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Phylogenetic and taxonomic status of the coral *Goniopora stokesi* and related species (Scleractinia: Poritidae) in Japan based on molecular and morphological data

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Abstract

Background: *Goniopora stokesi* is an uncommon species mainly found in tropical and subtropical regions but was also reported in temperate regions of Japan. This species has two unique characteristics. First, it does not typically attach to hard substrates, i.e., it is free-living. Although non-free-living colonies were reported, it is unclear whether their morphologies exhibit intraspecific or interspecific differences. Second, they can asexually form daughter colonies, which are secondary colonies that grow on the surface (coenosac) of a parent colony and subsequently detach. To date, this specific characteristic has not been reported in Japan, and it is not clear whether this species occurs in Japan.

Results: To clarify the taxonomic status of this species, we investigated its reproductive mechanism in Japan and morphologically and genetically analyzed specimens collected from both subtropical and temperate regions. We found that this species forms daughter colonies in Okinawa, Japan and that free-living colonies in the temperate region, which were formerly recognized as *G. stokesi*, likely constitute a morphological variation of a separate species. In addition, all non-free-living colonies with *G. stokesi*-like morphologies were also morphological variations of other species.

Conclusions: Overall, free-living colonies with large, deep calices, thin walls, and well-developed epitheca on the underside were *G. stokesi*, whereas other free-living and all non-free-living *G. stokesi*-like colonies belonged to other species, such as *Goniopora djiboutiensis*.

Keywords: Evolution; Phylogeny; rDNA; COI; Temperate region

Background

The genus *Goniopora* (Cnidaria: Scleractinia: Poritidae) is common throughout the Indo-Pacific, including tropical and temperate regions. However, there are very few ecological and molecular studies of *Goniopora* (e.g., Heyward 1985). Taxonomically, *Goniopora* is easily distinguished from confamilial genera such as *Porites* and *Alveopora*, by having three septal cycles (vs. two in *Porites* and *Alveopora*) and relatively larger corallites (Veron and Pichon 1982). Also, *Goniopora* has a genus-specific septal formula (called 'the gonioporoid pattern'; Bernard 1903; Veron and Pichon 1982). In the field, *Goniopora* may be confused with *Alveopora* because the polyps of both genera have very long columns, although Veron and Pichon (1982) pointed out that *Goniopora* polyps have 24 tentacles, whereas *Alveopora* polyps have 12. Despite the morphological similarities, the two genera are phylogenetically distant (Romano and Cairns 2000; Fukami et al. 2008).

Goniopora stokesi Milne Edwards and Haime, 1851 (Cnidaria: Scleractinia: Poritidae), which is widely distributed in subtropical and tropical regions from the Red Sea through the Indo-West Pacific, has the largest polyps

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of any species of the genus (Veron 2000). Based on descriptions of this species (Milne Edwards and Haime 1851; Milne Edwards 1860), the colony is hemispherical in shape, and calices are about 5 mm wide and 6 mm deep. The walls are very thin and porous with rugged tops. The underside of the corallum is covered by an epitheca, a thin layer of calcium carbonate. A notable characteristic of this species is that several daughter colonies (or polyp balls) are asexually formed from living tissues on the surface of the parental colony and are released around the parental colony (Scheer 1960; Rosen and Taylor 1969). This is a unique and peculiar reproductive mechanism among zooxanthellate scleractinian corals. In addition, unlike other species of the same genus, *G. stokesi* is typically found on soft substrates (e.g., mud or sand) and does not usually attach to hard substrates, i.e., it is free-living (Veron and Pichon 1982). This unique reproductive mechanism may be the result of adaptations to specific environmental conditions (Rosen and Taylor 1969).

However, some non-free-living colonies of *G. stokesi* were reported (Nemenzo 1955; Veron and Pichon 1982), which are difficult to distinguish from related species. According to Veron and Pichon (1982), *G. stokesi* is similar to *Goniopora lobata* Milne Edwards and Haime, 1851, *Goniopora djiboutiensis* Vaughan, 1907, and *Goniopora columna* Dana, 1846, all of which have corallites that are similar to *G. stokesi* in size and distribution patterns. In addition, *Goniopora pendulus* Veron, 1985, which is distributed only in the West Pacific (Veron 2000), is also morphologically similar to *G. stokesi* in the characters of the corallites and the size of the polyps (Veron 1985, 2000). Veron (2000) grouped three species (*G. pendulus*, *G. djiboutiensis*, and *G. stokesi*) into the same category, group 1, which represents massive species with large (>5 mm in diameter) corallites. Although *G. lobata* and *G. columna* were included in group 2, with branching or columnar species having large (>5 mm in diameter) corallites, the variation in the colony shape of these species was similar to that in *G. djiboutiensis* or *G. stokesi* (Veron and Pichon 1982). It is hard to define species boundaries among scleractinian corals because of the variability and size of morphological features of the skeleton. Species identification is therefore difficult because of a lack of characteristic morphological features. Thus, it remains unknown whether non-free-living colonies of *G. stokesi* truly represent intraspecific morphological variation or whether they belong to different species.

In Japan, *G. stokesi* was reported from subtropical regions of the Ryukyu Archipelago (Okinawa Prefecture), including Tanegashima Island (Kagoshima) (Veron 1992), and also from three temperate regions of Amakusa (Kumamoto), Tosashimizu (Kochi) (Veron 1992), and Miyake Island (Tokyo) (Tribble and Randall 1986). However, the unique asexual reproductive mechanism of forming daughter colonies has not yet been reported in Japan (Nishihira and Veron 1995). To the best of our knowledge, none of the skeletal samples used in those studies were stored in a known repository. It therefore remains unclear whether temperate colonies can be identified as *G. stokesi* and whether colonies in subtropical and temperate regions belong to the same species. In particular, the colony reported as *G. stokesi* by Nishihira and Veron (1995) appears to have much smaller calices than the typical colonies of *G. stokesi*. The possibility that some colonies identified as *G. stokesi* in Japan belong to different species cannot be excluded.

Recent molecular analyses have helped resolve several issues regarding coral taxa (e.g., phylogenetic positions and species boundaries) (Fukami et al. 2008; Forsman et al. 2009; Benzoni et al. 2010; Kitahara et al. 2010). In this study, we investigated *G. stokesi* and morphologically similar specimens collected from subtropical and temperate Japan using molecular and skeletal morphological analyses to clarify its phylogenetic and taxonomic status in Japan.

Methods

Sampling

Samples were collected from eight subtropical and six temperate sites. The subtropical sites were Iriomote Island (IR), Ishigaki and Taketomi Islands (IS), Oura Bay (OU), Sesoko Island (SS), Nakijin (NK), Kikai Island (KK), Amami-Oshima Island (AO), and Tanegashima Island (TN); while temperate sites were Amakusa (AM), Miyazaki-Oshima (MO), Otsuski (OT), Yokonami (YO), Shirahama (SR), and Kushimoto (KS) (Figure 1). We surveyed all localities where *G. stokesi* was previously recorded (see 'Background'), excluding Tosashimizu and Miyake Island, although Tosashimizu is geographically close (20 km) to Otsuki (Figure 1). Sampling was performed by scuba diving (two to more than ten occasions per site, at depths of 5 to 30 m). We selected massive colonies with large corallites (approximately 5 mm in diameter), but we did not identify them in the field because it is difficult to identify species from living colonies. During sampling, we took photographs of the living colonies. Samples (100 to 500 cm^3) were collected using hammer and chisel. A portion of each sample (<1 cm^3) was maintained in CHAOS solution (Fukami et al. 2004) to dissolve proteins for DNA analyses, and the remnants of the samples were bleached for morphological analyses.

Species identification

Species identification of *Goniopora* is difficult because of the limited number of skeletal characters and highly variable skeletal and polyp morphologies. To minimize the risk of misidentification in this study, we examined and summarized (data not shown) the original descriptions (for all species listed in this study) and related references

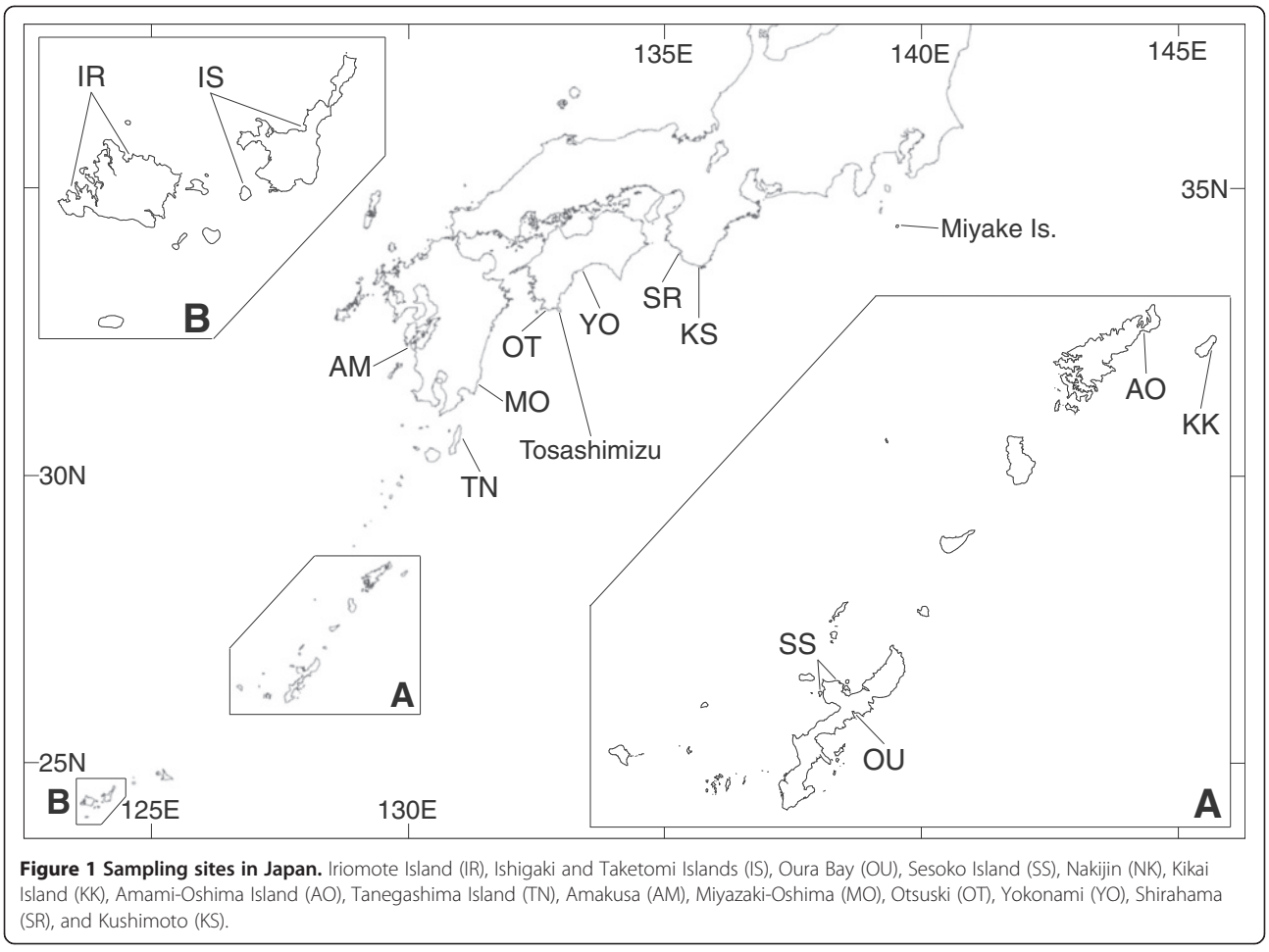

Figure 1 Sampling sites in Japan. Iriomote Island (IR), Ishigaki and Taketomi Islands (IS), Oura Bay (OU), Sesoko Island (SS), Nakijin (NK), Kikai Island (KK), Amami-Oshima Island (AO), Tanegashima Island (TN), Amakusa (AM), Miyazaki-Oshima (MO), Otsuki (OT), Yokonami (YO), Shirahama (SR), and Kushimoto (KS).

(Milne Edwards and Haime 1851; Milne Edwards 1860; Ortmann 1888; Bernard 1903; Bedot 1907; Vaughan 1907; Faustino 1927; Crossland 1952; Nemenzo 1955; Wells 1955; Veron and Pichon 1982; Veron 1985, 2000; Nishihira and Veron 1995). Specimens identified in this study are referred to as morphospecies (Oliver and Beattie 1996). Traditionally, species identification of *Goniopora* is based on skeletal characters such as the shapes of the coralla, septa, columella, and wall, and the size of the calices; but recently, Veron and Pichon (1982), Nishihira and Veron (1995), and Veron (2000) added polyp characters to support species identification.

Molecular analyses

Total DNA was extracted from coral tissue dissolved in CHAOS solution using a phenol/chloroform extraction method (see Fukami et al. 2004). Mitochondrial (mt)DNA sequences between the end of NADH dehydrogenase subunit 5 (ND5) and the first half of cytochrome oxidase subunit I (COI), including three intergenetic regions (IGRs), $tRNA^{Trp}$, and ATPase 8 were amplified using a polymerase chain reaction (PCR) with the primers ZCO1 and ZCO1R for the COI segment (Forsman et al. 2009) and Cs-F15 and Cs-R15 for the other segments (Lin et al. 2011). Because ND5 and COI were the primary components of the DNA sequences, we refer to them as ND5-COI in this study. Internal transcribed spacers (ITSs) of the nuclear ribosomal DNA (including partial 18S, ITS-1, 5.8S, ITS-2, and partial 28S) were also amplified by the PCR using primers 1S and 2SS (Wei et al. 2006). PCR conditions for these two markers were 94°C for 30 s followed by 30 or 35 cycles at 94°C for 30 s, 55 or 60°C for 45 s, and 72°C for 90 s, with a final phase of 72°C for 5 min. For the mitochondrial region, PCR products were treated with shrimp alkaline phosphatase and exonuclease I at 37°C for 40 min followed by 80°C for 20 min. Then the DNA sequences were determined by direct sequencing using ABI3730 or ABI310 sequencers (Applied Biosystems, Alameda, CA, USA). PCR products of the nuclear marker were also directly sequenced, but when they obtained sequences had double peaks in the chromatogram, they were subcloned into the TA vector (Promega, Madison, WI, USA) or TOPO10 (Invitrogen, Grand I., NY, USA) and were sequenced using ABI3730 or ABI310. All DNA sequences obtained in this study were submitted to DDBJ (accession numbers AB748660-AB748793 and AB764138, as listed in Table 1).

Table 1 List of identification of species of *Goniopora* based on the literature and the accession numbers of DNA sequences

Species	Morphological features for 'cf'	Sample number	Location	Accession number		Measurement	Free-living	Development of epitheca
				rDNA	mtDNA			
G. columna		IS3	Ishigaki	AB748660				
G. columna		IS4	Ishigaki	AB748661	AB748754			
G. columna		IR29	Iriomote		AB764138			
S. stokesi		NK1	Nakijin	AB748662	AB748755	Yes	Yes	Yes
G. stokesi		OU12	Oura Bay	AB748663	AB748756		Yes	Yes
G. stokesi		OU14	Oura Bay	AB748664			Yes	Yes
G. stokesi		OU17	Oura Bay	AB748665	AB748757		Yes	Yes
G. stokesi		OU19	Oura Bay	AB748666	AB748758		Yes	Yes
G. stokesi		OU20	Oura Bay	AB748667	AB748759	Yes	Yes	Yes
G. stokesi		OU22	Oura Bay	AB748668			Yes	Yes
G. stokesi		OU24	Oura Bay	AB748669	AB748760		Yes	Yes
G. stokesi		OU26	Oura Bay	AB748670			Yes	Yes
G. stokesi		OU41	Oura Bay	AB748671			Yes	Yes
G. stokesi		OU43	Oura Bay	AB748672	AB748761	Yes	Yes	Yes
G. stokesi		IR27	Iriomote	AB748673-4	AB748762	Yes	Yes	
G. stokesi		IR65	Iriomote	AB748675	AB748763	Yes	Yes	Yes
G. cf. *stokesi*	Shallow calices, colony attached to substrate	MO82	Miyazaki	AB748686	AB748768	Yes		
G. lobata		KS28	Kushimoto	AB748741	AB748788	Yes		
G. lobata		KS57	Kushimoto	AB748742				
G. lobata		SR44	Shirahama	AB748743				
G. lobata		YO1	Yokonami	AB748744	AB748789	Yes		
G. lobata		YO10	Yokonami	AB748745-6				
G. lobata		MO36	Miyazaki	AB748747-8	AB748790	Yes		
G. lobata		AM48	Amakusa	AB748749				
G. lobata		IR42	Iriomote	AB748750	AB748791	Yes		
G. cf. *lobata*	With pali	SR14	Shirahama	AB748751	AB748792	Yes		
G. cf. *lobata*	With pali	AO58	Amami	AB748752	AB748793	Yes		
G. cf. *lobata*	Wide columella	MO67	Miyazaki	AB748687	AB748769	Yes		
G. cf. *lobata*	Wide columella	IS20	Taketomi	AB748753		Yes		
G. djiboutiensis		KS9	Kushimoto	AB748695				
G. djiboutiensis		SR62	Shirahama	AB748696				
G. djiboutiensis		OT30	Otsuki	AB748697	AB748775	Yes		
G. djiboutiensis		AM30	Amakusa	AB748698				
G. djiboutiensis		AM74	Amakusa	AB748699	AB748776	Yes	Yes	
G. djiboutiensis		KK16	Kikai	AB748700				
G. djiboutiensis		KK60	Kikai	AB748701				
G. djiboutiensis		SS35	Sesoko	AB748702	AB748777	Yes		
G. djiboutiensis		OU2	Oura Bay	AB748703				
G. djiboutiensis		OU7	Oura Bay	AB748704				
G. djiboutiensis		OU21	Oura Bay	AB748705	AB748778	Yes		

Table 1 List of identification of species of *Goniopora* based on the literature and the accession numbers of DNA sequences *(Continued)*

G. djiboutiensis		OU28	Oura Bay	AB748706			
G. djiboutiensis		OU33	Oura Bay	AB748707			
G. djiboutiensis		IR13	Iriomote	AB748708-9			
G. djiboutiensis		IR22	Iriomote	AB748710-1	AB748779	Yes	
G. djiboutiensis		IR67	Iriomote	AB748712	AB748780		
G. cf. *djiboutiensis*	Small columella	KS31	Kushimoto	AB748713			
G. cf. *djiboutiensis*	Small columella	KS51	Kushimoto	AB748714			
G. cf. *djiboutiensis*	No pali, small calice	SR23	Shirahama	AB748715-6			
G. cf. *djiboutiensis*	Deep calice	SR24	Shirahama	AB748717			
G. cf. *djiboutiensis*	Irregular shape of corallum	OT24	Otsuki	AB748688	AB748770	Yes	
G. cf. *djiboutiensis*	Deep calice	OT29	Otsuki	AB748718-20	AB748781	Yes	
G. cf. *djiboutiensis*	Deep calice	MO16	Miyazaki	AB748721			
G. cf. *djiboutiensis*	Deep calice	MO23	Miyazaki	AB748689	AB748774	Yes	
G. cf. *djiboutiensis*	Deep calice	MO40	Miyazaki	AB748722			
G. cf. *djiboutiensis*	Deep calice	MO53	Miyazaki	AB748723	AB748782	Yes	
G. cf. *djiboutiensis*	Deep calice	MO63	Miyazaki	AB748724-5			
G. cf. *djiboutiensis*	Thin wall	AM26	Amakusa	AB748726-7			
G. cf. *djiboutiensis*	Irregular shape of corallum	AM75	Amakusa	AB748728-9	AB748783	Yes	Yes
G. cf. *djiboutiensis*	Small, deep calice	AM89	Amakusa	AB748730	AB748784	Yes	Yes
G. cf. *djiboutiensis*	Thin wall	TN40	Tanegashima	AB748731-2			
G. cf. *djiboutiensis*	No pali	TN41	Tanegashima	AB748690	AB748771	Yes	
G. cf. *djiboutiensis*	No pali	TN55	Tanegashima	AB748733-4	AB748785		
G. cf. *djiboutiensis*	No pali	TN95	Tanegashima	AB748735	AB748786	Yes	
G. cf. *djiboutiensis*	No pali	TN113	Tanegashima	AB748736	AB748787	Yes	
G. cf. *djiboutiensis*	Small columella	TN115	Tanegashima	AB748737-8			
G. cf. *djiboutiensis*	Deep calice	KK4	Kikai	AB748691	AB748772		
G. cf. *djiboutiensis*	No pali	KK11	Kikai	AB748692	AB748773	Yes	
G. cf. *djiboutiensis*	No pali	KK22	Kikai	AB748693			
G. cf. *djiboutiensis*	No pali	KK27	Kikai	AB748694			
G. cf. *djiboutiensis*	Deep calice at top of colony	IS19	Taketomi	AB748739			
G. cf. *djiboutiensis*	Deep calice at top of colony	IR3	Iriomote	AB748740			
G. pendulus		OT14	Otsuki	AB748676-80	AB748764	Yes	
G. pendulus		OT19	Otsuki	AB748681	AB748765	Yes	
G. pendulus		OT27	Otsuki	AB748682			
G. pendulus		OT31	Otsuki	AB748683	AB748766	Yes	
G. pendulus		TN11	Tanegashima	AB748684	AB748767	Yes	
G. pendulus		TN42	Tanegashima	AB748685			

For outgroups, we used two colonies of *G. columna*, the corallites which are similar in size to those of *G. djiboutiensis* and *G. lobata* but which forms thick branches or short columns, because our preliminary data showed that this species was genetically distant from other species analyzed in the study.

DNA sequences were aligned with Sequencher version 5.1 (Gene Codes, Ann Arbor, MI, USA) and SeaView

version 4.3.0 (Gouy et al. 2010). Phylogenetic trees were reconstructed using the neighbor-joining (NJ), maximum-likelihood (ML), and Bayesian-inference (BI) techniques. For the NJ, ML, and BI methods, we assumed a model of nucleotide evolution obtained using the Akaike information criterion (AIC) as implemented in MrModeltest 2.2 (Nylander 2004). The most appropriate models of nucleotide evolution were K81 (Kimura 3-parameter model) with unequal base frequencies (K81uf) for the ND5-COI marker and TVM with equal base frequencies (TVMef) for the ITS markers. For NJ, PAUP* (Swofford 2002) was used to estimate the topologies for both the ND5-COI and ITS markers and conduct a bootstrap analysis (with 1,000 replicates). PAUP was also used to reconstruct the best ML tree using a heuristic search and the tree-bisection-reconnection branch-swapping method for each marker. The software, GARLI version 0.951 (Zwickl 2006), was used to search for optimal ML topologies and conduct bootstrap analyses (500 replicates) for each marker. MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) was also used to conduct Bayesian analyses. Four parallel chains of 4×10^6 to 5×10^6 generations were run for each marker. Trees were sampled every 100 generations, and 10^4 'burn-in' trees were excluded from the consensus tree. The average standard deviation of split frequencies after 4×10^6 generations was 0.002069 for ND5-COI, and after 5×10^6 generations was 0.013847 for ITS. Because parsimoniously informative sites (13 characters) of ND5-COI were fewer than those (83 characters) of the ITS, we analyzed more samples for ITS to infer clearer phylogenetic relationships.

Morphological analyses

The skeletal morphology of each specimen was examined using a VHX-1000 digital microscope (Keyence, Osaka, Japan) to measure diameters of both the columella and calice, and using vernier calipers to measure the depth of the calice and thickness of the walls. Columella and calice diameters, the depth of the calice, the width of the walls, the number of septa, development of the pali, and the septal arrangement (presence of a gonioporoid pattern), all of which were evaluated from 5 mature corallites per colony (excluding those near the edge of a colony, which tended to be deformed), were measured or counted (Figure 2). For development of pali, we classified them into three categories: ≥6 pali, 1 to 5 pali, and no pali. For the septal arrangement, we also classified them in three categories: a clear gonioporoid pattern, an irregular (imperfect) gonioporoid pattern, and no gonioporoid pattern. For columella (Co) and calice (Ca) diameters, we measured the longest diameter (long diameter, L) and the dimension orthogonal to the longest diameter (short diameter, S) (referred to as LCo, LCa, SCo, and SCa, respectively) (Figure 2A). In addition, ratios of S/L of the columella and calice (SCo/LCo, SCa/LCa) and the columella/calice ratio using the L (LCo/LCa) were calculated. For calice depth (DC) and wall thickness (TW), we measured three different parts for each corallite (Figure 2B), and the calculated mean value of each three data points was used for the analyses. A principal component (PC) analysis (PCA) was performed with the software Stata v.11 (StataCorp, College Station, TX, USA). The first two PCs were plotted to examine whether any group of specimens could be distinguished. Then the groups that were found in the PCA plot were compared to the morphospecies or genetic groups inferred from the molecular analyses. Comparisons between morphospecies and morphometric data were also performed by a one-way analysis of variance (ANOVA) for parametric data or a Kruskal-Wallis one-way ANOVA for nonparametric data, and all pairwise multiple comparisons were performed with Tukey's test (parametric) or Dunn's test (nonparametric) using SigmaStat 3.0.1 (SPSS, Chicago, IL, USA).

Results

Species identification

In total, 74 samples (excluding two colonies of *G. columna* as outgroups) were collected from 14 locations in Japan, and seven morphospecies, including *G. stokesi* (13 colonies), *G.* sp. cf. *stokesi* (1), *G. lobata* (8), *G.* sp. cf. *lobata* (4), *G. djiboutiensis* (16), *G.* sp. cf. *djiboutiensis* (26), and *G. pendulus* (6), were identified (Figures 3 and 4; hereafter 'sp.' is not shown when referring to the morphospecies). Species lists and locations are summarized in Table 1. Several specimens had morphological characters atypical of the species. It is likely that some represented intraspecific morphological variations, but it was difficult to determine whether they were intraspecific morphological variations or different species. Therefore, we added 'confer (cf.)' followed by the species name where specimens were similar to a species but differed in more than one character from a species (Table 1). The morphospecies with the largest number of collected samples (26 colonies) was *G.* cf. *djiboutiensis*. *G. pendulus* is similar to *G. lobata* and *G. djiboutiensis* in skeletal morphology but differs in its irregular polygonal calices and reduced septa. The morphologies of *G. stokesi* clearly differed from those of the other morphospecies (see below discussion for details).

G. stokesi and morphologically similar colonies

Thirteen colonies of *G. stokesi* with a typical colony shape (i.e., free-living with large polyps) (Figure 3) were collected from three sites (IR, NK, and OU) in Okinawa; such colonies were not found at TN (Figure 1). The *G. stokesi* collected in this study had elongated polyps (>5 cm) with long tapering tentacles (about 1 cm). The polyps were pale brown which gradually turned white toward the

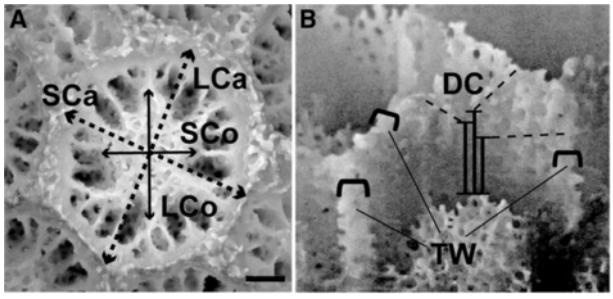

Figure 2 Measurements of morphological characters of *Goniopora*. (A) Corallite characters: long diameter of calice (LCa), short diameter of calice (SCa), long diameter of columella (LCo), and short diameter of columella (SCo). **(B)** Vertical section of corallites: depth of walls (DW) and thickness of walls (TW). Bar = 1 mm.

tip of the tentacles and had white oral discs. They were found only on mud in waters deeper than 18 m, even where there were several rocky patches available at the same depth.

Table 2 summarizes field observations of *G. stokesi* at Okinawa. At OU, more than one half of the colonies observed in the field (approximately 20 colonies) had daughter colonies (connected to parental colonies by tissue) approximately 1 cm in diameter on the colony surface (Figure 3B,D). We also observed several small colonies (<2 cm in diameter) around larger colonies (3 to 10 cm in diameter). Furthermore, all of these colonies were free-living and found on mud, although four of the thirteen colonies that we collected were attached to a small piece of dead coral skeleton. Tissues around the bottom of the colonies were typically swollen.

In the temperate region, we found only three colonies (AM74, AM75, and AM89) with free-living forms at AM (Figure 5A1,A2 and Table 2), where the existence of *G. stokesi* had previously been reported (Nishihira and Veron 1995). We did not find these forms at any other study site in the temperate region. Their skeletal morphologies, however, indicated that they were not *G. stokesi*, but rather *G. djiboutiensis* or *G.* cf. *djiboutiensis* (Figures 4 and 5), because these three colonies had smaller corallites (<5 mm) and thicker walls than those of *G. stokesi*

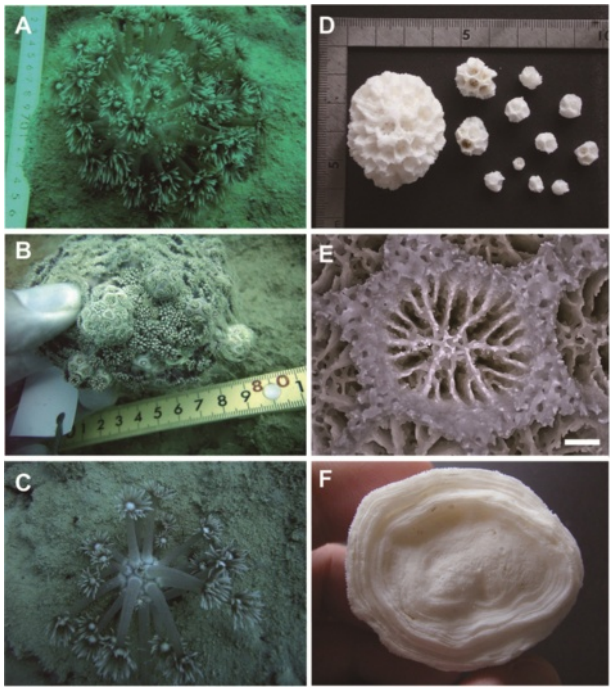

Figure 3 *Goniopora stokesi* at Oura Bay. (A) Living specimen with elongated polyps. **(B)** Living specimens with shrunken polyps and several daughter colonies attached. **(C)** Living daughter colony with elongated polyps. **(D)** Skeletons of parent and daughter colonies. **(E)** Corallites of parent colony. **(F)** An epitheca on the underside with concentric circles. Bar = 2 mm.

Figure 4 *Goniopora* spp. examined in this study. Polyps **(A)** and corallites **(B)** of *G. pendulus* (OT31), *G.* cf. *stokesi* (MO82) **(C**, **D)**, *G. djiboutiensis* (OT30) **(E, F)**, *G.* cf. *djiboutiensis* (KK11) **(G**, **H)**, *G. lobata* (KS28) **(I**, **J)**, and *G.* cf. *lobata* (SR14) **(K**, **L)**. Each bar = 1 mm.

(see below discussion and Tables 3 and 4 for results of the morphological analyses). In addition, there was no well-developed epitheca on the underside of each corallum (Figure 5A2).

However, at IR and MO we found three *G. stokesi*-like specimens that were completely attached to hard substrates. Polyps of two of these three colonies looked very similar to those of *G. stokesi* in the water (Figure 5B1, C1) upon comparison with polyps of *G. stokesi* at OU and photographs of living specimens in the literature (Veron 2000), but their skeletal features were not similar to those of *G. stokesi*. Based on skeletal characters, they were identified as either *G. djiboutiensis* or *G.* cf. *djiboutiensis* (Figure 5A3, C2). Another colony had skeletal characters similar to those of *G. stokesi*, excluding the width of the columella (Figure 4D, Tables 3 and 4); although the morphology of the polyps (evenly extended with terete tentacles of even length; Figure 4C; Veron (2000)) was similar to that of *Goniopora tenuidens*. We treated this colony as *G.* cf. *stokesi*.

Table 2 Summary of ecological and morphological features of *Goniopora stokesi* and *G. stokesi*-like specimens

	G. stokesi			*G. stokesi*-like specimens			
Site[a]	**OU**	**NK**	**IR**	**AM**	**IR**	**MO**	**MO**
No. of colonies collected	OU12, OU14, OU17, OU19, OU20, OU22, OU24, OU26, OU41, OU43	NK1	IR27, IR65	AM74 AM75 AM89	IR22	MO53	MO82
Sampling date	May and October 2009	August 2010	June and August 2011	December 2011	June 2011	March 2011	February 2011
No. of colonies observed in the field	>20	>20	2	3	1	1	1
Living form	Free-living	Free-living	Free-living	Free-living	Attached	Attached	Attached
Depth (m)	18 to 29	20	21 to 29	3 to 10	19	10	6
Daughter colonies	Yes	Yes	No	No	No	No	No
Polyp morphology	*G. stokesi*	*G. stokesi*	*G. stokesi*	*G. djiboutiensis*	*G. stokesi*	*G. stokesi*	*G. tenuidens*
Skeletal morphology	*G. stokesi*	*G. stokesi*	*G. stokesi*	*G. djiboutiensis* or *G.* cf. *djiboutiensis*	*G. djiboutiensis*	*G.* cf. *djiboutiensis*	*G.* cf. *stokesi*

[a]Site abbreviations are defined in Figure 1. See Table 1 for more information.

Molecular analyses

The molecular-based phylogenetic tree based on ND5-COI (Figure 6) classified the species analyzed in this study into two main clades (clades I and II). Clade I included specimens from temperate regions identified as *G.* cf. *stokesi* (1 colony), *G. pendulus* (3), *G.* cf. *djiboutiensis* (3), and *G.* cf. *lobata* (1). In the same clade, specimens from subtropical regions identified as *G. stokesi* (9), *G. pendulus* (1), and *G.* cf. *djiboutiensis* (3) were retrieved. In clade II, specimens from temperate regions included those identified as *G. lobata* (3), *G.* cf. *lobata* (1), *G. djiboutiensis* (2), and *G.* cf. *djiboutiensis* (4), and those from

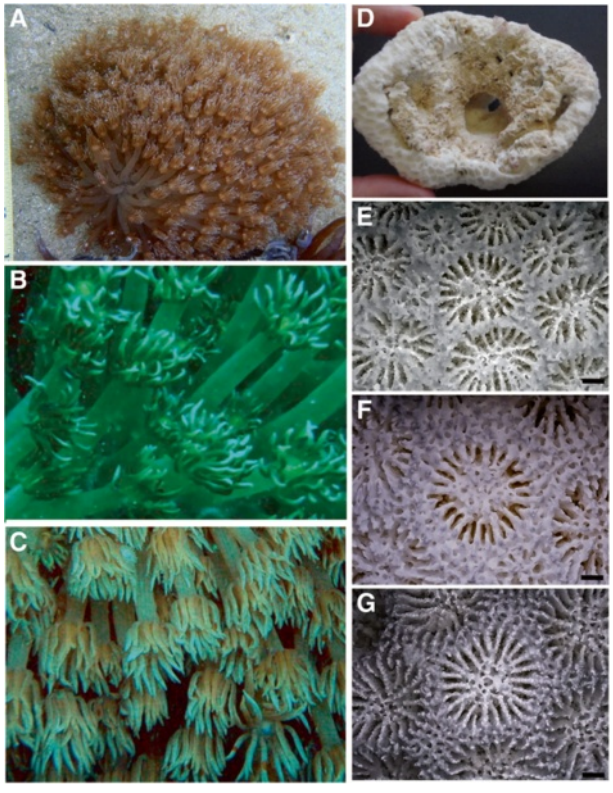

Figure 5 *Goniopora* spp. with living morphologies that are similar to that of *G. stokesi*. Living specimen **(A1)**, underside of the corallum **(A2)**, and corallite structures **(A3)** of a free-living form of *G. djiboutiensis* (AM74) at AM. Living specimen **(B1)** and corallite structures **(B2)** of *G. djiboutiensis* (IR22). Living specimen **(C1)** and corallite structures **(C2)** of *G.* cf. *djiboutiensis* (MO53). Polyps of colonies IR22 and MO53 are similar to those of *G. stokesi*. Each bar = 2 mm.

Table 3 Morphological variables of *Goniopora* used in the analysis (mean ± SD)

	Description/value									Between ITS clades		
ITS clade type	Ia	Ib	Ib	Ib	Ib	II	II	II	II	Ia	Ib	II
No. of colonies	5	1	4	4	1	5	6	4	3	5	10	18
Morphospecies	*G. stokesi*	*G.* cf *stokesi*	*G. pendullus*	*G.* cf *djiboutiensis*	*G.* cf *lobata*	*G. djiboutiensis*	*G.* cf *djiboutiensis*	*G. lobata*	*G.* cf *lobata*			
Measured variable												
LCa (μm)	5313 ± 840.8	4781 ± 437.6	4313 ± 438.5	4726 ± 461.7	3925 ± 144.2	4721 ± 630.1	4730 ± 483.1	4268 ± 440.2	3847 ± 282.6	5313 ± 840.8	4486 ± 501.4	4477 ± 593.4
LCo (μm)	2853 ± 663.3	3279 ± 448.9	2477 ± 405.5	2804 ± 470.7	2388 ± 224.5	3081 ± 566.8	2975 ± 527.7	2349 ± 470.9	2370 ± 218.7	2853 ± 663.3	2678 ± 488.5	2764 ± 583.3
DC (mm)	3.40 ± 0.91	3.47 ± 0.36	2.32 ± 0.98	2.09 ± 0.67	2.68 ± 0.69	1.21 ± 0.51	2.10 ± 0.72	2.22 ± 0.56	1.34 ± 0.15	3.40 ± 0.91	2.38 ± 0.87	1.76 ± 0.72
TW (mm)	0.89 ± 0.15	0.91 ± 0.07	1.30 ± 0.28	1.23 ± 0.22	1.14 ± 0.27	1.34 ± 0.35	1.30 ± 0.28	1.22 ± 0.23	1.27 ± 0.29	0.89 ± 0.15	1.22 ± 0.26	1.29 ± 0.29
Counted variable												
No. of septa	22.6 ± 1.55	21.4 ± 1.67	19.5 ± 1.67	23.3 ± 2.47	23.2 ± 1.10	23.9 ± 0.67	23.3 ± 1.95	22.9 ± 1.68	23.9 ± 1.36	22.6 ± 1.55	21.6 ± 2.67	23.5 ± 1.55
Derived variable												
LCo/LCa[a]	0.54 ± 0.08	0.69 ± 0.08	0.57 ± 0.08	0.59 ± 0.08	0.61 ± 0.05	0.65 ± 0.06	0.63 ± 0.09	0.55 ± 0.09	0.62 ± 0.06	0.54 ± 0.08	0.60 ± 0.08	0.62 ± 0.09
SCa/LCa	0.87 ± 0.07	0.73 ± 0.09	0.84 ± 0.09	0.81 ± 0.08	0.79 ± 0.22	0.87 ± 0.05	0.86 ± 0.08	0.80 ± 0.08	0.82 ± 0.09	0.87 ± 0.07	0.81 ± 0.11	0.84 ± 0.08
SCo/LCo[a]	0.81 ± 0.11	0.70 ± 0.15	0.57 ± 0.13	0.71 ± 0.12	0.78 ± 0.09	0.80 ± 0.08	0.84 ± 0.11	0.72 ± 0.12	0.79 ± 0.14	0.81 ± 0.11	0.70 ± 0.14	0.79 ± 0.12

[a]The parametric analysis (see 'Methods'). LCa, long diameter of calice; LCo, long diameter of columellae; DC, depth of calices; TW, thickness of walls; LCo/LCa, ratio of LCo and LCa; SCa/LCa, ratio of SCa and LCa; SCo/LCo, ratio of SCo and LCo.

Table 4 Multiple pairwise comparisons of morphological variables of *Goniopora*

	Description/value									Between ITS clades		
ITS clade type	Ia	Ib	Ib	Ib	Ib	II	II	II	II	Ia	Ib	II
Serial number	1	2	3	4	5	6	7	8	9			
Morphospecies	*G. stokesi*	*G.* cf *stokesi*	*G. pendullus*	*G.* cf *djiboutiensis*	*G.* cf *lobata*	*G. djiboutiensis*	*G.* cf *djiboutiensis*	*G. lobata*	*G.* cf *lobata*			
Measured variable												
LCa (μm)	3, 5, 8, 9	9	1,	9	1,	9	9	1,	1, 2, 4, 6, 7	Ib, II	Ia	Ia
LCo (μm)		9				9	9		2, 6, 7	Ib	Ia, II	Ib
DC (mm)	6	6, 9	6	6	6	1 to 5, 7, 8	6	6	2	Ib, II	Ia, II	Ia, IB
TW (mm)										Ib, II	Ia	Ia
Counted variable												
No. of septa			6, 7, 9			3	3		3	II	II	Ia, Ib
Derived variable												
LCo/LCa[a]	2, 6, 7, 9	1, 8				1, 8	1, 8	2, 6, 7	1	Ib, II	Ia	Ia
SCa/LCa												
SCo/LCo[a]	2	1, 6, 7, 9	7	7		2	2, 3, 4, 8	7	2	Ib	Ia, II	Ib

[a]The parametric analysis (see 'Methods'). Serial numbers are only shown for pairwise comparisons that significantly differed ($p < 0.05$). LCa, long diameter of calice; LCo, long diameter of columellae; DC, depth of calices; TW, thickness of walls; LCo/LCa, ratio of LCo and LCa; SCa/LCa, ratio of SCa and LCa; SCo/LCo, ratio of SCo and LCo.

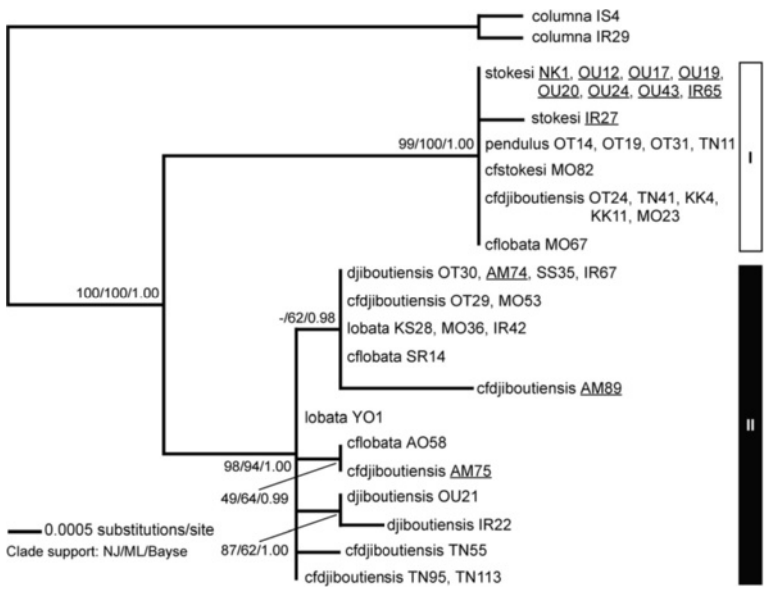

Figure 6 Maximum-likelihood (ML) phylogenetic tree of the NADH dehydrogenase subunit 5 (ND5)-cytochrome oxidase subunit 1 (COI) dataset. See Table 1 for species names and colony numbers. Bootstrap values (%) for the neighbor-joining (NJ) and ML analyses, and probability values for the Bayesian inferences (BIs), are shown on the branches. Underlining of a sample number indicates a free-living form.

subtropical regions included *G. lobata* (1), *G.* cf. *lobata* (2), *G. djiboutiensis* (4), and *G.* cf. *djiboutiensis* (3). Thus, in the ND5-COI tree, *G. stokesi* was indistinguishable from the other species.

In the ITS tree (Figure 7), *G. stokesi* (clade Ia), which partially corresponded to clade I of the ND5-COI tree, was genetically separate from the other clades (Ib, II). Species groups (*G. lobata*, *G.* cf. *lobata*, *G. djiboutiensis*, and *G.* cf. *djiboutiensis*), which corresponded to clade II of the ND5-COI tree, also formed a single clade (clade II in the ITS tree). Although clade II in the ITS tree appeared to be divided into two subclades (Figure 7), the division of these subclades was not related to differences in morphospecies, i.e., alleles from a single colony were separately included in both subclades.

Morphological analyses

Seven morphometric variables (LCa, SCa, LCo, SCo, the number of septa, CD, and TW) were scored from 33 specimens (165 corallites). A PCA biplot of the morphometric data for these specimens is shown in Figure 8. The first two PCs accounted for 66.2% of the total variance. PC1 was positively correlated with all parameters, especially LCo, SCo, LCa, and SCa ($r > 0.8$). No groups were clearly visible in the PCA plot, but at least two groups were likely separable when we applied these plots to the clades in the ITS tree (Figure 8A) and morphospecies (Figure 8B). *G. stokesi* and *G. djiboutiensis* (or clade II) were particularly likely to belong to different groups, although *G. pendulus* (or clade Ib) overlapped between them.

In the specimens we observed, the epitheca had only developed on the underside of *G. stokesi*; however, we were unable to determine the presence of the epitheca in one large colony of *G. stokesi* because we did not collect part of the underside and did not check for its presence in the field.

Then, we performed multiple comparisons of each variable or character. Development of pali and septal arrangements were too variable even within a single morphospecies, and their comparisons showed no significant differences among morphospecies (data not shown). Values of short diameter of either columella or calice (SCo or SCa) were directly proportional to their long diameter (LCo or LCa) (data not shown); therefore, we only used LCo and LCa in this study. The dataset for a total of eight variables is shown in Tables 3 and 4. Pairwise multiple comparisons showed that *G. stokesi* was significantly separable from *G. pendulus* (clade Ib), *G.* cf. *lobata* (Ib), *G. lobata* (II), and *G.* cf. *lobata* for LCa ($p < 0.05$, Dunn's test), from *G.* cf. *djiboutiensis* (II) for DC ($p < 0.05$, Dunn's test), from *G.* cf. *stokesi* (Ib), *G. djiboutiensis* (II), *G.* cf. *djiboutiensis* (II), and *G. lobata* (II) for LCo/LCa ($p < 0.05$, Tukey's test), and from *G.* cf. *stokesi* for SCo/LCo ($p < 0.05$, Tukey's test). For other species, *G. djiboutiensis* (II) was significantly ($p < 0.05$, Dunn's test) separable from all others, including *G.* cf. *djiboutiensis*, for DC. However, other variables did not show sufficient differences to separate them from one another. In

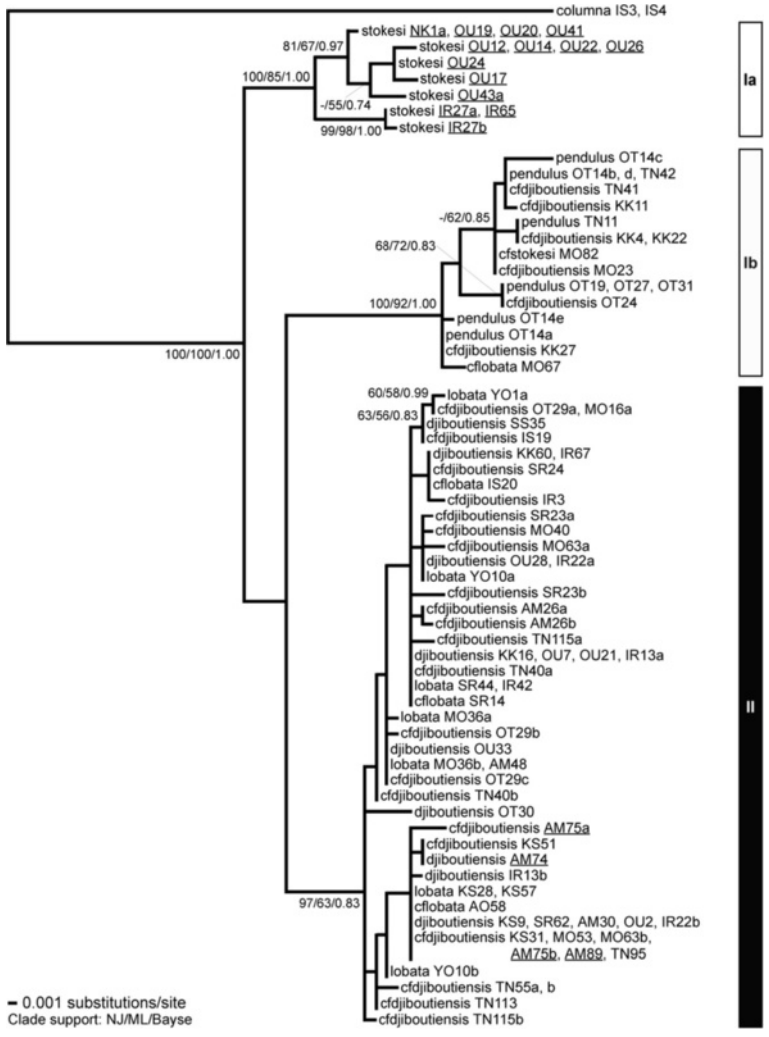

Figure 7 Maximum-likelihood (ML) phylogenetic tree using the internal transcribed spacer (ITS) dataset. See Table 1 for species names and colony numbers. Bootstrap values (%) for the neighbor-joining (NJ) and ML analyses, and probability values for the Bayesian inferences (BIs), are shown on the branches. Underlining of a sample number indicates a free-living form.

summary, combinations of several morphological variables could at least separate *G. stokesi* from the others, whereas other morphological variables that were able to separate other morphospecies could not be identified except for DC for *G. djiboutiensis*.

Discussion

Phylogeny and taxonomy of *G. pendulus*, *G. djiboutiensis*, and *G. lobata*

There were no significant differences in the morphological variables of corallites (SCo, LCo, SCa, LCa, DC, TW, and number of septa) between *G. pendulus* and *G. lobata*, even though the original description (Veron 1985) of *G. pendulus* stated that corallites and columellae of *G. pendulus* were larger than those of *G. lobata*. Nevertheless, *G. pendulus* could be separated from *G. lobata* based on a reduced septal number and the length of the polyps and tentacles (much longer in *G. pendulus* than in *G. lobata*; Veron (1985)). Although each of these two characters varied within and between species (e.g., long tentacles were observed in some colonies of *G.* cf. *djiboutiensis*), their combination could largely separate these species.

We could not clearly distinguish among the four morphospecies (*G. djiboutiensis*, *G.* cf. *djiboutiensis*, *G. lobata*, and *G.* cf. *lobata*) based on either morphological or phylogenetic analyses. In particular, *G.* cf. *djiboutiensis* and *G.* cf. *lobata* were included in both clades Ib and II in the ITS tree (Figure 7). Considering that these clades were clearly separate, it is highly possible that *G.* cf. *djiboutiensis* and *G.* cf. *lobata* in the two clades (Ib and II) represent different species. Because *G. pendulus*, the only one that did not appear in other clades, was the main species in clade

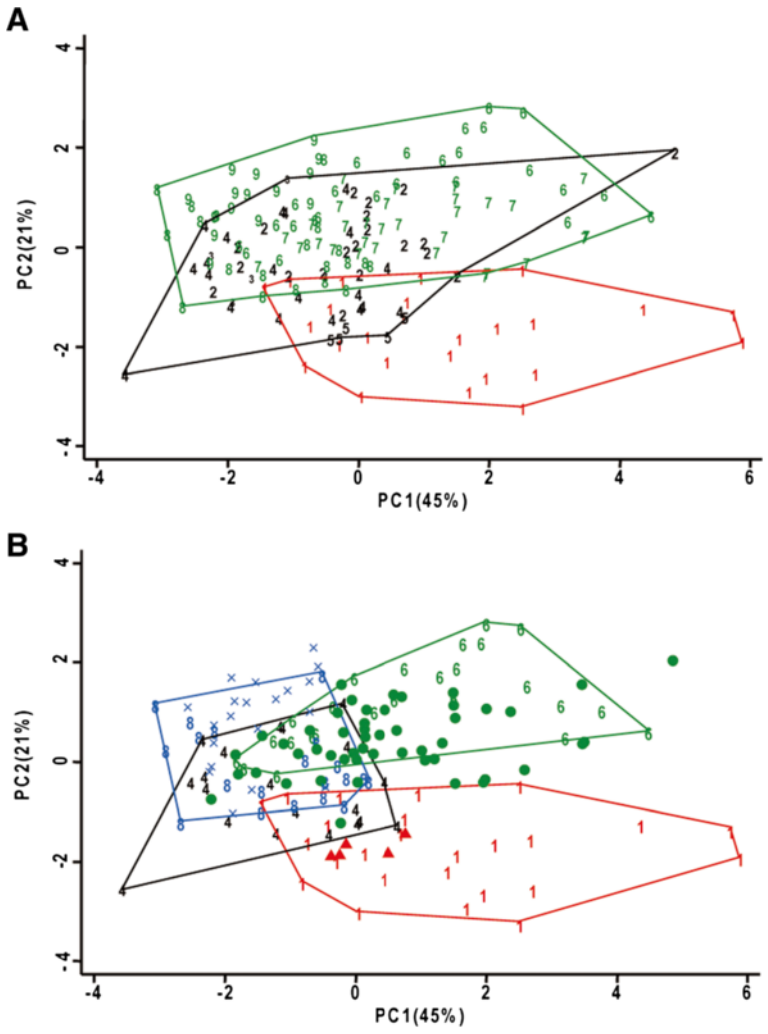

Figure 8 Results from a principal component analysis of morphometric data from *Goniopora* spp. Grouping by clades in the internal transcribed spacer (ITS) tree **(A)** and grouping by morphospecies **(B)**. In A, the group in red represents clade I, the group in black represents clade Ib, and the group in green represents clade II. Numbers are defined as follows; 1, *G. stokesi*; 2, *G.* cf. *djiboutiensis* (clade Ib); 3, *G.* cf. *lobata* (Ib); 4, *G. pendulus*; 5, *G.* cf. *stokesi*; 6, *G. djiboutiensis*; 7, *G.* cf. *djiboutiensis* (II); 8, *G. lobata*; and 9, *G.* cf. *lobata* (II). In B, the group in red represents *G. stokesi*, the group in black represent *G. pendulus*, the group in green represents *G. djiboutiensis*, and the group in blue represents *G. lobata*. Symbols are defined as follows: red triangles, *G.* cf. *stokesi*; green circles, *G.* cf. *djiboutiensis*; and blue crosses, *G.* cf. *lobata*.

Ib, the two morphs, *G.* cf. *djiboutiensis* and *G.* cf. *lobata*, in clade Ib (not in clade II) might represent morphological variations of *G. pendulus*. For morphospecies in clade II, *G. djiboutiensis* had very shallow corallites, which could be a good character to separate it from the others (Tables 3 and 4). In general, it was quite difficult to identify these *Goniopora* species based on macro-morphologies that have typically been used in the taxonomy of this group. In the future, a more-detailed morphological analysis of microstructures using scanning electron microscopy is necessary (if they are separate species), as has been done with other families of scleractinian corals (Benzoni et al. 2007; Budd and Stolarski 2011). Crossing experiments may also be useful in detecting species boundaries.

Reproductive mechanism of *G. stokesi*

It was reported that *G. stokesi* releases daughter colonies asexually in the Maldives, Nicobar Islands, Seychelles, and Australia (Scheer 1960; Rosen and Taylor 1969; Veron and Pichon 1982). However, this type of asexual reproduction has never been observed in Japan (Nishihira and Veron 1995). This is the first report of the formation of daughter colonies by *G. stokesi* in Japan. We also found that this species inhabits only muddy habitats at water depths of >18 m in Okinawa, Japan, whereas in the Seychelles, it inhabits protected sandy areas such as lagoons at 1 m in depth (Rosen and Taylor 1969). As noted by Rosen and Taylor (1969), this unique reproductive mechanism of releasing daughter colonies is associated with colonization

of unstable soft substrates. Indeed, we observed daughter colonies only on top of the mud. They had swollen tissues, which prevented them from becoming buried. It is possible that such daughter colonies could be affected by strong currents associated with rocky environments, thus limiting their distribution to more-protected environments, such as deeper areas or inside lagoons. Additionally, in OU, many daughter colonies were found on and around parent colonies in spring (May) and autumn (October), suggesting that this asexual reproduction may frequently occur throughout the year. It is clear that the formation of asexual daughter colonies is an important reproductive mechanism in this species, while sexual reproduction has never been observed.

Regarding the molecular phylogeny based on the ITS, all samples of *G. stokesi* formed a single clade and were clearly separated from morphologically closely related species, including *G. djiboutiensis*, *G. lobata*, and *G. pendulus* (Figure 7). This result strongly supports the idea that *G. stokesi* is a genetically isolated species from *G. stokesi*-like colonies (see below).

G. stokesi-like colonies and free-living forms

We found that colonies attached to hard substrates and with a similar morphology (tentacles or skeletons) to *G. stokesi* did not actually belong to this species (Table 2). Several previous studies reported that *G. stokesi* unusually attaches to hard substrates (Nemenzo 1955; Veron and Pichon 1982; Veron 2000). However, our data suggest that (at least in Japan) colonies that attach to hard substrates are not *G. stokesi*. Further analyses of such colonies from locations outside Japan are necessary to clarify whether *G. stokesi* attaches to hard substrates, such as other corals.

We also found three free-living colonies in AM in the temperate region. In general, any free-living forms of *Goniopora* were presumably *G. stokesi*. However, their morphology differed from that of typical *G. stokesi*. The underside of the coralla was slightly hollow (Figure 5A2) or may have been broken and lacked an epitheca (Figure 3F). In addition, other colonies with the same skeletal characters as these three colonies, which were identified as *G. djiboutiensis* or *G.* cf. *djiboutiensis*, were found attached to rocks near sand. This suggests that they are intraspecific morphological variations of *G. djiboutiensis* and that these free-living colonies were not adapted to living on sand but had accidentally grown there, probably after breaking off a rock.

There are two types of free-living corals. One type consists of corals that are permanently free-living, such as fungiids, while the other type consists of those that are secondarily and accidentally free-living (Pichon 1974). Secondarily and accidentally free-living forms were observed in some species from other genera (*Acropora*, Riegl et al. (1996); *Pavona* and *Porites*, Glynn (1974), Scoffin et al. (1985); and *Siderastrea*, Lewis (1989)), which are possibly an adaptation to a disturbed habitat (Lewis 1989). They are known to have spheroidal growths like rolling stones, which are called coralliths (Glynn 1974). It was reported that the formation of coralliths needs periodic movement by waves and currents or disturbance by browsing fish (Murray 1885; Glynn 1974). However, coralliths differ from what was observed in the present study. Free-living forms in AM did not become coralliths because the underside of these corals was completely dead (no living tissue). Species of the genus *Goniopora* share common morphological characteristics, such as long tentacles and thick tissues, which can prevent them from being buried in sand. Because of these characteristics, they can survive on sand without becoming corallith forms.

Conclusions

Our investigation revealed that *G. stokesi* is free-living, forms daughter colonies, and in Okinawa, Japan, was found on the mud below 18 m of water. This is the first report of asexual reproduction by *G. stokesi* in Japan. Molecular phylogenetic analyses using ITSs (but not mitochondrial markers) revealed that *G. stokesi* was genetically isolated from other morphologically related species. We identified several other colonies that looked like *G. stokesi*, but in fact belonged to other species. Overall, *G. stokesi* is distinguishable from morphologically related species by a combination of the following characters: being free-living; living on soft substrates in protected environments; and having large, deep calices, thin walls, and a well-developed epitheca on the underside of the corallum. However, because the morphological features of *Goniopora* species can overlap, it is important to also use genetic techniques to confirm species boundaries. Currently, molecular phylogenetic analyses using ITS markers (in comparison with ND5-COI) are the most useful means of separating these morphospecies and recognizing morphological variations in each genetic group, although it is necessary to look for additional markers to distinguish morphospecies within clades. Based on our results, novel morphological characteristics, including detailed polyp morphologies or microstructures, may be identified in the future. Because *Goniopora* species are sometimes abundant even on the coast of Japan, it is important to further elucidate their detailed taxonomy to estimate species diversity and/or regional specificity.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YFK carried out the molecular phylogenetic and morphological studies, collected samples, and drafted the manuscript. MO and DU participated in the design of the study and collected samples. KM participated in the design and coordination of the study. HF conceived of the study, collected samples, and participated in its design and coordination. All authors read and approved the final manuscript.

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