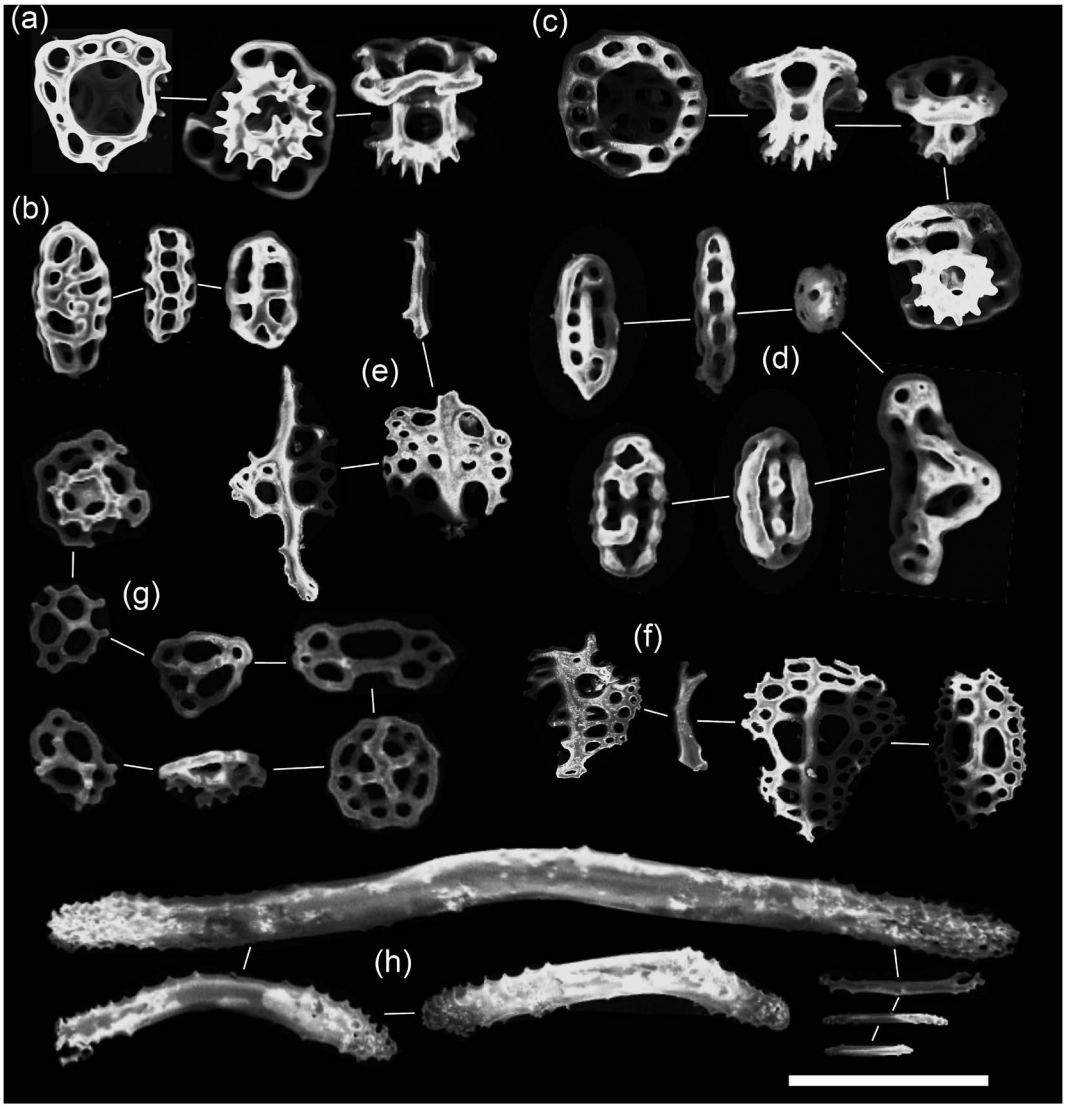

Fig. 2. Scanning electron micrographs of ossicles of *Holothuria* (*Microthele*) *whitmaei* (specimen: Okinawa 3): (a) Tables and (b) ellipsoid buttons in the dorsal body wall, (c) tables and (d) ellipsoid buttons in the ventral body wall, perforated or branched rods in the papillae and tube feet (e and f, respectively), and (g) tables and (h) rods in the tentacles. The scale bar corresponds to 100 μm for (a), (b), (c), (d) and (g), and 200 μm for (e), (f) and (h).

black teatfish specimens. But there were large variability in shapes among the individuals of white teatfish as same as among individuals of black teatfish, and it was difficult to find out differences between the two species.

Sequence analysis

All specimens of black and white teatfish were located in the clades of *H. whitmaei* and *H. fuscogilva*, respectively, in the neighbor-joining tree (Fig. 5). For Japanese specimens, the number of substitutions between the most distant pairs of haplotypes were 6 and 7 for white (*H. fuscogilva*) and black (*H. whitmaei*) teatfish, respectively (Fig. 6). The variability of haplotypes for the Japanese black teatfish specimens almost represented those of the range of other haplotypes for *H. whitmaei* from various locations in the world, while haplotypes of the Japanese white teatfish specimens only covered a limited part of the whole Pacific haplotypes of *H. fuscogilva*, and far distant from a haplotype from the Seychelles.

Discussion

Body color of the Japanese black teatfish specimens was entirely dark (black or nearly black) in both dorsal and ventral sides, which is identical to the characteristics of *H. whitmaei* (Bell 1887, Uthicke et al. 2004). Color patterns of the Japanese white teatfish specimens were a mixture of pale and dark colors. Mixed patterns of pale and dark colors can be seen in both *H. fuscogilva* and *H. nobilis*; however, pale color in *H. nobilis* is limited to the lateral body, appearing as round blotches around teats, and its ventral color is entirely dark (Selenka 1867, Cherbonnier 1988,

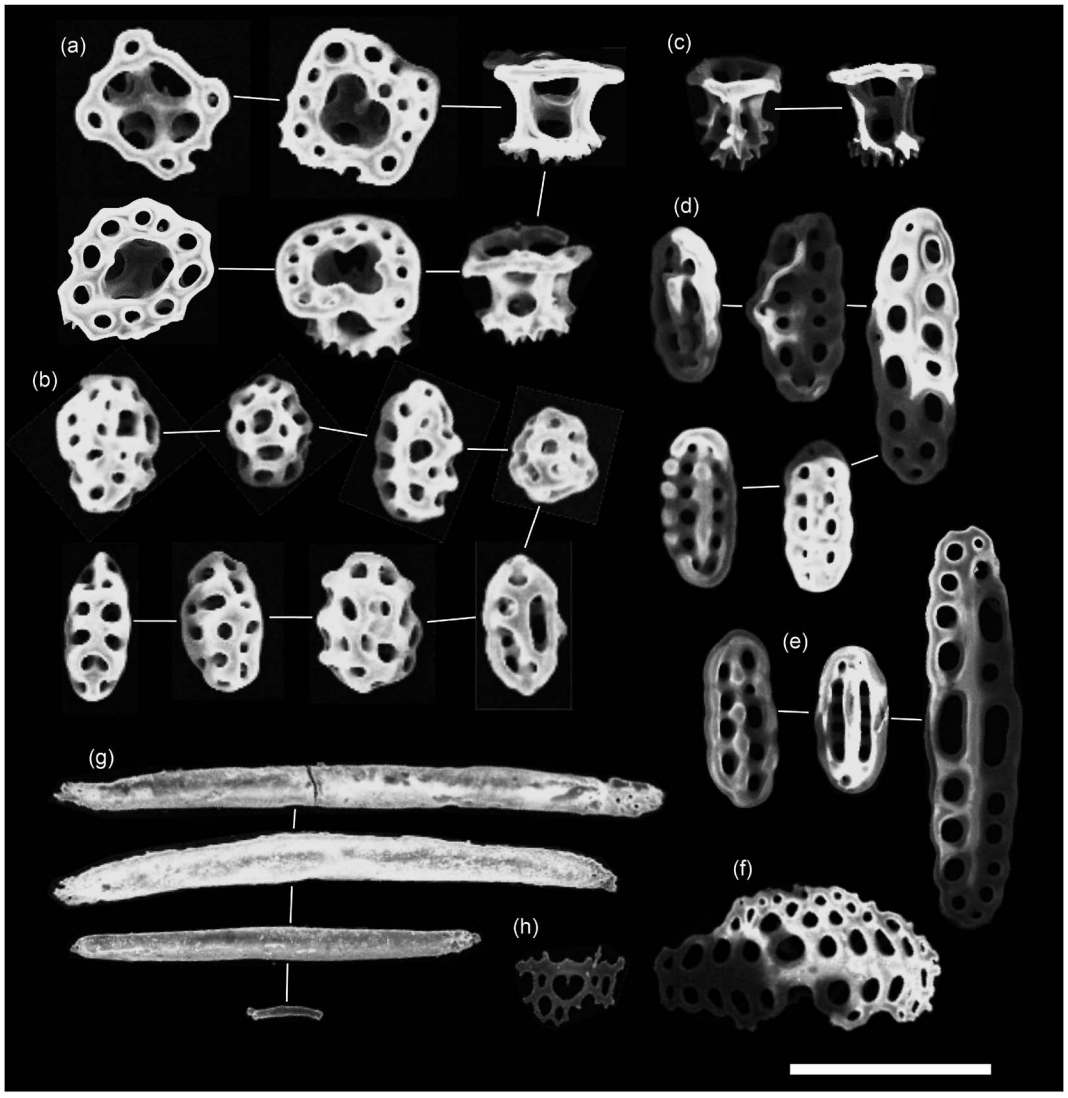

Fig. 3. Scanning electron micrographs of ossicles of *Holothuria* (*Microthele*) *fuscogilva* (specimen: Okinawa 2): (a) Tables and (b) ellipsoid buttons in the dorsal body wall, (c) tables and (d) simple buttons in the ventral body wall, (e) simple buttons and (f) a perforated plate in the tube feet, and (g) rods and (h) a perforated plate in the tentacles. The scale bar corresponds to 100 μm for (a), (b), (c), (d), (e), and (f), and 200 μm for (g) and (h).

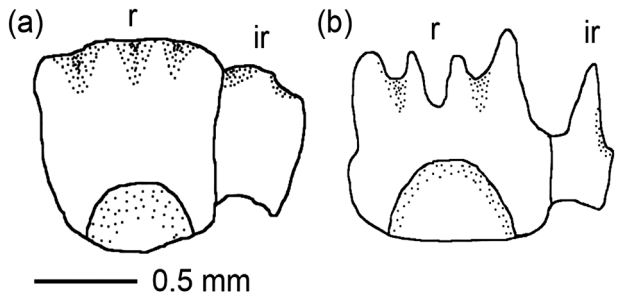

Fig. 4. Radial (r) and interradial (ir) elements in calcareous rings of (a) *Holothuria* (*Microthele*) *whitmaei* (specimen: Okinawa 4) and (b) *H.* (*M.*) *fuscogilva* (specimen: Okinawa 2).

Uthicke et al. 2004). As the ventral color is ivory to goldenrod in our white teatfish specimens from Japan, the color patterns are different from those of *H. nobilis* and identical to those of *H. fuscogilva* (Cherbonnier 1980, Uthicke et al. 2004). Cuvierian tubules are usually absent or degenerate in *H. fuscogilva*, while present in *H. whitmaei* and *H. nobilis* (Cherbonnier 1980, Uthicke et al. 2004). The condition of Cuvierian tubules in our white and black specimens was almost consistent with the above descriptions for *H. fuscogilva* and *H. whitmaei*/*H. nobilis*, respectively, except that one black specimen lacked this organ. The absence of Cuvierian tubules in the black specimen seems to be an exceptional case, suggesting plasticity of this trait. Cuvierian tubules in the three species of teatfish are generally small and non-adhesive (Uthicke et al. 2004), and are not ejected against disturbance, not like other holothurians. As same as previously suggested for *H. fuscogilva* (Uthicke et al. 2004), this lack of a role as a defense mechanism may have generated the plasticity of this trait during

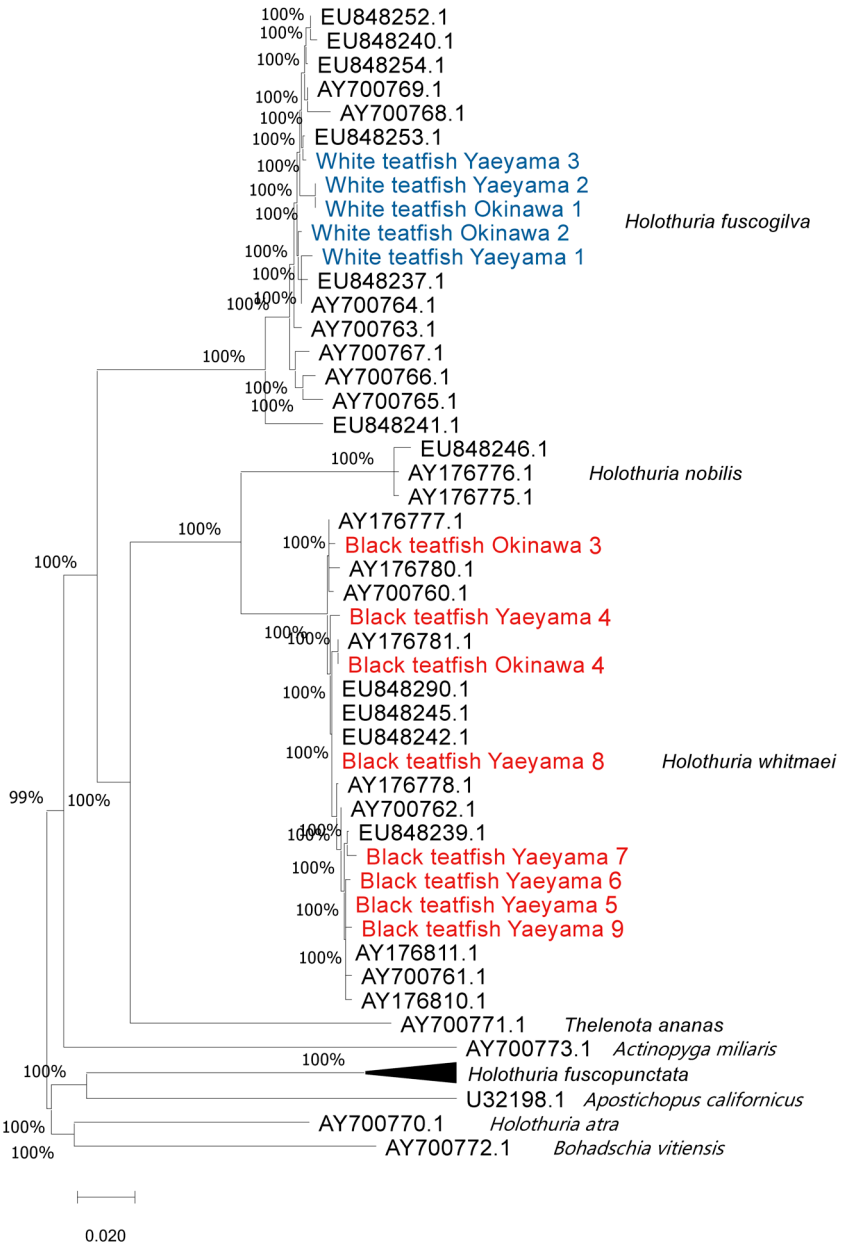

Fig. 5. A neighbor-joining tree of 54 sequences of mitochondrial cytochrome c oxidase subunit I, which include black (red letters) and white (blue letters) teatfish collected in this study, and three species of teatfish and several other holothurians referred to from the GenBank repository. The evolutionary distances were computed using the Kimura 2-parameter method.

evolution also for *H. whitmaei*.

The composition of ossicles in our specimens was identical with that of subgenus *Holothuria* (*Microthele*) described previously (Cherbonnier 1988, Conand 1998). One clear difference of ossicles between the black and white teatfish specimens were the absence and presence of simple buttons, respectively, in the ventral body wall and tube feet. According to the previous works, *H. fuscogilva* usually, but not always, has simple buttons in addition to ellipsoid buttons in the ventral body wall (Cherbonnier 1980, Massin 1999, Uthicke et al. 2004), while *H. whitmaei* and *H. nobilis* usually have only ellipsoid buttons (Uthicke et al. 2004). But *H. whitmaei* also occasionally has simple buttons (Uthicke et al. 2004). Therefore, the shapes and compositions of buttons in our black and white teatfish specimens can be regarded as typical for those of *H. whitmaei*/*H. nobilis* and *H. fuscogilva*, respectively, but cannot be treated as definitive identification keys for the species. Another difference of ossicles between our black and white teatfish specimens was the mean size of tables (diameter of the disc, and height of the pillars), which has not been reported previously. However, the size ranges were overlapped between the two types, and it seems difficult to use this feature for identifying species. Although Cherbonnier (1980) pointed out the presence of large tables specific to *H. fuscogilva* which have nodules on the edge

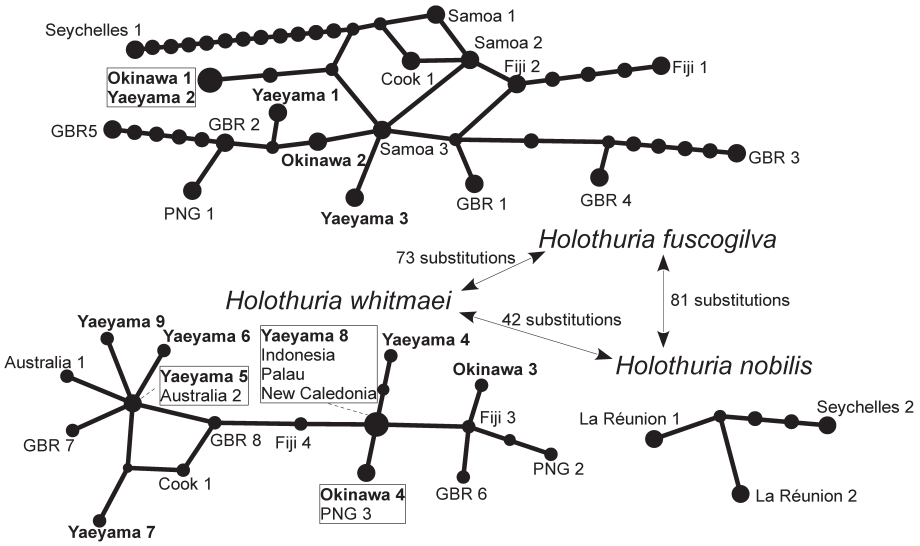

Fig. 6. TCS networks for *Holothuria* (*Microthele*) *fuscogilva*, *H.* (*M.*) *whitmaei* and *H.* (*M.*) *nobilis*, where each neighboring black dot represents one substitution. The number of substitutions between the nearest haplotype pairs between different species was shown with arrows. GBR: Great Barrier Reef, PNG: Papua New Guinea, Cook: Cook Island.

of the disc, such tables were not found in our specimens. There were no obvious differences in shapes of calcareous rings between our specimens and previous reports for *H. whitmaei* and *H. fuscogilva* (Cherbonnier 1980, 1988).

The result of the sequence analysis showed that our specimens of black and white teatfish were genetically clustered with known sequences of *H. whitmaei* and *H. fuscogilva* from GenBank, respectively, and clearly distinguished from the clade of *H. nobilis*. Considering the morphological and genetical agreement, our specimens of black and white teatfish in Japan were identified as *H. whitmaei* and *H. fuscogilva*, respectively. The previously known distribution area of another black teatfish *H. nobilis* is the Indian Ocean (Uthicke et al. 2004), and as far as our data show is suggested not to be present in Japan. The *H. nobilis* that were previously reported from Japan by Minami (2011) undoubtedly corresponds to *H. whitmaei*, because it showed entirely black color patterns (Dr. Y. Minami pers. comm.).

The Japanese haplotypes of both *H. fuscogilva* and *H. whitmaei* were located inside the whole haplotype networks of the same species. Therefore, it was unlikely that Japanese populations of these species form genetically independent populations from that in other areas, regardless of the location at the northern limit of the teatfish distribution (Uthicke et al. 2004). Although our sample sizes were small, Japanese haplotypes of *H. whitmaei* dispersed over the whole haplotype network of this species (Fig. 6), which suggests that populations in Japan and in other Pacific regions form an almost genetically identical population. On the other hand, the whole population of *H. fuscogilva* contains a higher haplotype diversity than the other two species, as previously reported by Uthicke et al. (2004), and some haplotypes from Great Barrier Reef, Fiji and Seychelles were genetically far distant from the Japanese haplotypes obtained in this study. These results imply that only a limited part of the whole genetic diversity of *H. fuscogilva* occurs in Japan. Although high genetic dispersal over a short time scale has been suggested previously (Uthicke & Benzie 2003), *H. fuscogilva* seems to possess regional variability of haplotype composition (Uthicke et al. 2004) including Japan, implying the importance of conservation of local populations.

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