

The stoloniferous octocoral, *Hanabira yukibana*, gen. nov., sp. nov., of the southern Ryukyus has morphological and symbiont variation

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Abstract

Stoloniferan octocorals (Cnidaria: Anthozoa: Octocorallia: Alcyonacea) are a relatively unexplored fauna in the Ryukyus (southern Japan), known to be a tropical marine region of high biodiversity and endemism of species. Specimens of stoloniferous octocorals were collected during fieldwork along the coasts of two islands (Okinawa and Iriomote) in the Okinawa Prefecture. Despite their phenotypic polyp variation, this study shows their morphological and molecular uniqueness, leading to the description of a new genus with a single species: *Hanabira yukibana*, gen. nov., sp. nov. They are placed within the Clavulariidae and form a sister clade basally to the genus *Knopia* Alderslade & McFadden, 2007 and species of *Clavularia* Blainville, 1830. The polyps of this new species show morphological variation in both shape and sclerite density, but there is conformity in the typical overall petal shaped tentacles, which have fused pinnules (pseudopinnules). Depending on the densities of their sclerites and their photosynthetic endosymbiotic algae (zooxanthellae) of the family Symbiodiniaceae, there is a characteristic sheen present in the

tentacles. Moreover, the zooxanthellae hosted by our specimens form a clear, small-scale biogeographic pattern; all *H. yukibana* specimens from Okinawa Island contained zooxanthellae of the genus *Cladocopium* Lajeunesse & H.J. Jeong, 2018 (= former *Symbiodinium* 'Clade C') and all specimens from Iriomote Island hosted zooxanthellae of the genus *Durusdinium* Lajeunesse, 2018 (= former *Symbiodinium* 'Clade D'). These results show the potential for variation among the Symbiodiniaceae floras within octocorals, something that has not yet been investigated for the large majority of zooxanthellate octocoral species.

Keywords

28S rDNA – COI – endemism – Iriomote Island – mtMutS – ND6 – Okinawa Island – phenotypic variation – stoloniferous octocorals – Symbiodiniaceae – taxonomy

Introduction

The Ryukyu Islands are the southernmost region of Japan and are a tropical marine region of high biodiversity and species endemism (Hughes et al., 2002; Cowman et al., 2017; Reimer et al., 2019). It was estimated that a large part (70%) of the marine biodiversity in Japanese waters has remained undescribed (Fujikura et al., 2010). Coral reefs are the most biologically diverse of shallow-water marine ecosystems (Roberts et al., 2002) and contain cryptic, usually small, inconspicuous species that are easily overlooked and therefore poorly observed and known (Wolf et al., 1983; Reaka-Kudla, 1997; Hoeksema, 2017). Octocorals of the subordinal group Stolonifera are characterized by their growth form by having polyps that are connected through stolons (Fabricius & Alderslade, 2001; Daly et al., 2007; McFadden & Ofwegen, 2012). Many stoloniferans have small inconspicuous colonies, which is one reason that such species are poorly known. Stoloniferan octocorals are a relatively unexplored fauna in the Ryukyus. This is especially true concerning molecular and ecological research, such as the examination of octocoral-Symbiodiniaceae associations. Previous research has revealed biogeographical patterns in the presence of

Symbiodiniaceae genera occurring in different octocoral hosts in different geographic regions (Oppen et al., 2005; Goulet et al., 2008), but this is much less explored within smaller geographic areas, such as across islands of the southern Ryukyus.

Stolonifera are known to be polyphyletic, appearing in various lineages within the Octocorallia radiation (McFadden & Ofwegen, 2012; Conti-Jerpe & Freshwater, 2017). Small and relatively under-studied stoloniferous octocoral colonies may occupy unique phylogenetic positions within the Octocorallia, as new species, genera, and families have recently been erected (McFadden & Ofwegen, 2012; Lau et al., 2018). Currently, there are seven families of Alcyonacea considered to belong to the subordinal group Stolonifera: Acrossotidae Bourne, 1914; Arulidae McFadden & Ofwegen, 2012; Coelogorgiidae Bourne, 1900; Cornulariidae Dana, 1846; Clavulariidae Hickson, 1894; Pseudogorgiidae Utinomi & Harada, 1973; and Tubiporidae Ehrenberg, 1828. The family Clavulariidae comprises 28 genera and over 60 species (Cordeiro et al., 2019). Recent studies confirm the polyphyly of the family Clavulariidae and there is still no clear understanding about the morphological distinctions that separate the different molecular clades within this family

(McFadden & Ofwegen, 2012; Benayahu et al., 2017; Conti-Jerpe & Freshwater, 2017). Thus, there is a need for more research on Stolonifera that combines molecular, morphological, and ecological datasets, in order to gain a better understanding of this family.

Recent observations and collections in the north-western Pacific, including those from around the island of Okinawa (or Okinawa-jima, hereafter Okinawa Island), have revealed a high abundance of stoloniferous octocoral species on coral reefs that are either unrecorded or possibly undescribed (Okinawa Churashima Foundation and Biological Institute on Kuroshio, 2016; Lau et al., 2018) and mostly these octocorals are not only small in colony size with few polyps, but some species also have polyps which are only ~2–3 mm in diameter (Lau et al., 2018). Here, we use a combined approach of morphological and molecular data to describe one new monotypic genus within the family Clavulariidae based on recent collections from shallow waters surrounding Okinawa and Iriomote Islands (or Iriomote-jima, hereafter Iriomote Island). We also investigate the identity of their endosymbiotic Symbiodiniaceae and their relations to morphological polyp variations and geographic locations.

Material and methods

Specimen collection

Clavulariid specimens (total $n = 38$) were collected from locations in Okinawa Prefecture, Japan, around Okinawa Island ($n = 18$) from March to October 2011, and May 2017 to August 2018, and around Iriomote Island ($n = 20$), 25–27 July 2017. A total of 18 sites were visited, at Okinawa ($n = 11$) and Iriomote ($n = 7$) Islands (fig. 1, table 1). Specimens were collected in depth ranges of 10–35 m by means of SCUBA and all material was preserved in

99% ethanol. High-resolution *in situ* images were taken with an Olympus Tough TG-4 in an Olympus PT-056 underwater housing. Vouchers and type material have been deposited at the National Museum of Nature and Science, Tokyo, Japan.

Morphological examinations

A detailed morphological species description was made by examination of colony and polyp morphology and embedded sclerites (Appendix). Sclerites were isolated by following the same protocol as described in Lau et al. (2018). In addition, polyps were fixed in 20% formalin and embedded in methylene blue (1%) to visualise and accentuate polyp morphology and tentacle pinnules. Sclerites were mounted on scanning electron microscope (SEM) stubs and coated with Pd/Au for imaging on a JEOL JSM6490LV SEM operated at high vacuum at 15kV.

DNA extraction, amplification, and sequencing

DNA was extracted from polyps using a DNeasy Blood and Tissue kit (Qiagen, Tokyo). PCR amplification and sequencing of the COI, mtMutS and 28S rDNA gene regions of the octocorals followed the protocols in Lau et al. (2018). In addition, we sequenced a ~600 bp fragment of the ND6 subunit mitochondrial gene using primers Alc_715_Car 5'-GRGCYAAACAGTAGAGG-3' and Alc_1303_Car 5'-AGGTGWATTTGGCTGCTKAGC-3' (Reijnen, 2016; modified from Reijnen et al., 2014). Reactions and PCR conditions for ND6 were similar to those used for mtMutS, except for an annealing temperature of 50°C. For Symbiodiniaceae, the nuclear Internal Transcribed Spacer (ITS) region of ribosomal DNA (~600 bp fragment) was amplified, using primer pair zITSf 5'-GGAAGTAAAAGTCGTAACAAG-3' and ITS4 5'-TCCGCTTATTGATATGC-3' (White et al., 1990; Rowan & Powers, 1992; Hunter

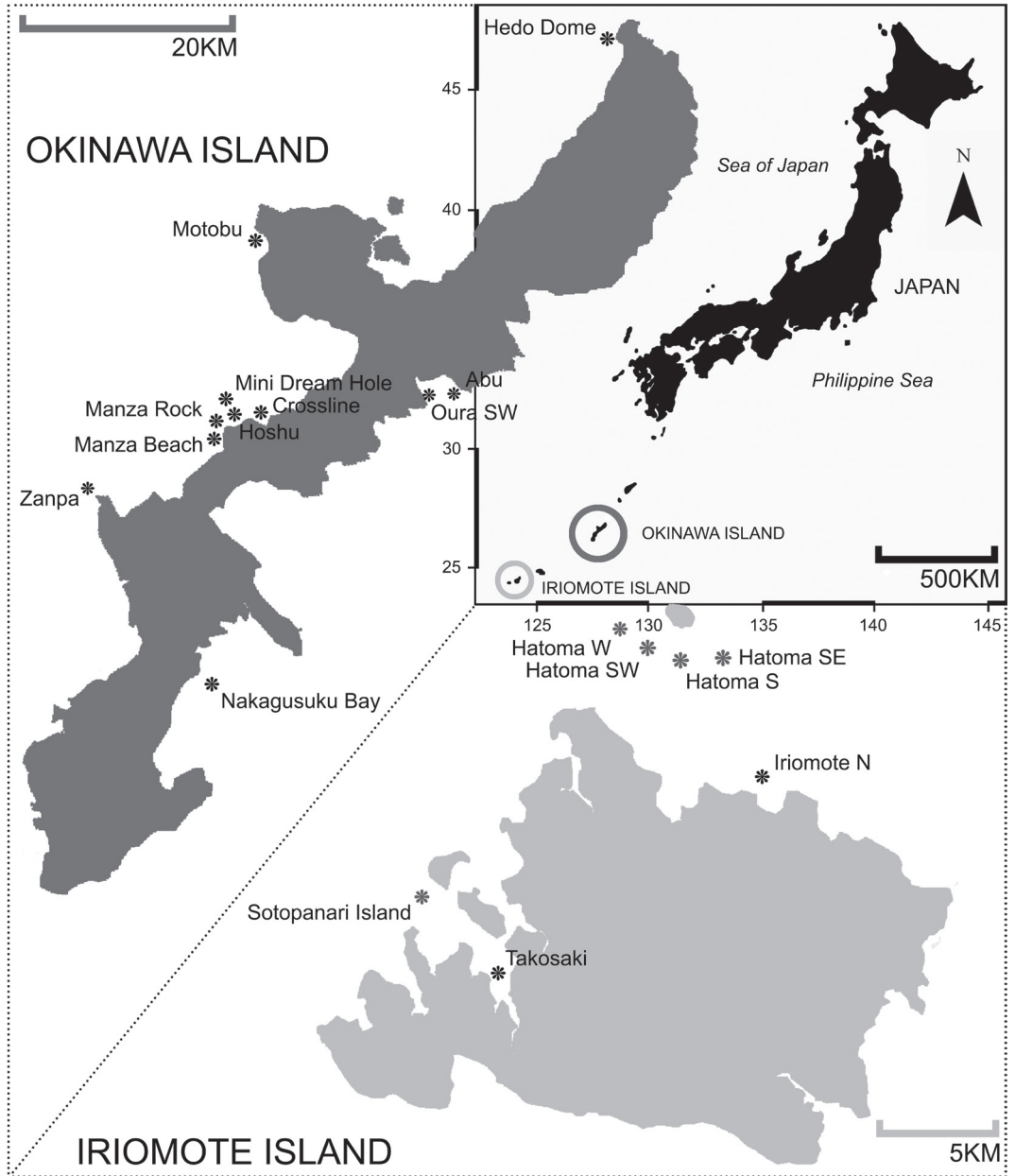


FIGURE 1 Map of sampling locations in Okinawa Prefecture around Okinawa Island (11 sites) and Iriomote Island (seven sites); collection sites *Hanabira yukibana*, gen. nov., sp. nov., (*) and *Clavularia* spp. (*) specimens.

et al., 1997). Reactions were carried out in a 25µl reaction volume containing 0.25 µl (5 U/ul) Taq DNA polymerase, 0.5 µl (2.5 mM) dNTP's, 2.5 µl (10x) Coral Load Buffer, 1.25 µl

(25 mM) MgCl₂, 16.5 µl RNase-free water, 1 µl (10 pMol/ul) of each primer, and 1 µl DNA template. The amplification protocol consisted of 5 min of initial denaturation at 95°C

TABLE 1 Overview of clavulariid octocoral specimens from Iriomote and Okinawa Islands used in this study, including GenBank accession numbers, locality and symbiont (for *Hanabira yukibana*, gen. nov., sp. nov., specimens only) information. Catalogue number: NSMT=National Museum of Nature and Science, Tokyo, Japan. Voucher number: OKA = Okinawa Island; IRI = Iriomote Island. N.a. = not available.

Clavulariidae Genus/ species	Catalogue/ voucher numbers	Symbiodini- aceae genus	Location	GPS (DDM)	GenBank accession numbers					
					28S rDNA	COI	mtMutS	ND6 ITS		
<i>Hanabira yukibana</i>	Paratype 1	NSMT-Co 1625	<i>Durusdinium</i>	Takosaki, Iriomote Is.	24-327010, 123-738290	MK185353	MK523659	MK523711	MK523689	MK524066
	Holotype	NSMT-Co 1626	<i>Durusdinium</i>	Takosaki, Iriomote Is.	24-327010, 123-738290	MK185363	MK523660	MK523712	MK523690	MK524072
	Paratype 2	NSMT-Co 1627	<i>Durusdinium</i>	Takosaki, Iriomote Is.	24-327010, 123-738290	N.a.	N.a.	N.a.	N.a.	MK524067
	Paratype 3	NSMT-Co 1628	N.a.	Takosaki, Iriomote Is.	24-327010, 123-738290	MK185346	MK523661	MK523713	MK523691	N.a.
	Paratype 4	NSMT-Co 1629	<i>Durusdinium</i>	Takosaki, Iriomote Is.	24-327010, 123-738290	N.a.	MK523662	MK523714	MK523692	MK524073
	Paratype 5	NSMT-Co 1630	<i>Durusdinium</i>	Takosaki, Iriomote Is.	24-327010, 123-738290	MK185364	MK523663	N.a.	MK523693	MK524071
	Paratype 6	NSMT-Co 1631	N.a.	Takosaki, Iriomote Is.	24-327010, 123-738290	N.a.	N.a.	N.a.	N.a.	N.a.
	Paratype 7	NSMT-Co 1632	<i>Durusdinium</i>	Takosaki, Iriomote Is.	24-327010, 123-738290	MK185348	MK523664	MK523715	MK523694	MK524070
	Paratype 8	NSMT-Co 1633	N.a.	Takosaki, Iriomote Is.	24-327010, 123-738290	MK185365	MK523665	MK523716	MK523695	N.a.
Paratype 9	NSMT-Co 1634	<i>Durusdinium</i>	Takosaki, Iriomote Is.	24-327010, 123-738290	MK185366	MK523666	MK523717	MK523696	MK524068	

Paratype 10	NSMT-Co 1635	<i>Durusdinium</i>	Takosaki, Iriomote Is.	24-327010, 123-738290	MK185359	MK523667	MK523718	MK523697	MK524069
Paratype 11	NSMT-Co 1636	N.a.	Takosaki, Iriomote Is.	24-327010, 123-738290	MK185360	MK523668	MK523719	MK523698	N.a.
Paratype 12	NSMT-Co 1637	<i>Durusdinium</i>	Iriomote N, Iriomote Is.	24-407805, 123-854199	MK185358	MK523669	MK523720	MK523699	MK524065
Paratype 13	NSMT-Co 1638	<i>Cladocopium</i>	Abu - Oura Bay, Okinawa Is.	26-537756, 128-079521	MK185368	MK523670	MK523721	MK523700	MK524063
Paratype 14	NSMT-Co 1639	<i>Cladocopium</i>	Hoshu - Manza, Onna, Okinawa Is.	26-504659, 127-846604	MK185345	MK523671	MK523722	MK523701	MK524061
Paratype 15	NSMT-Co 1640	<i>Cladocopium</i>	Hoshu - Manza, Onna, Okinawa Is.	26-504659, 127-846604	MK185350	MK523672	MK523723	MK523702	MK524060
Paratype 16	NSMT-Co 1641	<i>Cladocopium</i>	Zanpa - Cape Zanpa, Okinawa Is.	26-441517, 127-711417	MK185351	MK523673	MK523724	MK523703	MK524054
Paratype 17	NSMT-Co 1642	<i>Cladocopium</i>	Hedo Dome - Cape Hedo, Kunigami, Okinawa Is.	26-852091, 128-250450	MK185367	MK523674	MK523725	MK523704	MK524059
Paratype 18	NSMT-Co 1643	<i>Cladocopium</i>	Manza Beach - Manza, Onna, Okinawa Is.	26-502957, 127-841909	MK185361	MK523675	MK523726	MK523705	MK524062
Paratype 19	NSMT-Co 1644	<i>Cladocopium</i>	Manza Beach - Manza, Onna, Okinawa Is.	26-502957, 127-841909	N.a.	MK523676	MK523727	MK523706	MK524058

TABLE 1 Overview of clavulariid octocoral specimens from Iriomote and Okinawa Islands (cont.)

Clavulariidae Genus/ species	Catalogue/ voucher numbers	Symbiodini- aceae genus	Location	GPS (DDM)	GenBank accession numbers				
					28S rDNA	COI	mtMutS	ND6	ITS
Paratype 20	NSMT-Co 1645	<i>Cladocopium</i>	Manza Rock - Manza, Onna, Okinawa Is.	26.504522, 127.843748	MK185357	MK523677	MK523728	MK523707	MK524057
Paratype 21	NSMT-Co 1646	<i>Cladocopium</i>	Manza Rock - Manza, Onna, Okinawa Is.	26.504522, 127.843748	MK185352	MK523678	MK523729	MK523708	MK524056
Paratype 22	NSMT-Co 1647	<i>Cladocopium</i>	Hoshu - Manza, Onna, Okinawa Is.	26.504659, 127.846604	MK185355	MK523679	MK523730	MK523709	MK524055
Paratype 23	NSMT-Co 1648	<i>Cladocopium</i>	Crossline, Seragaki, Onna, Okinawa Is.	26.508734, 127.881453	MK185349	MK523671	MK523731	MK523710	MK524064
Paratype 24	NSMT-Co 1649	<i>Cladocopium</i>	Mini Dream Hole - Manza, Onna, Okinawa Is.	26.509833, 127.854006	N.a.	N.a.	MK523732	MK523702	MK524053
Paratype 25	NSMT-Co 1650	<i>Cladocopium</i>	Nakagusuku Bay - Nakagusuku, Okinawa Is.	26.262778, 127.825278	MK185356	MK523680	MK523727	N.a.	MK524052
Paratype 26	NSMT-Co 1651	N.a.	Oura sw - near Cape Henoko, Futami, Oura Bay, Nago City, Okinawa Is.	26.529373, 128.047446	N.a.	N.a.	N.a.	N.a.	N.a.
Paratype 27	NSMT-Co 1652	N.a.	Motobu - off Yamakawa, Motobu Town, Okinawa Is.	26.679389, 127.879222	N.a.	N.a.	N.a.	N.a.	N.a.

<i>Clavularia inflata</i>	Paratype 28	NSMT-Co 1653	N.a.	Motobu - off Yamakawa, Motobu Town, Okinawa Is.	26.679389, 127.879222	N.a.	N.a.	N.a.	N.a.
		OKA170530-01	N.a.	Abu - Oura Bay, Okinawa Is.	26.537756, 128.079521	MK523652	MK523657	MK523736	N.a.
		OKA170530-02	N.a.	Abu - Oura Bay, Okinawa Is.	26.537756, 128.079521	MK523651	MK523656	MK523735	MK523683
		IRI170725-02	N.a.	Sotopanari Is., Iriomote Is.	24.360951, 123.706404	MK523649	MK523654	MK523733	MK523681
		IRI170725-04	N.a.	Sotopanari Is., Iriomote Is.	24.360951, 123.706404	MK185334	MK185370	MK523737	MK523684
		IRI170726-37	N.a.	Hatoma SE, Hatoma Is., Iriomote Is.	24.452411, 123.832658	MK185343	MK185376	N.a.	MK523685
		IRI170726-43	N.a.	Hatoma SW, Hatoma Is., Iriomote Is.	24.459608, 123.803265	MK185344	MK185380	N.a.	MK523686
		IRI170727-67	N.a.	Hatoma W, Hatoma Is., Iriomote Is.	24.464737, 123.800546	MK523653	MK523658	MK523738	MK523687
		IRI170727-74	N.a.	Hatoma S, Hatoma Is., Iriomote Is.	24.452878, 123.815600	MK185340	MK185379	N.a.	MK523688
	<i>Clavularia</i> sp.								

followed by 39 cycles of 30 sec at 94°C, annealing at 53°C for 45 sec, extension at 72°C for 2 min and a final extension at 72°C for 10 min. PCR products were purified using the standard ExoSAP protocol and sent for bidirectional sequencing on an ABI 3730XL (Fasmac, Kanagawa, Japan). Sequences were assembled and edited using Geneious R11 (Kearse et al., 2012) and BioEdit (Hall, 1999). COI, mtMutS and ND6 were checked for introns, exons and stop-codons in AliView (Larsson, 2014).

Molecular phylogenetic analyses

Multiple sequence alignments were performed using MAFFT 7 (Katoh & Standley, 2013) under default parameters. To determine the phylogenetic position of clavulariid specimens ($n = 6$), consensus sequences for markers 28S rDNA, COI and mtMutS were aligned to a reference dataset of 120 octocoral genera ($n = 128$), including *Parasphaerasclera rotifera* and *Eleutherobia grayi* as outgroup (total $n = 134$). Alignments of 895 bp for 28S rDNA, 786 bp for COI and 950 bp for mtMutS were separately run in maximum likelihood (ML) analyses (supplementary figs. S1-S3). To investigate morphological and symbiont variability in the clavulariid specimens, another analysis was performed on a dataset with more clavulariid specimens. This dataset included consensus sequences for markers 28S, COI, mtMutS and ND6. The four markers were aligned to a reference dataset of four octocoral genera ($n = 14$), including *Parasphaerasclera rotifera* and *Eleutherobia grayi* as outgroup (total $n = 37$). Alignments of 895 bp for 28S rDNA, 786 bp for COI, 950 bp for mtMutS, 534 bp for ND6 (available for all octocoral specimens), and 677 bp for ITS-rDNA (Symbiodiniaceae only) were separately run in ML analyses. The ITS-rDNA dataset for Symbiodiniaceae was aligned with reference taxa *Cladocopium* spp. and *Durusdinium* spp., using *Gerakladium* sp. as outgroup (total $n = 25$) (supplementary figs.

S4-S8). All new sequences generated in this study were deposited in GenBank (table 1). ML and Bayesian inference analyses were performed on the Naturalis OpenStack computing cloud using PhylOstack (Dooreweerd, 2016).

Alignments of the different octocoral markers were concatenated using SequenceMatrix 1.8 (Gaurav et al., 2011), resulting in a 2631 bp dataset (three markers) of 121 taxa ($n = 134$) and a 3165 bp dataset (four markers) of five taxa ($n = 37$). ML analyses were run with RAX-ML 8 (Stamatakis, 2014), using the GTRCAT model. The best ML tree was calculated using the $-D$ parameter. A multi-parametric bootstrap search was performed, which automatically stopped based on the extended majority rule criterion. The Bayesian inference was performed with ExaBayes 1.5 (Aberer et al., 2014) using the GTR substitution model. Four independent runs with each four Monte Carlo Markov Chains were run for 1,000,000 generations during which convergence (with a standard deviation of split frequencies $< 2\%$) had been reached. Bootstrap supports and posterior probabilities were depicted on the branches of the best ML tree using P4 (Foster, 2004). The resulting tree was visualized in FigTree 1.4.2 (Rambout, 2014). Additionally, average distance estimations within species and within genera were computed using MEGA7 (Kumar et al., 2016) by analysing pairwise measures of genetic distances (uncorrected P) among sequences.

Results

Morphological study

The present investigation revealed the morphological distinction of a new genus and species *Hanabira yukibana*, gen. nov., sp. nov. (Appendix). Several morphological characters separate this monotypic genus from other genera, based on colony and sclerite morphology.

which is the main means of identification in octocoral species delimitation. The main distinguishing characters of the polyps include the typical petal-shaped tentacles and tentacle pinnules, which are fused together (pseudopinnules). Other characteristics include the morphological variation seen in the polyps of *H. yukibana* in both tentacle shape and sheen (fig. 2). We divided all specimens into the three main polyp variations, based mainly on the shape of the tentacles; variation A, petal shaped, variation B, tongue shaped and variation C, feather shaped (figs. 2, 5). The sclerites seen in *H. yukibana* are different from those in closest sister genera *Clavularia* and *Knopia* in composition, types and size; the most typical sclerites in *H. yukibana* are platelets with a distinct waist in combination with small, smooth rods (figs. 3, 5).

Molecular phylogenetic analyses

This study has added 90 *Hanabira yukibana* sequences to the public reference database, representing a species for which no barcodes have been sequenced before. An additional 32 sequences have been added for *Clavularia* sp., of which 13 belong to a possibly undescribed species. For the family Symbiodiniaceae, 13 *Cladocopium* spp. and nine *Durusdinium* spp. sequences were added. The phylogenies resulting from the ML analyses of the separate host markers (supplementary figs. S4-S8) were highly congruent with those from the combined markers (figs. 4, 5). ML and Bayesian analyses of the combined datasets yielded almost identical tree topologies. Paratypes 26–28 were not analysed molecularly, as no sub-samples were available.

Hanabira yukibana from Okinawa

Sequences of *Hanabira yukibana*, from Okinawa and Iriomote Islands grouped together in a well-supported clade (100%/1.00). The new species grouped basally to two members

of Clavulariidae (100%/1.00), the closest sister genera *Clavularia* and *Knopia* (fig. 4), supporting the morphological data that justify the description of a new genus and species.

When examining morphological and symbiont variability in *H. yukibana*, there are two groups with moderately high support values (60%/0.90 and 45%/0.99) within the main clade of its specimens (fig. 5), which could suggest multiple species or subspecies. The genetic distances (uncorrected *p*, expressed as percentage) between these groups and the main clade of *H. yukibana* specimens were 0.01% and 0.17% for COI and 0.00% and 0.00% for mtMutS (supplementary table S1). These values are well below average values typical of differences among congeneric octocoral species, but comparable to distances among conspecific octocoral species (McFadden et al., 2011).

This observation is supported by values of the genetic differences within all *Hanabira yukibana* specimens; 0.09% and 0.00% for COI and mtMutS, respectively (supplementary table S2), values below average within-species comparisons (McFadden et al., 2011). Thus, the major ramification and distances indicate a separation of this species from *Clavularia* at the generic level and neither support the distinction of multiple species nor subspecies.

Morphological characteristics support this separation at the generic level, as there are clear differences in sclerite features between *Knopia*, *Clavularia* and *Hanabira* (figs. 3, 5). The main difference is the contrast in sclerite types between the three genera. *Hanabira* specimens lack the spindle sclerite types seen in *Clavularia*, while in comparison with *Knopia* the *Hanabira* specimens have rodlets as an additional sclerite type. Hence, in concurrence with the molecular data, polyp morphology also does not support multiple species nor subspecies: the three groups of *H. yukibana* specimens seen in the phylogenetic

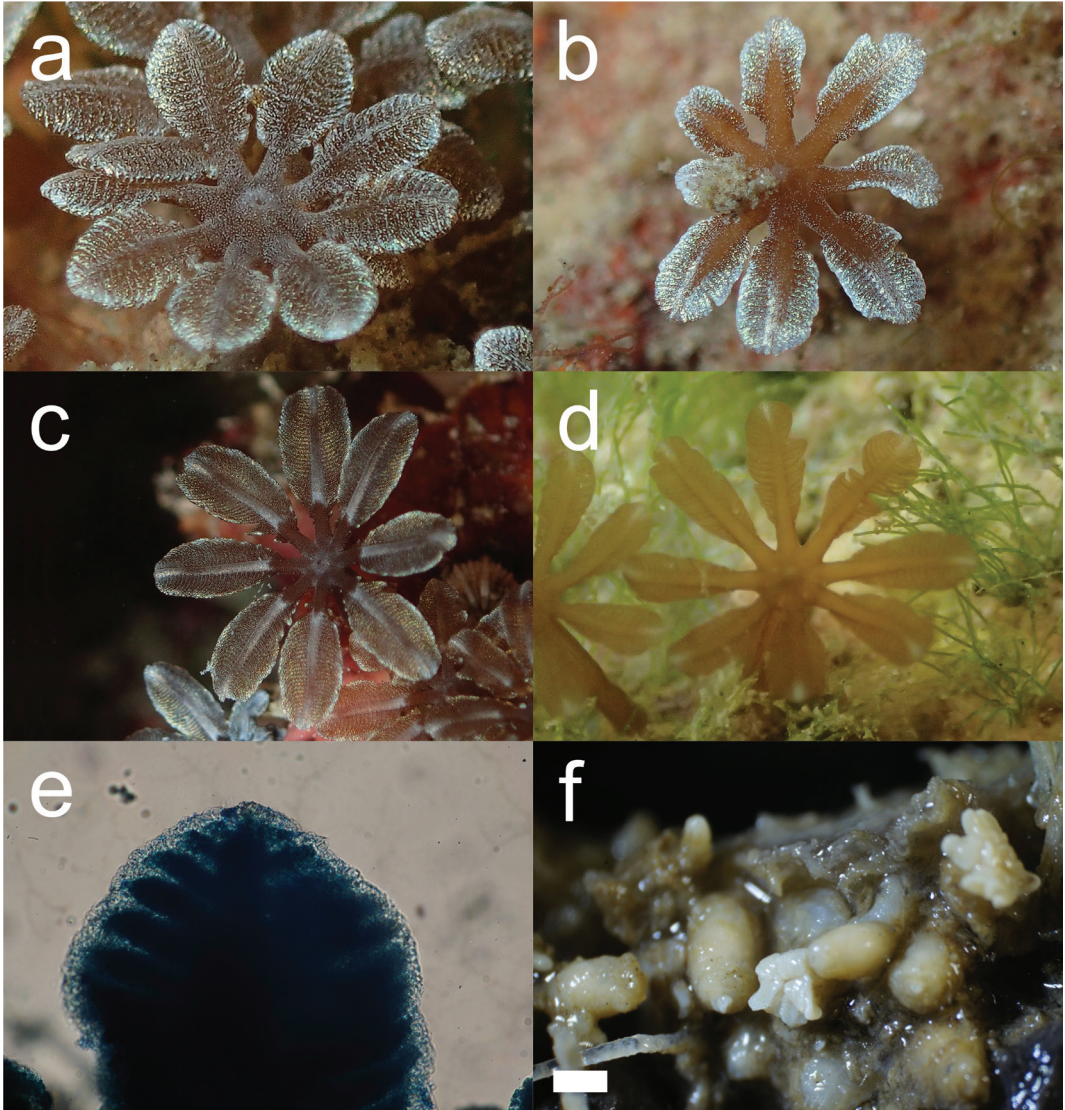


FIGURE 2 *In situ* photographs of the three main polyp variations seen in *Hanabira yukibana*, gen. nov., sp. nov.; variation A: a) NSMT Co 1626, holotype and b) NSMT Co 1625, paratype 1; variation B: c) NSMT Co 1637, paratype 12; variation C: d) NSMT Co 1633, paratype 8. Images of *Hanabira yukibana*, gen. nov., sp. nov., holotype, NSMT Co 1626: e) stained with methylene blue to accentuate pseudopinnules of the tentacles and f) colony preserved in ethanol. Scale bar: 1 mm.

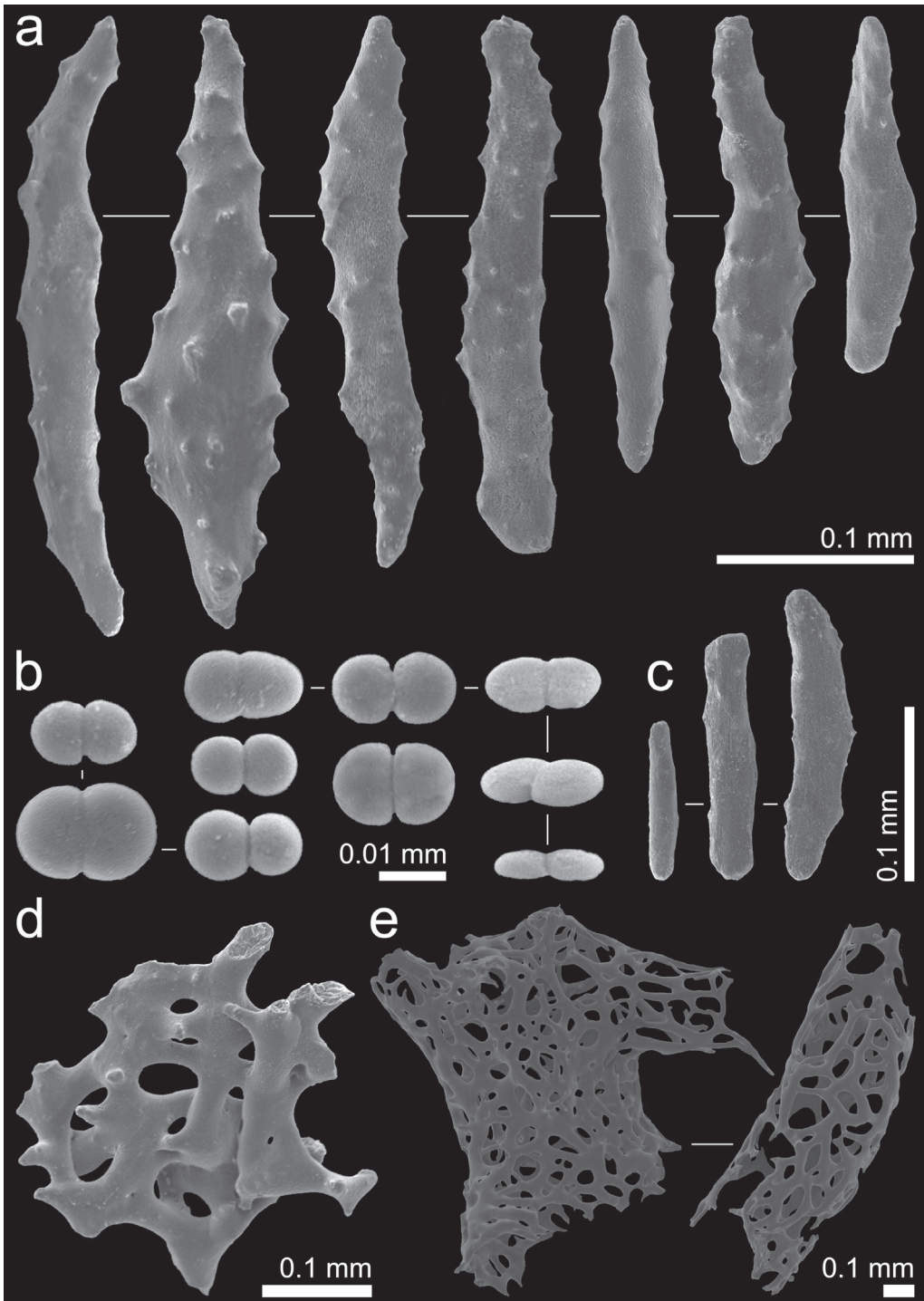


FIGURE 3 Sclerite types seen in *Hanabira yukibana*, gen. nov., sp. nov., NSMT Co 1626, holotype; a) spindles of calyx (scale bar: 0.1 mm), b) anthocodial platelets (scale bar: 0.01 mm), c) anthocodial rods (scale bar: 0.1 mm), d) fused clump of stolon (scale bar: 0.1 mm). NSMT Co 1637, paratype; e) fragments of stolon (scale bar: 0.1 mm).

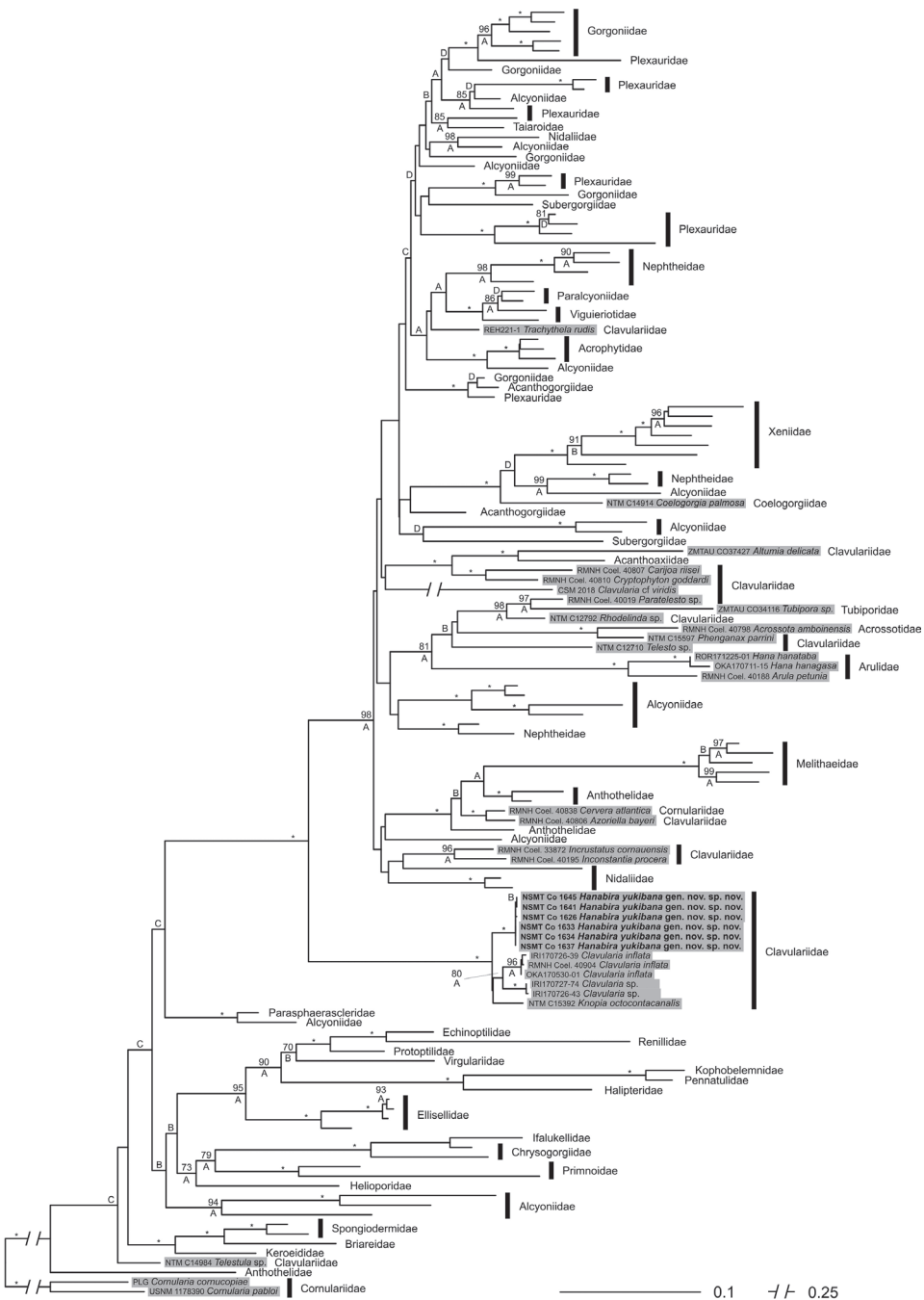


FIGURE 4 Phylogenetic relationships among 134 species of octocorals, including *Hanabira yukibana*, gen. nov., sp. nov., using the combined 28S rDNA+CO1+mtMutS dataset. The best maximum likelihood tree is shown, with values at branches representing bootstrap probabilities shown when >70%. Bayesian posterior probabilities are shown at branches when >0.80 (A = 1.00; B = 0.95–0.99; C = 0.90–0.94; D = 0.80–0.89). * represents 100/1.00 for both analyses. Stoloniferous species are highlighted in grey boxes and non-stoloniferous octocorals are shown with family classification only. *Comularia* spp. were used as outgroup.

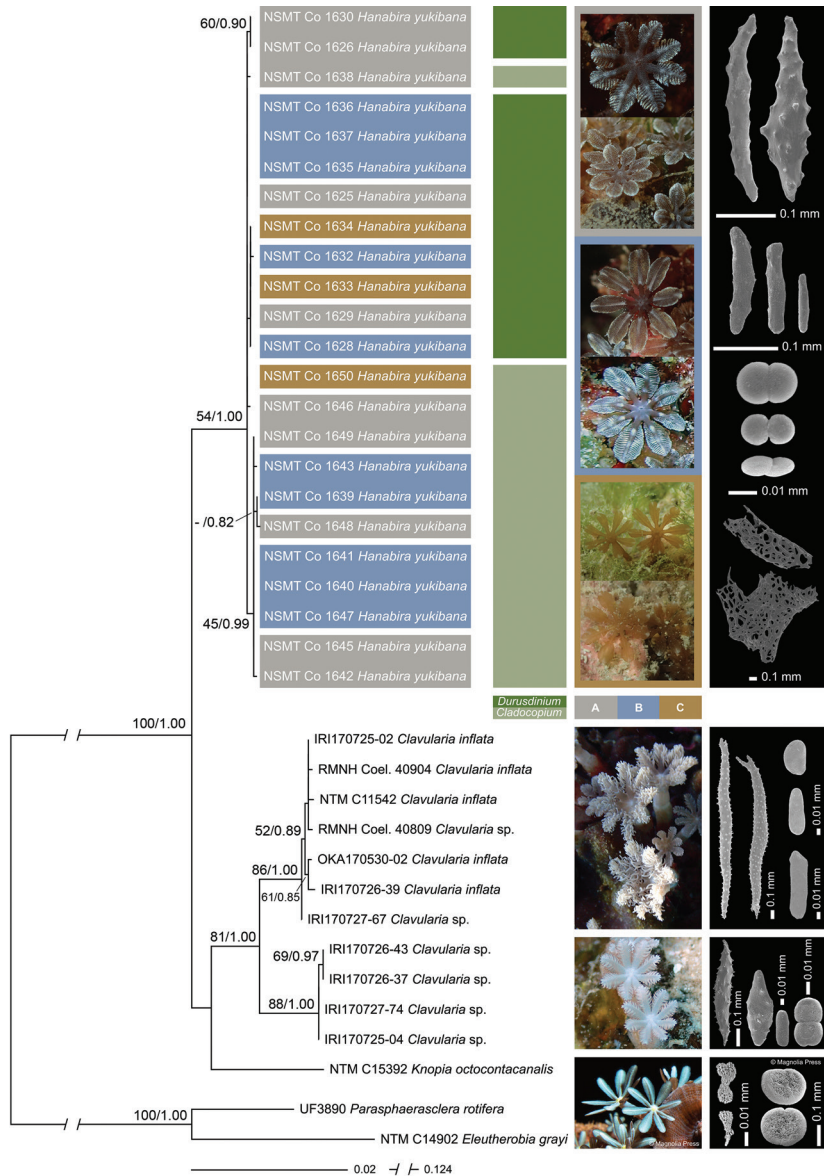


FIGURE 5 Combined 28S rDNA+COI+mtMutS+ND6 phylogenetic reconstruction for 35 clavulariid specimens, including *Hanabira yukibana*, gen nov., sp. nov., and *Clavularia* spp. from Okinawa (OKA) and Iriomote (IRI) Islands and sister taxa, *Knopia octocontacanalisis* Alderslade & Mcfadden, 2007. *Parasphaerasclera rotifera* and *Eleutherobia grayi* were used as outgroup. The best maximum likelihood tree is shown, with values at branches representing bootstrap probabilities (>50%) and posterior probabilities from the Bayesian inference (>0.50), respectively. Polyp variation for *H. yukibana* is illustrated for the three groups by colour and corresponding *in situ* photographs. Photograph credit: *in situ* image NTM C15392 *Knopia octocontacanalisis*, by Frances Dipper (modified from Alderslade & McFadden, 2007, reproduced with permission from copyright holder). Mutualistic Symbiodiniaceae (genera *Cladocopium* and *Durusdinium*) found in *H. yukibana* specimens are displayed in green shades and sclerite types unique to each genus are also shown. Photograph credit: sclerite images NTM C15392 *Knopia octocontacanalisis*, modified from Alderslade & McFadden, 2007, reproduced with permission from copyright holder.

tree did not coincide with the three main polyp morphologies (variation A–C) and the polyp morphologies were scattered randomly through all groups (fig. 5).

Hanabira yukibana-Symbiodiniaceae associations

Hanabira yukibana specimens were analysed for the presence of Symbiodiniaceae zooxanthellae to examine possible relationships with polyp morphology. With the exception of four specimens from Iriomote Island, ITS sequences of Symbiodiniaceae were obtained for all examined specimens; paratypes 3, 6, 8 and 11 were zooxanthellate, but no sequences were obtained. There was a clear biogeographical distinction in the presence of Symbiodiniaceae in the hosting octocorals. All *H. yukibana* specimens examined from Okinawa Island hosted genus *Cladocopium* ($n = 12$), while all specimens from Iriomote Island hosted *Durudinium* ($n = 8$). The Symbiodiniaceae genus present was unrelated to neither polyp morphology (fig. 5) nor depth.

Discussion

Analyses of separate gene regions (28S rDNA, COI and mtMutS) resulted in different positions of *Knopia* in the obtained phylograms. Nonetheless, *Hanabira* was consistently a strongly supported lineage in all the separate gene region analyses (supplementary figs. S1–S7). Concatenation of all four gene regions (28S rDNA, COI, mtMutS and ND6), which gives the highest resolution, demonstrated that *Knopia octocontacanalisis* and *Clavularia* spp. were positioned basally to *Hanabira yukibana* specimens, with *Knopia* being its closest sister taxon (figs. 4, 5). Exclusion of gene region ND6 did not result in any differences in branch topology. Therefore, we recommend utilising such four region analyses

for future studies of octocorals (Reijnen et al., 2014; Reijnen, 2016; Lau et al., 2018).

Sclerite morphology also demonstrated similarity to both genera *Clavularia* and *Knopia*. *Clavularia* has similar sclerite types: platelets and rods in the anthocodiae and spindles in the calyx. However, sclerites differ significantly in size of the different types; sclerites seen in *Clavularia* have a much larger size range. The platelets are almost twice as large, ~ 0.02 mm in *Hanabira* and from ~ 0.04 to 0.05 mm in *Clavularia*, the rodlets in *Hanabira* range from ~ 0.08 – 0.17 mm and 0.23 – 0.26 mm in *Clavularia* and spindles are ~ 0.16 – 0.32 mm in *Hanabira* and ~ 1 mm in *Clavularia* (Schenk, 1896; Fabricius & Alderslade, 2001).

In contrast, *Knopia* has only two sclerite types, platelets and small scale-like sclerites, which are scattered amongst the platelets, and neither type is seen in *Hanabira* or *Clavularia*. The sclerites seen in *Knopia* are of similar size as those seen in *Hanabira*; platelets are ~ 0.011 – 0.025 mm, but they have a more oval, circular or kidney-shaped outline when compared to platelets seen in *H. yukibana* (Alderslade & McFadden, 2007). No comparison can be made between the three genera concerning the sclerites in the stolon, as these have not been described for *Knopia* (Alderslade & McFadden, 2007), nonetheless, the fused clumps seen in *Hanabira yukibana* are similar to the fused clumps seen in *Clavularia*, and are also similar in size (both ~ 0.05 mm in width of the fused sclerites) (Fabricius & Alderslade, 2001).

Polyp morphology of *Hanabira* also shows resemblance to that of both *Knopia* and *Clavularia*, even though the polyps in *H. yukibana* are much smaller (diameter expanded polyps ~ 2 – 3 mm) than seen in both *Knopia* (tentacle length ~ 5 mm; Alderslade & McFadden, 2007) and *Clavularia* (tentacle length up to 20 mm; Fabricius & Alderslade, 2001). Overall shape of the polyps show affinity to *Clavularia*, although tentacle pinnules show a likeness with

Knopia; both *Knopia* and *Hanabira* have pseudopinnules (fig. 2e).

The molecular results (figs. 4, 5) show that the relationship between *Hanabira yukibana* and *Clavularia* spp. is consistent, however the location of the monotypic genus *Knopia* is not well-supported in either ML or Bayesian inferences; 42/0.59, respectively, for the 3-marker dataset (28S rDNA+COI+mtMutS) and 48/ - for the 4-marker dataset (28S rDNA+COI+mtMutS+ND6). Both in colony form and sclerites, *Knopia* is different from the genera *Hanabira* and *Clavularia*, but as a result of the unresolved phylogenetic location of *Knopia*, it remains unclear how *Hanabira* and *Clavularia* are related to *Knopia*.

It is noteworthy that there is a well-supported division within *Clavularia* spp. (fig. 5), in which one lineage of clavulariids (88%/1.00) branches off from *Clavularia inflata* (86%/1.00). Therefore, these specimens were investigated morphologically (supplementary figs. S10-S11). The SEM images of the sclerites of this group appear to be different from the sclerites described for *Clavularia inflata* Schenk, 1896. This difference is also supported by values of genetic distances between this group and *Clavularia inflata* specimens, which are above the average of conspecific octocorals (supplementary table S3). Therefore, we suggest that these *Clavularia* specimens may represent an undescribed species, here designated as *Clavularia* sp. It is clear that *Clavularia* is in need of a revision that investigates this genus both morphologically and molecularly.

Phenotypic variation is not uncommon in octocorals. Morphological variation in octocorals has been investigated in Caribbean gorgonians, specifically with regard to the branching structure and sclerite morphology (Brazeau & Lasker, 1988; Sánchez et al., 2007; Prada et al., 2008; Sánchez, 2016), and also polyp characters, e.g., for xeniids, differing in pinnules and rows of pinnules (McFadden

et al., 2017). However, phenotypic variation in polyp morphology in octocorals is yet to be thoroughly examined for most groups, and morphological variation has not been studied sufficiently in other related genera to determine if the degree of variation in polyp morphology observed in *Hanabira* is of outstanding significance or not. In this study the morphological variations seen in the polyps of *H. yukibana* could not be explained by any clear pattern of their algal symbionts. Previously, comparisons of Symbiodiniaceae within various cnidarian species have been used to clarify intra-specific physiological (Goulet et al., 2005, 2008) or distributional differences (Kamezaki et al., 2013). Earlier studies have examined biogeographic patterns for octocoral-zooxanthellae associations and these studies generally have comprised large datasets with wide taxonomic coverage spanning wide geographical regions (e.g., the Great Barrier Reef (GBR), the far eastern Pacific and the Caribbean). These studies have demonstrated that *Clavularia* spp. in the Caribbean, Torres Strait, and central GBR host *Durusdinium* (Goulet et al., 2004; Oppen et al., 2005; Goulet et al., 2008), and these are the only data available on stoloniferan octocoral-Symbiodiniaceae relationships.

The current work represents the first in depth small-scale examination of stoloniferan octocoral and Symbiodiniaceae relationships for Okinawan waters. *Hanabira yukibana* specimens were found on both the east and west coasts of Okinawa Island, but were only found at two out of seven locations around Iriomote Island. There were no outstanding noteworthy conditions at these two locations, Takosaki and Iriomote N, which could explain the occurrence of *H. yukibana*. *Durusdinium* was found in all *H. yukibana* specimens collected at Iriomote Island. *Durusdinium* includes species that are known extremophiles, adapted to survive in regions with large fluctuations in temperature, and many *Durusdinium* spp.

tend to be resistant to bleaching (LaJeunesse et al., 2018). Iriomote Island has a recent history of extreme bleaching events; from the 1980s to 2001, there were five bleaching events recorded (Research Institute for Subtropics, 1999; Ministry of the Environment and the Japanese Coral Reef Society, 2004), with more events in 2016–2017 (Kayanne et al., 2017). On the other hand, members of *Cladocopium* are more adapted to a comparatively wider range of temperatures and irradiances, similar to what would be observed in more northern Okinawan waters. Thus, the observed *Hanabira yukibana*-Symbiodiniaceae relationship patterns at both islands fit well with this generalization. It is recommended for future octocoral research to continue investigating Symbiodiniaceae associations with the prospect of understanding octocoral-algal symbioses as global climate change will continue to severely decimate coral reefs. To fully understand the current *Hanabira*-Symbiodiniaceae relationship, it would be worthwhile to investigate the occurrence of *Hanabira yukibana* around Miyako Island, situated southwest of Okinawa Island and northeast of Iriomote Island.

Understanding octocoral-Symbiodiniaceae relationships is becoming increasingly important, as octocorals constitute a large portion of the biodiversity in coral reef ecosystems, and are commonly the dominant benthos (Daly et al., 2007; Parrin et al., 2016). Additionally, previous research has shown that octocorals are able to mitigate bleaching through intra-colony migration of symbionts deeper into the colony (Parrin et al., 2016; Parrin, 2016) or into the stolons, as seen in the stoloniferan *Phenganax parrini* (Netherton et al., 2014), suggesting some capability to resist or even survive bleaching at least in some species (Dias & Gondim, 2015).

Finally, this study is an addition to the existing literature on the character of pseudopinnules in stoloniferan octocoral polyps. As

discussed by Alderslade & McFadden (2007), the possession of pinnated tentacles is one of the major diagnostic features of the subclass Octocorallia, and thus the true diagnostic utility of this feature needs further examination. In conclusion, the molecular, morphological and ecological information of this study supplements the few studies that emphasize the need for a thorough investigation of the polyphyletic family Clavulariidae.

Acknowledgements

We would like to thank Dr. T. Naruse (Iriomote Field Station, University of the Ryukyus) for help in the field and logistics at Iriomote Island. We thank Dr. S. Kunihiro for providing specimen NSMT-Co 1631 from Takosaki, Iriomote Island. In Okinawa we thank MISE members for their help in fieldwork, Y. Kushida for providing a specimen and image NSMT-Co 1650 from Nakagusuku Bay, Okinawa Island. We would like to thank Dr. M. Nonaka and Ms. H. Yamamoto (Research Center, Okinawa Churashima Foundation) for help in the field at Motobu Town, and Dr. M. Obuchi (Endo Shell Museum, Manazuru) and Dr. T. Fujii (Kagoshima University) for providing specimen NSMT-Co 1651 from Oura Bay, Nago City. We thank Prof. J. Tanaka for contributing photographic records. Prof. E. Hirose (University of the Ryukyus) is thanked for providing access to a scanning electron microscope (SEM) used in this work. P. Biondi and G. Masucci (University of the Ryukyus) are thanked for their help in logistics. B.J. van Heuven and Dr. B.T. Reijnen (Naturalis Biodiversity Center) are thanked for their help with SEM facilities. Dr. B.T. Reijnen is also thanked for the use of the modified ND6 primer set. Finally, we would like to thank Prof. C.S. McFadden (Harvey Mudd College) and Dr. L.P. van Ofwegen (Naturalis Biodiversity Center) for their inspirational drive, ever-insightful

help, and expert octocoral advice. Finally, we thank the reviewers for their thorough and helpful comments that have contributed to a better manuscript. This work was partly funded by Okinawa Churashima Foundation (Y. Imahara). The first author was supported by a MEXT, Japan Student Services Organization (JASSO) scholarship. All material included in this manuscript was collected under approved collection permits and ethics guidelines.

Supplementary material

Supplementary material is available online at: <https://brill.figshare.com/s/cf7fd24d89132c80288e>

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RECEIVED: 2 NOVEMBER 2018 | REVISED AND

ACCEPTED: 21 FEBRUARY 2019

EDITOR: B.W. HOEKSEMA

APPENDIX

Systematic account

Class Anthozoa Ehrenberg, 1831

Subclass Octocorallia Haeckel, 1866

Order Alcyonacea Lamouroux, 1812

Family Clavulariidae Hickson, 1894

Genus *Hanabira*, gen. nov.

Clavulariinae gen. Okinawa Churashima Foundation and Biological Institute on Kuroshio, 2016.

Type species. Hanabira yukibana, sp. nov., by original designation and monotypy.

Diagnosis. Colony with anthocodiae that retract into cylindrical to barrel-shaped calyces, which do not retract into the stolons. Tentacles with pinnules arranged adjacent to one another along either side of the tentacle rachis and are fused together (pseudopinnules). Sclerites of anthocodiae are platelets with a distinct median waist and small smooth rods, with the tentacles having only the platelet type. Sclerites of calyces are small spindles, which are larger than the anthocodial rods and are prickly and warty. Sclerites of stolon are a tubular network of fused sclerites. Zooxanthellate.

Etymology. From the Japanese language 'hanabira' (花弁), meaning 'petal'. After the flower petal-like shape of the polyp tentacles. Gender: feminine.

Remarks. The main morphological differences with closest sister taxa, genera *Clavularia* and *Knopia*: the tentacle rachis of *Clavularia* contains smooth to warty rods and long, narrow spindles (~1.0 mm), which are prickly or complexly warted, and sometimes branched. The calyces of genus *Clavularia* contain similar spindles, but can be twice as long (Fabricius & Alderslade, 2001). The anthocodiae of *Hanabira* do not contain warty rods and large, prickly or warted spindles are completely lacking.

Hanabira yukibana, sp. nov.

Figs. 2, 3

Clavulariidae gen. sp. Imahara et al., 2017.

Material examined. Holotype: NSMT-Co 1626, Takosaki, Iriomote Island (24.327010, 123.738290), 11 m depth, coll. YWL, 25 July 2017.

Paratypes from Takosaki, Taketomi Town, Iriomote Island (24.327010, 123.738290): Paratype 1: NSMT-Co 1625, 11 m depth, coll. YWL, 25 July 2017. Paratype 2: NSMT-Co 1627, 11 m depth, coll. YWL, 25 July 2017. Paratype 3: NSMT-Co 1628, 11 m depth, coll. YWL, 25 July 2017. Paratype 4: NSMT-Co 1629, 10 m depth, coll. YWL, 25 July 2017. Paratype 5: NSMT-Co 1630, 10 m depth, coll. YWL, 25 July 2017. Paratype 6: NSMT-Co 1631, 12.7 m depth, coll. S Kunihiro, 25 July 2017. Paratype 7: NSMT-Co 1632, 14 m depth, coll. YWL, 25 July 2017. Paratype 8: NSMT-Co 1633, 12 m depth, coll. YWL, 25 July 2017. Paratype 9: NSMT-Co 1634, 13 m depth, coll. YWL, 25 July 2017. Paratype 10: NSMT-Co 1635, 11 m depth, coll. YWL, 25 July 2017. Paratype 11: NSMT-Co 1636, 13 m depth, coll. YWL, 25 July 2017.

Paratype 12: NSMT-Co 1637, Iriomote N, Taketomi Town, Iriomote Island (24.407805, 123.854199), 31 m depth, coll. YWL, 25 July 2017.

Paratypes from Okinawa Island: Paratype 13: NSMT-Co 1638, Abu, Oura Bay, Nago City (26.537756, 128.079521), 14 m depth, coll. YWL, 04 Jun 2017. Paratype 14: NSMT-Co 1639, Hoshu, Manza, Onna Village (26.504659, 127.846604), 13 m depth, coll. YWL, 24 Jun 2017. Paratype 15: NSMT-Co 1640, Hoshu, Manza, Onna Village (26.504659, 127.846604), 14 m depth, coll. YWL, 24 Jun 2017. Paratype 16: NSMT-Co 1641, Zanpa, Cape Zanpa, Yomitan Village (26.441517, 127.711417), 21 m depth, coll. YWL, 24 Jun 2017. Paratype 17: NSMT-Co 1642, Hedo Dome, Cape Hedo, Kunigami Village (26.852091, 128.250450), 34 m depth, coll. YWL, 11 Jul 2017. Paratype 18: NSMT-Co 1643, Manza Beach, Manza, Onna Village (26.502957, 127.841909), 17 m depth, coll. YWL, 16 Jul 2017. Paratype 19: NSMT-Co 1644, Manza Beach, Manza, Onna Village (26.502957,

127.841909), 17 m depth, coll. YWL, 16 Jul 2017. Paratype 20: NSMT-Co 1645, Manza Rock, Manza, Onna Village (26.504522, 127.843748), 16 m depth, coll. YWL, 12 Sep 2017. Paratype 21: NSMT-Co 1646, Manza Rock, Manza, Onna Village (26.504522, 127.843748), 11 m depth, coll. YWL, 12 Sep 2017. Paratype 22: NSMT-Co 1647, Hoshu, Manza, Onna Village (26.504659, 127.846604), 33 m depth, coll. JDR, 13 Nov 2017. Paratype 23: NSMT-Co 1648, Crossline, Seragaki, Onna Village (26.508734, 127.881453), 32 m depth, coll. JDR, 14 Nov 2017. Paratype 24: NSMT-Co 1649, Mini Dream Hole, Manza, Onna Village (26.509833, 127.854006), 29 m depth, coll. JDR, 14 Nov 2017. Paratype 25: NSMT-Co 1650, Nakagusuku Bay, Nakagusuku Town (26.2627778, 127.825278), 17 m depth, coll. Y Kushida, 04 Aug 2018. Paratype 26: NSMT-Co 1651, Oura SW, near Cape Henoko, Futami, Oura Bay, Nago City (26.529373, 128.047446), ca. 10m depth, coll. M Obuchi & T Fujii, 4 Mar 2011. Paratype 27: NSMT-Co 1652, Motobu, off Yamakawa, Motobu Town (26.679389, 127.879222), 15 m depth, coll. Y Imahara, 27 Oct 2011. Paratype 28: NSMT-Co 1653, Motobu, off Yamakawa, Motobu Town (26.679389, 127.879222), 15 m depth, coll. Y Imahara, 27 Oct 2011.

Description. The holotype colony is attached to a sponge, which was fragmented into three pieces during sampling, although, this species is not always an epibiont and can be attached to other hard substrates. Small groups of polyps are attached by stolons to the sponge tissue (total ~10 polyps). Polyps are approximately 2–3 mm in diameter expanded and are spaced apart irregularly (from ~0.5 mm up to ~2 cm) and connected through flat and ribbon-like stolons that are 1 mm at the widest and 0.2 mm at the narrowest point. Anthocodiae can retract fully into cylindrical to barrel-shaped calyces, which are 1.5–2.5 mm in height and up to 1 mm in width; calyces do not retract into the stolon. Tentacles have pseudopinnules arranged adjacent to one another along either side of the tentacle rachis (~18 pseudo-pairs); when stained with methylene

blue, the outline of the tentacles can be observed. A structure that seems to be the pinnule axis can be seen, although notches, which can distinguish the adjacent pinnules, are not observed in the contour of the tentacle (fig. 2e). In life the tentacles are elliptical to petal-shaped and the polyps have a pale, green-gold and white sheen. This could be caused by refraction on the minute sclerites, as described in Alderslade & McFadden (2007) (fig. 2a). Sclerites of anthocodiae are platelets with a distinct median waist (0.01–0.02 mm) and small smooth rods (0.1–0.2 mm), with the tentacles only having the type of platelets as seen in the anthocodiae (fig. 3b, c). Sclerites of calyces are rods, which are larger than the anthocodial rods, that are prickly and warty (0.2–0.3 mm) (fig. 3a). Sclerites of the stolon are a tubular network of fused sclerites (fig. 3d, e). The polyps are zooxanthellate.

Morphological variation. Polyp density in *H. yukibana* is variable, as is also the broadness of the stolons (0.1–1.1 mm). Tentacles are not all elliptical in life; some specimens have a more pointed shape at the distal part of the tentacle. The number of pseudopinnules ‘paired’ alongside the tentacle rachis varies within a range of 15–31 ‘pairs’. The colour of the polyps is also very variable; in exception of paratypes 15, 16 and 25, all specimens have a clear recognisable sheen. The sheen is thought to be caused by refraction from the platelet sclerites in the tentacles (Alderslade & McFadden, 2007); usually green-gold and/or white. Paratypes 15, 16 and 25 do not lack the minute platelet sclerites but instead have a lower platelet density in the tentacles and only have this typical sheen (white) at the far distal part of their tentacles. Live specimens were overall brown in colour.

Etymology. From the Japanese language ‘yukibana’ (雪花), meaning ‘snow flower’; denoting the resemblance of the sheen of the polyps (including tentacles) to the shimmer of snowflakes or snow crystals.

Habitat. Colonies encrust hard substrates with their stolons. Common substrates are rock, sponges, coral rubble and shells.

Distribution. Southwestern Japan, southern Ryukyu Islands, around Okinawa Island and the

north and west coasts of Iriomote Island in the East China Sea. Specimens were collected from depths of 10–35 m.