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Redescription of *Synactinernus flavus* for the First Time After a Century and Description of *Synactinernus churaumi* sp. nov. (Cnidaria: Anthozoa: Actiniaria)

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Two species of *Synactinernus* sea anemones were found in Japanese waters. *Synactinernus flavus* Carlgren, 1918, the only described species of this genus, is rediscovered from off the Goto Islands a century after the original description. *Synactinernus flavus* was once synonymized with *Isactinernus quadrilobatus* Carlgren, 1918; however, we show that, based on morphological (including examination of type specimens) and molecular (using nuclear 18S rDNA) evidence, these species are completely different. The other species, *Synactinernus churaumi* sp. nov., was found off Ishigaki Island and Okinawa Island by a remotely operated vehicle (ROV), and had been kept for 15 years in a tank at the Okinawa Churaumi Aquarium. There are clear differences between these two species; therefore, we describe the second species and revise the diagnosis of *Synactinernus*.

Key words: Endocoelanthae, Anenthemonae, Actinernidae, mesogleal thickening, mesenterial arrangement, fertile mesentery, Goto Islands, Okinawa Churaumi Aquarium

INTRODUCTION

The family Actinernidae Stephenson, 1922 comprises four genera and seven species (Fautin, 2013; Daly and Fautin, 2018). This family is characterized by the following three characteristics: more than 20 mesenteries; mesenteries of the second and younger cycles born in the endocoels of the first cycle; and actinopharynx with siphonoglyphs both on dorsal and ventral sides (Stephenson, 1922; Carlgren, 1949; Uchida, 2007). The peculiar mesenterial arrangement shared by Actinernidae and Halcuriidae Carlgren, 1918 in the suborder Endocoelantheae Carlgren, 1925 is traditionally regarded as the most important character for the classification of Actiniaria; for this reason, Carlgren (1925) established the suborder to accommodate these two families, and distinguished them from all other actinarians. Although this classification by Carlgren (1925) was followed until quite recently, Rodríguez et al. (2014) revealed that Endocoelantheae actiniarians are closely related to the family Edwardsiidae Andres, 1881 of the suborder Anenthemonae Rodriguez and Daly, 2014, by molecular phylogeny, and revised the suborders of Actiniaria. Rodríguez et al. (2014) concluded that Endocoelantheae should be a part of the larger suborder Anenthemonae, but maintained the taxon combining Actinernidae and Halcuriidae by reviving the superfamily Actinernoidea Stephenson, 1922.

The genus *Synactinernus* was described in Carlgren (1918) monotypically with a single species, *Synactinernus flavus* Carlgren, 1918, collected from Japan. However, there have been no newly collected specimens of this genus for approximately a century (Fautin, 2013); hence, the study of *Synactinernus* has not advanced. Meanwhile, Fautin and den Hartog (2003) stated that *S. flavus* is a junior synonym of *Isactinernus quadrilobatus* Carlgren, 1918. However, the genus *Synactinernus* remains valid to this day (Fautin et al., 2007; Fautin, 2016). This contradiction results from the difficulty to grasp the real nature of *S. flavus*; thus, new specimens are needed to resolve this problem. Yanagi (2006) and Uchida (2007) recognized *S. flavus* as a junior synonym of *I. quadrilobatus* following Fautin and den Hartog (2003), and considered *Actinernus robustus* (Hertwig, 1882),

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Isactinernus quadrilobatus, and *Synhalcurias elegans* (Wassilieff, 1908) as the only three currently valid species of Actinernidae from Japanese waters.

During the 21st Century, several specimens identified as *S. flavus* were collected from Goto-nada Sea, near the type locality. By analyzing these newly collected specimens, we discovered several morphological differences from *I. quadrilobatus* and realized that *S. flavus* cannot be accommodated in *Isactinernus* or any other genera of Actinernidae. Molecular phylogenetic analyses suggest that *Synactinernus* is less closely related to *Isactinernus* than the other genera in Actinernidae. Moreover, we also found other specimens of undescribed species of *Synactinernus* from off Okinawa Island. Some new characteristics of this new species suggest that the diagnosis of the genus *Synactinernus* is in need of revision. In addition, we observed some ecological features of *Synactinernus* anemones: transverse fission of *S. flavus* for the first time in Actinernoidea sea anemones and aggregation of *S. churaumi* sp. nov., the first observation of the ecology of Actinernidae in nature.

MATERIALS AND METHODS

Sample collection and preservation

Six specimens of *Synactinernus* were collected in this study: three each of *Synactinernus flavus* and *Synactinernus churaumi* sp. nov. Of the three specimens of *S. flavus*, two were collected from south off the Goto Islands, Nagasaki Prefecture, using a biological dredge during a cruise of R/V *Nagasaki-Maru*, and the other was from Uragami, Wakayama Prefecture, by a net for coral fishing (Fig. 1). Of the three specimens of *Synactinernus churaumi* sp. nov., two were collected from the sea northwest of Ishigaki Island and the other was from the sea east of Okinawa Island, Okinawa Prefecture (Fig. 1); all were collected by a remotely operated vehicle LEO (Kowa Corporation), operated from Dai-2 *Kuroshio-Maru*. All but the one from Uragami were kept alive in aquarium tanks at Okinawa Churaumi Aquarium (Okinawa, Japan) for several years before fixation. Prior to fixation, *in vitro* images of living polyps were taken to record external form of the oral disc, as well as the color and size of the polyps. From three of the five specimens—one of *S. flavus* from the Goto-nada Sea (NSMT-Co 1660) and two *S. churaumi*, one from off Ishigaki Island (NSMT-Co 1661) and one from off Okinawa Island (CMNH-ZG 09733)—tentacle tissues were dissected and preserved in 99% ethanol for DNA extraction; the remaining whole polyps were fixed in 10–20% (v/v) formalin sea water solution after approximately half a day of anesthe-

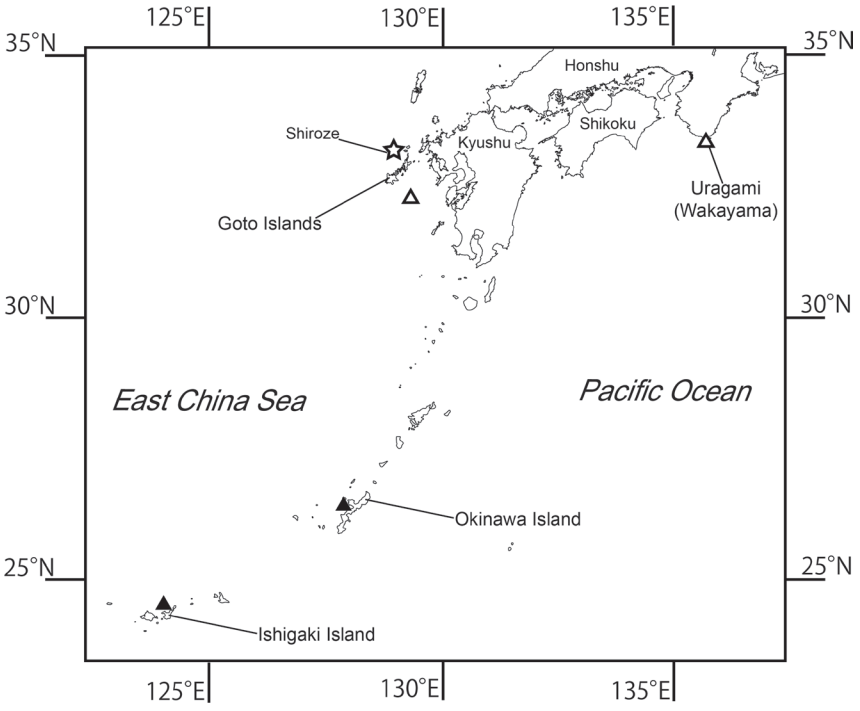

Fig. 1. Localities of *Synactinernus*. White triangles indicate the localities of *S. flavus*, and black ones indicate those of *S. churaumi* sp. nov. The white star shows the type locality of *S. flavus* (UUZM 232).

Table 1. Specimens used in the molecular analyses. GenBank accession numbers of newly obtained sequences of 18S and 16S are shown in bold.

Family	Species	Locality	Voucher	Accession number	
				18S	16S
Actinernidae	*Synactinernus flavus*	off the Goto Islands, Nagasaki Pref.	NSMT	**LC484633**	**LC484639**
	Synactinernus flavus	off the Goto Islands, Nagasaki Pref.	N/A	**LC484634**	**LC484640**
	Synactinernus flavus	Uragami, Wakayama Pref. 100–130 m depth	CMNH	**LC484635**	–
	Synactinernus churaumi sp. nov.	Northwest off Ishigaki Island, Okinawa Pref.	NSMT	**LC484636**	**LC484641**
	Synactinernus churaumi sp. nov.	Northwest off Ishigaki Island, Okinawa Pref.	N/A	**LC484637**	**LC484642**
	"*Isactinernus quadrilobatus*"	Papua New Guinea	AMNH	KJ483024	(KJ482968)
	Isactinernus quadrilobatus	off Hamajima, the sea of Kumano, 350 m depth	NSMT	**LC484638**	**LC484643**
	Actinernus robustus	In Ryukyu Trough, 1500 m depth	N/A	**LC484632**	–
	Actinernus elongatus	Antarctica	AMNH	KJ483023	(KJ482966)
	Synhalcurius elegans	Seto, Japan	N/A	KJ483021	–
Halcuriidae	*Halcurius pilatus*	Chile	AMNH	KJ483020	(KJ482967)
Edwardsiidae (outgroup)	*Nematostella vectensis*	(For outgroup)	KUNHM	AF254382	(AY169370)

tization using magnesium chloride solution. For another two specimens—one each of *S. flavus* (the other one from the Goto Islands) and *S. churaumi* (the other one from Ishigaki Island)—only tentacle tissues were dissected for molecular analyses (Table 1), with the rest of the bodies still kept alive in a tank, along with additional other specimens, at the Okinawa Churaumi Aquarium for exhibition. The other specimen of *S. flavus* from Uragami had been kept alive at the Kushimoto Marine Park (Wakayama, Japan), and processed using the same method as described above.

For morphological comparison and molecular analyses with *Synactinernus* species, we also examined specimens of *Isactinernus quadrilobatus* and *Actinernus robustus*: *I. quadrilobatus* (NSMT-Co 1662), collected on 8 March 2016, from the Kumano-nada Sea off Hamajima Island, Mie Prefecture, around a depth of 350 m, by the fishing boat *Kiei-Maru*; *I. quadrilobatus* (CMNH-ZG 09734), collected on 18 April 2018, from the East China Sea off Koshikijima Island, Kagoshima Prefecture, around a depth of 380 m, by the fishing boat *Koei-Maru*; and *A. robustus* (CMNH-ZG 09735), collected on 28 April 2002, from Okinawa trough off Kumejima Island, Okinawa Prefecture (27°02.89′N, 126°59.09′E), around a depth of 1550 m, using an ORE beam trawl during a cruise of R/V *Tansei-Maru*. The specimens were preserved by the same method described earlier in this section. The specimens examined have been deposited at either the National Museum of Nature and Science, Tokyo (NSMT) or the Coastal Branch of Natural History Museum and Institute, Chiba (CMNH).

Examination of type specimens

Observation of type specimens were performed at the Museum of Zoology, Lund University (MZLU) and the Museum of Evolution Zoology, Uppsala University (UUZM) in September–October 2014, the Zoological Museum, University of Copenhagen (ZMUC) in October 2013, and the Natural History Museum, London (BM) in March 2016. We observed the specimens of *I. quadrilobatus* (LO L14/3033: MZLU, UUZM 102a: UUZM, ANT-000087: ZMUC), *S. flavus* (UUZM 232: UUZM), and *A. robustus* (BM 89-11-25-30: BM). The type specimens were photographed and their external and gross internal morphological characteristics were examined.

Preparation of histological sections

Histological sections were prepared following standard protocols. The specimens of *Synactinernus flavus* (NSMT-Co 1660) and *S. churaumi* sp. nov. (NSMT-Co 1661) were dissected to obtain some tissues. The tissues were dehydrated by ethanol and cleared in xylene, embedded in paraffin, sliced into serial sections (7–10 μm thick) using a microtome, mounted on glass slides, and stained with hematoxylin and eosin (Presnell and Schreibman, 1997). Because *S. churaumi* was too large to be mounted on slide glasses, the specimen was cut into blocks including some mesenteries before embedding. For identification and comparison, the specimens of *A. robustus* and *I. quadrilobatus* were processed in the same way as *Synactinernus* specimens.

Cnidae observation

Cnidae were observed in the tentacle, actinopharynx, column, and filament. Tissue from each organ was placed on slide glasses and mounted using 50% (v/v) glycerin seawater solution. Images of the cnidae were obtained by differential interference contrast microscopy (Yanagi et al., 2015). The length and width were measured using the software ImageJ ver. 1.49 (Rasband, 1997–2012). Cnidae nomenclature followed Mariscal (1974).

Phylogenetic analyses

DNA was extracted from the tissues of three specimens of *S. flavus* (NSMT-Co 1660, CMNH-ZG 09732, and the specimen preserved only tentacle tissue) and *S. churaumi* sp. nov. (NSMT-Co 1661, and the specimen only tissue) preserved in 99% EtOH following a guanidine extraction protocol (Sinniger et al., 2010) or by ChargeSwitch gDNA Micro Tissue Kit (Invitrogen). PCR amplifications were performed for mitochondrial 16S rDNA primers, 16Sant0a and 16SbmoH (Sinniger et al., 2005), and nuclear 18S rDNA primers, 18SA and 18SB (Medlin et al., 1988). The PCR reaction was performed in a 10-μL reaction volume, consisting of 0.4 μL of forward and reverse primers (25 μM), 2.0 μL of EmeraldAmp PCR Master Mix (TaKaRa), and 3.4 μL of distilled water. The amplification conditions followed Sinniger et al. (2005) for 16S rDNA and Medlin et al. (1988) for 18S rDNA. The PCR products were processed with Exonuclease I and shrimp alkaline phosphate (Exo-SAP) prior to sequencing. Sequencing reaction was performed using PCR primers (16S) or PCR primers and internal primers (18S). We used four primers (Apakupakul et al., 1999); two forwards, 18SC and 18SO and two reverses, 18SL and 18SY, and BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1 (Applied Biosystems). Sequencing was performed by ABI 3130xL or 3500xL Genetic Analyzer (Applied Biosystems). The two sequences of 16S and six sequences of 18S were individually assembled by GeneStudio ver. 2.2.0.0 (http://genestudio.com). The new sequences obtained in this study have been deposited in GenBank (Table 1).

For phylogenetic analyses, sequence data of four species of Actinernoidea (Actinernidae and Halcuriidae) and *Nematostella vectensis* Stephenson, 1935 (belonging to Edwardsiidae in Edwardsioidea) as an outgroup, were obtained from GenBank (Table 1). The data set was aligned by MAFFT ver. 7.402 (Katoh and Standley, 2013) under the default settings. Ambiguously aligned regions were eliminated by Gblocks ver. 0.91b (Castresana, 2002): type of sequences was DNA; the parameter was default except allowing small final blocks and gap positions within the final blocks. Next, the file was processed by Kakusan 4 (Tanabe, 2011) to test its substitution models for analyses of both RAxML and MrBayes (the alignment is available from the corresponding author upon request). Maximum-likelihood (ML) analyses were performed by RAxML-VI-HPC (Stamatakis, 2006), with the GTR+Γ evolutionary model recommended by Kakusan 4 and evaluated by 100 bootstrap replicates. Bayesian inference (BI) was conducted by using MrBayes ver. 3.2.6 (Ronquist and Huelsenbeck, 2003) with HKY85_Gamma as the substitution parameter. Two independent runs of the Markov Chain Monte Carlo were carried out simultaneously for 3,000,000 generations, sampling trees every 100 generations and calculating average standard deviation of split frequencies (ASDSFs) every 100,000 generations. As ASDSF was calculated based on the last 75% of the samples, the initial 25% of the sampled trees were discarded as burn-in.

Finally, two resultant trees were combined by FigTree ver. 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) and low bootstrap (<50) and posterior probability values (<0.90) were manually deleted on each node.

RESULTS

Description

Order **ACTINIARIA** Hertwig, 1882
Suborder **ANENTHEMONAE** Rodríguez and Daly, 2014
Superfamily **Actinernoidea** Stephenson, 1922
Family **Actinernidae** Stephenson, 1921
Genus ***Synactinernus*** Carlgren, 1918
(New Japanese name: Kuroba-kawari-ginchaku-zoku)

Diagnosis (revised from Carlgren, 1918; revised parts are shown in *italics*). Actinernidae with cylindrical body, which is distally drawn out into eight distinct lobes, *all eight of same size or* four larger and four smaller alternating. Column without papillae. No sphincter. Tentacles in at least two

cycles without distinct mesogloeal thickenings, *all same length or* largest at apices of the lobes, numerous. Longitudinal muscles of tentacles ectodermal, radial muscles of oral disc chiefly ectodermal, strong. Two broad siphonoglyphs *on actinopharynx. 36 macrocnemes* cyclic in arrangement, beyond them weak mesenteries of unequal size in upper part of the body. Retractors weak, *parietal* muscles *weak or* rather well developed.

Type species. *Synactinernus flavus* Carlgren, 1918.

Remarks. The genus *Synactinernus* was established in the family Actinernidae by Carlgren (1918) monotypically for *Synactinernus flavus* Carlgren, 1918. Recently, Fautin and den Hartog (2003) argued that *Synactinernus flavus*, the only species of *Synactinernus,* cannot be distinguished from *Isactinernus quadrilobatus* and synonymized *Synactinernus* with *Isactinernus.* However, *Synactinernus* was listed as a valid genus in later studies (Fautin, 2013, 2016). Therefore, the actual status of *S. flavus* and the validity of *Synactinernus* remained unclear. Additional specimens are needed to resolve this taxonomical problem, but no new specimen of *Synactinernus* had been found for a century after the original description of Carlgren (1918). The present study is the first revision of the genus *Synactinernus*, as we obtained a second specimen of *S. flavus* and the second species of this genus.

Our morphological analyses of the newly collected specimens of two *Synactinernus* species and *I. quadrilobatus* and of the type specimens of *I. quadrilobatus* showed that tentacles of *S. flavus* and *S. churaumi* sp. nov. are simple in shape, apparently lacking thickening of the aboral side (Figs. 2C, G, 3C, 6B, 7C), while those of *I. quadrilobatus*, both in our collection and type specimens, are apparently thickened at the base of the aboral side (Fig. 6C). This clear difference suggests that *Synactinernus* species can be distinguished from *I. quadrilobatus* by their tentacles. In addition, *Synactinernus* species had a fixed number (36) of macrocnemes (Figs. 3A, 7A), and this prominent feature in the mesentery structure should be a diagnostic characteristic of the genus, while *I. quadrilobatus* has many indistinguishable mesenteries. In contrast, the alternate arrangement of larger and smaller oral lobes of *S. flavus,* considered as an important morphological feature of this monotypic genus (Carlgren, 1918, 1949), turned not to be a diagnostic characteristic, because the new species *S. churaumi* has eight lobes of the same size.

The result of the current molecular phylogenetic analyses that *Synactinernus* formed a well-supported independent clade demonstrates the morphological deviation from the other genera of Actinernidae including the former belonging genus, *Isactinernus*.

Synactinernus is endemic to Japan and is distributed around a depth of 300 m in the East China Sea and the Pacific Ocean.

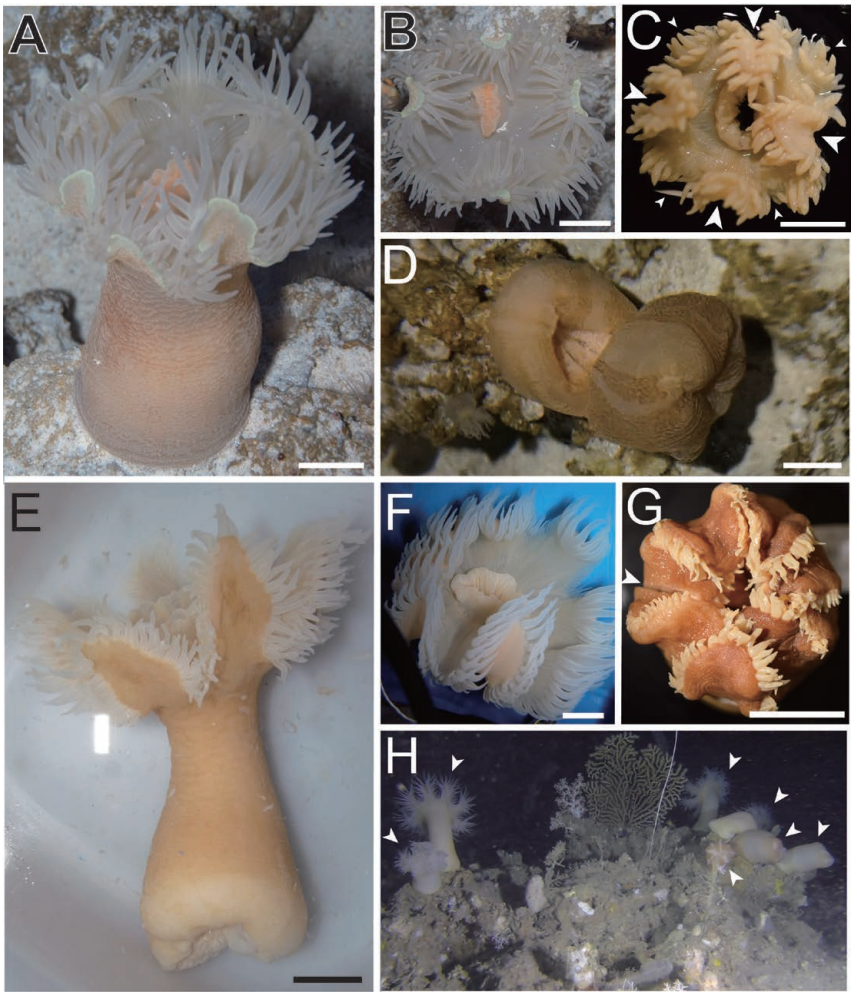

Fig. 2. External morphology and ecological observation of *Synactinernus flavus* **(A–D)** and *S. churaumi* sp. nov. (E–H). **(A)** Living specimen (NSMT-Co 1660) in the tank at the Okinawa Churaumi Aquarium. **(B)** Oral view of living specimen (NSMT-Co 1660). **(C)** Oral view of fixed specimen (NSMT-Co 1660). Large arrowheads indicate large lobes, and small arrowheads indicate small ones. **(D)** A living individual undergoing transverse fission in the tank at the Okinawa Churaumi Aquarium (Photograph by Toshiki Higa). **(E)** Lateral view of living specimen (NSMT-Co 1661). **(F)** Oral view of living specimen (NSMT-Co 1661). **(G)** Oral view of fixed specimen (NSMT-Co 1661). An arrowhead indicates a notch by our dissection. **(H)** Underwater photograph of an aggregation of *S. churaumi* off Okinawa Island (around a depth of 320 m taken by ROV of the Okinawa Churaumi Aquarium). Each individual is indicated by arrowheads. Scale bars indicate 1 cm in **(A–D)**; 5 cm in **(E–G)**.

Synactinernus flavus Carlgren, 1918
(New Japanese name: Kuroba-kawari-ginchaku)
(Figs. 2, 3, 5, 6; Table 2)

Synactinernus flavus Carlgren, 1918, p. 31, "Gote Island, Kin Shin 137 m" [sic., most likely "Goto Islands, Kyushu" in modern orthography].

Material examined. NSMT-Co 1660: specimen dis-

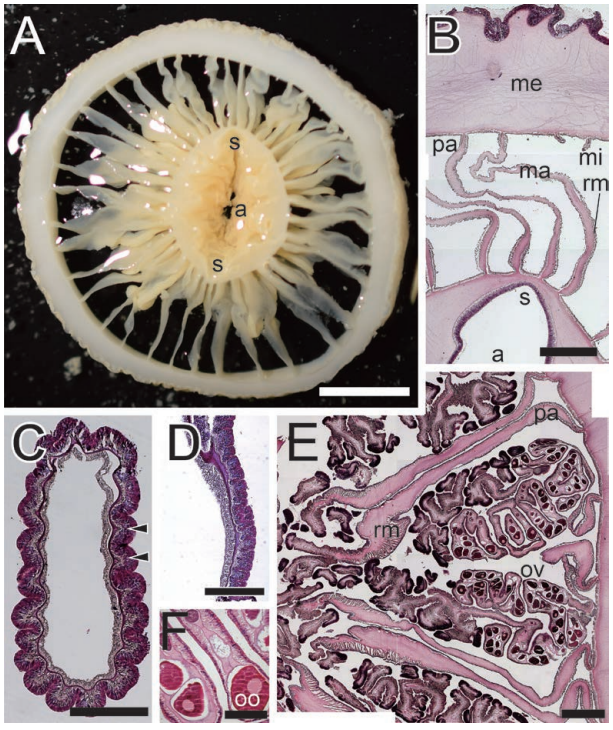

Fig. 3. Internal morphology of *Synactinernus flavus* (NSMT-Co 1660). **(A)** Transverse section of actinopharynx of the gross specimen. **(B)** Transverse section of macrocnemes and microcnemes. **(C)** Transverse section of the tentacle. Arrowheads indicate tentacular longitudinal muscle. **(D)** Longitudinal section of the most basal part of the tentacle. **(E)** Transverse section of filaments and gonads. **(F)** Enlarged view of gonad. Abbreviations: a, actinopharynx; ma, macrocneme; me, mesoglea; mi, microcneme; oo, oocyte; ov, ovary; pa, parietal muscle; rm, retractor muscle; s, siphonoglyph. Scale bars indicate 5 mm in **(A)**, 1 mm in **(B, E)**, 500 μm in **(C, D)**, and 200 μm in **(F)**.

sected, tissues embedded in paraffin, histological sections (16 slides) prepared, nematocysts prepared (four slides); originally collected on 19 November 2012, the Goto-nada Sea off southeastern Goto Islands, Nagasaki Prefecture (32°23.00′N, 129°03.00′E), at a depth of 304–504 m, using a biological dredge of R/V *Nagasaki-Maru* (Cruise No. 365), collected by Masanori Nonaka, and fixed by Takuma Fujii and Hiroko Takaoka from the tank at Okinawa Churaumi Aquarium on January 25, 2013. CMNH-ZG 09732: whole specimen attached to a rock; originally collected in January 2018, at Uragami, Wakayama Prefecture, at 100–130 m, by Isao Hirabayashi, and fixed by Takuma Fujii and Kensuke Yanagi from the tank at the Kushimoto Marine Park on May 22, 2018. UUZM 232 holotype: dissected specimen, 17 May 1914, off Goto Islands (33°41′N, 128°50′E [collection information listed on the label]), depth 110 m, collected by Sixten Bock (Fig. 1).

Description. *External anatomy*. Body cylindrical (Fig. 2A), up to ca. 5–8 cm in length and ca. 2–4 cm in width when alive, and 2–4 cm in length and 1–2 cm in width on specimen. Column surface comparatively smooth, without tenaculi, with numerous discontinuous, wavy wrinkles running in a transverse direction, pale surface with pale orange ectoderm layer. Ectoderm of column without nematocyst batteries, nematocysts very sparsely distributed containing numerous spirocysts. Upper part of column widely expanded and thrown into eight lobes, four larger and four smaller ones (Fig. 2B, C). Upper margin of the lobes fluorescent yellow in color (Fig. 2B). Tentacles simple, all marginal, ca. 2–5 mm in length, no thickenings including their aboral base, pale white in color, and ca. 120–200 in number on oral disk; inner and outer ones alternatingly bared. The tip of tentacles pointed. Pedal disk in aboral end, semitransparent, and mesenterial insertion visible. Oral disk diameter ca. 3–6 cm, same color as tentacles, mouth at center of oral disk, appar-

Table 2. Size and distribution of cnidae in *Synactinernus flavus* (NSMT-Co 1660), and *Synactinernus churaumi* sp. nov. (holotype, NSMT-Co 1661). Size range, mean, and SD are dictated as length × width. A–T indicate the figures of each cnidae in Fig. 5; *n* indicates number of nematocysts measured.

Cnidae	*Synactinernus flavus* NSMT-Co 1660						*Synactinernus churaumi* sp. nov. NSMT-Co 1661					
		Abundance	Size range (μm)	Mean (μm)	SD (μm)	n		Abundance	Size range (μm)	Mean (μm)	SD (μm)	n
Tentacle												
Spirocysts	**A**	numerous	18.5–45.7 × 3.0–9.8	32.3 × 5.9	6.74 × 1.53	88	**K**	numerous	17.8–56.8 × 2.6–8.3	33.5 × 4.8	10.41 × 1.74	105
Basitrichs	**B**	numerous	15.3–33.4 × 2.3–4.4	28.7 × 3.3	2.59 × 0.42	55	**L**	numerous	30.0–42.2 × 2.1–3.3	35.1 × 2.9	2.58 × 0.33	56
Actinopharynx												
Spirocysts	**C**	numerous	25.8–40.2 × 5.4–8.0	34.4 × 6.4	3.02 × 0.57	55	**M**	numerous	34.4–43.1 × 5.8–8.9	39.6 × 7.1	2.17 × 0.70	21
Basitrichs	**D**	numerous	23.8–39.6 × 2.6–4.2	31.1 × 3.5	3.32 × 0.35	65	**N**	numerous	26.5–42.5 × 3.2–4.6	35.7 × 3.9	2.27 × 0.34	80
Microbasic *p*–mastigophores	**E**	rare	23.0–23.9 × 4.0–5.0	23.4 × 4.5	0.42 × 0.47	2	**O**	numerous	34.3–39.5 × 5.6–6.8	36.4 × 6.3	1.56 × 0.38	12
Column												
Spirocysts	**F**	numerous	20.9–47.1 × 4.1–7.6	35.2 × 6.6	6.14 × 1.11	17	**P**	numerous	28.6–39.2 × 4.3–6.0	33.2 × 5.2	2.82 × 0.46	15
Basitrichs	**G**	numerous	27.9–42.7 × 2.9–5.0	34.9 × 3.9	2.50 × 0.40	100	**Q**	numerous	36.1–46.4 × 2.9–4.6	41.8 × 3.6	2.02 × 0.33	98
Filament												
Spirocysts	**H**	numerous	19.9–31.0 × 3.4–6.5	26.7 × 5.2	2.58 × 0.72	24	**R**	numerous	24.5–36.1 × 4.6–7.3	31.8 × 5.9	2.31 × 0.60	79
Basitrichs	**I**	numerous	28.5–38.5 × 2.5–4.3	32.3 × 3.7	1.90 × 0.39	59	**S**	numerous	21.6–36.3 × 2.4–3.7	27.8 × 3.1	3.82 × 0.38	29
Microbasic *p*–mastigophores	**J**	numerous	20.5–27.5 × 4.5–6.7	23.9 × 5.7	1.64 × 0.57	37	**T**	numerous	28.1–39.4 × 4.8–7.1	33.5 × 6.0	2.22 × 0.52	56

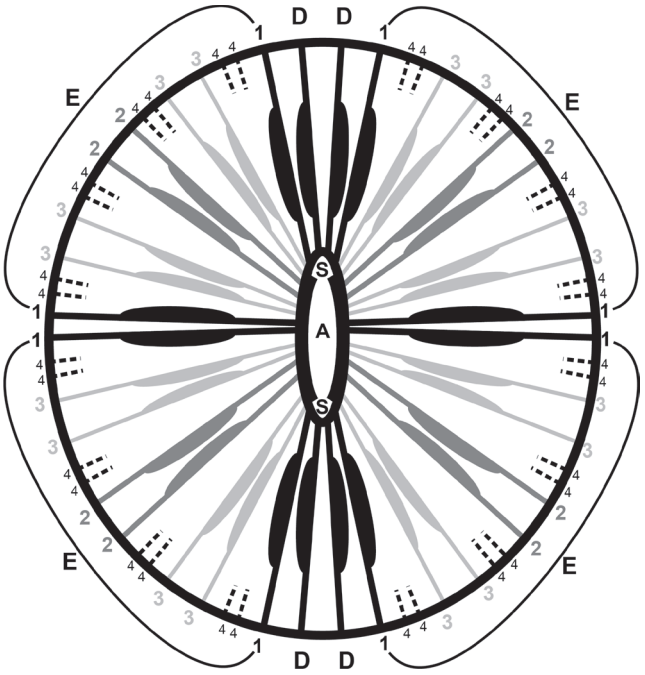

Fig. 4. Schematic representation of the mesenterial arrangement of *Synactinernus*. The cycle of mesenteries is indicated by numbers and coloration: D (black), directives; 1 (black), lateral mesenteries of the first cycle; 2 (dark grey), second cycle mesenteries; 3 (light grey), third cycle mesenteries; 4 (black broken line), fourth cycle mesenteries. Mesenteries of the first to third cycles are macronemes, and those of fourth and younger cycles are micronemes. The number of mesentery cycles reaches the sixth (*S. flavus*) or seventh (*S. churaumi* sp. nov.), although the fifth and younger cycles are omitted. All mesenteries of the second and younger cycles are developed in the endocoels of four pairs of lateral mesenteries in the first cycle (shown by E). Abbreviations: A, actinopharynx; S, siphonoglyph.

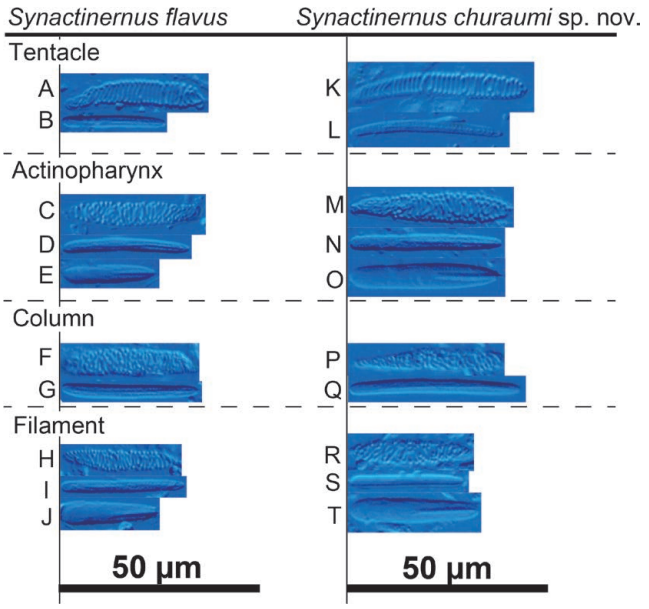

Fig. 5. Cnidom of *Synactinernus flavus* **((A–J)**, NSMT Co 1660) and *Synactinernus churaumi* sp. nov. (**(K–T)**, NSMT Co 1661). **(A, K)** Spirocyst in tentacles. **(B, L)** Basitrich in tentacles. **(C, M)** Spirocyst in actinopharynx. **(D, N)** Basitrich in actinopharynx. **(E, O)** Microbasic *p*-mastigophore in actinopharynx. **(F, P)** Spirocyst in column. **(G, Q)** Basitrich in column. **(H, R)** Spirocyst in filaments. **(I, S)** Basitrich in filaments. **(J, T)** Microbasic *p*-mastigophore in filaments.

ently swelled, lip-like.

Internal anatomy. 36 (18 pairs of) macrocnemes (Figs. 3A, 4) on actinopharynx; twelve, including four directives, in the first cycle; eight in the second cycle; 16 in the third cycle. Macrocnemes in the second cycle born in the endocoel of the first cycle mesenteries, an arrangement obeying the rule of mesenterial arrangement of Actinernidae (Fig. 4). Pairs of microcnemes, in the fourth cycle, observed in transverse section (Fig. 3A, arrowhead). Judged by the numbers of tentacles, mesenteries of *Synactinernus flavus* in the first–sixth cycles. All mesenteries perfect near the mouth, and each tentacle between either exo- or endocoelic. Tentacular longitudinal muscle ectodermal (Fig. 3C); tentacular circular muscle too weak to observe in histological sections (Fig. 3D). Retractor muscles comparatively weak and restricted actinopharynx or filament side (Fig. 3B, E). Muscle processes short, mostly simple or slightly branched, around 30–50 in each muscle pennon. Parietal muscles of macrocnemes weak, distinct with approximately 10 muscle fibers in upper part near actinopharynx (Fig. 3B), but indistinct in lower part near filaments (Fig. 3E). Mesoglea thickest in body wall and actinopharynx (Fig. 3A), reaching 2–3 mm in thickness, and far thicker than the ectoderm and endoderm. However, mesoglea thinner in mesenteries (Fig. 3E), and thinnest in tentacles (Fig. 3C, D). Actinopharynx, with siphonoglyphs on dorsal and ventral sides (Figs. 3A, 4), always connected to actinopharynx, and with 10 longitudinal grooves as deep as siphonoglyphs. Sphincter muscle absent. On the aboral end, basilar muscle absent. Dioecious, matured eggs in gonads in NSMT-1660 (Fig. 3F). Mesenteries in the first and second cycles only fertile (Fig. 3E).

Cnidom. Basitrichs, spirocysts, and microbasic *p*-mastigophores. See Table 2 and Fig. 5 for size and distribution.

Derivation of new Japanese name. “Kuroba” means the plant clover. The oral disk with four large and foursmall lobes resembles the four-leaf clover, which is considered a symbol of good luck.

Taxonomic remarks. We provide a detailed description of *S. flavus* based on the holotype and our new specimens in this study. We presented cnidom data of every part of the body (Table 2) and the figures of external features (Fig. 2), which were lacking in Carlgren (1918).

Almost all morphological features of the present specimen (NSMT-Co 1660) corresponded to Carlgren (1918) and the holotype (UUZM 232): cylindrical body (Figs. 2A, 6A); ca. 120–200 tentacles with no thickening even on base (Figs. 2B, C, 3D, 6B); about 10 longitudinal grooves on actinopharynx (Fig. 3A); mesoglea on the actinopharynx are thick (Fig. 3B); ectodermal but weak tentacular muscle (Fig. 3D); numerous spirocysts in column (Fig. 5F, Table 2). The size of spirocysts, 20.9–47.1 µm in length and 4.1–7.6 µm in width in our specimens (Table 2), is similar to that of the syntype, 26–38 µm in length and 3–5 µm in width (Carlgren, 1918).

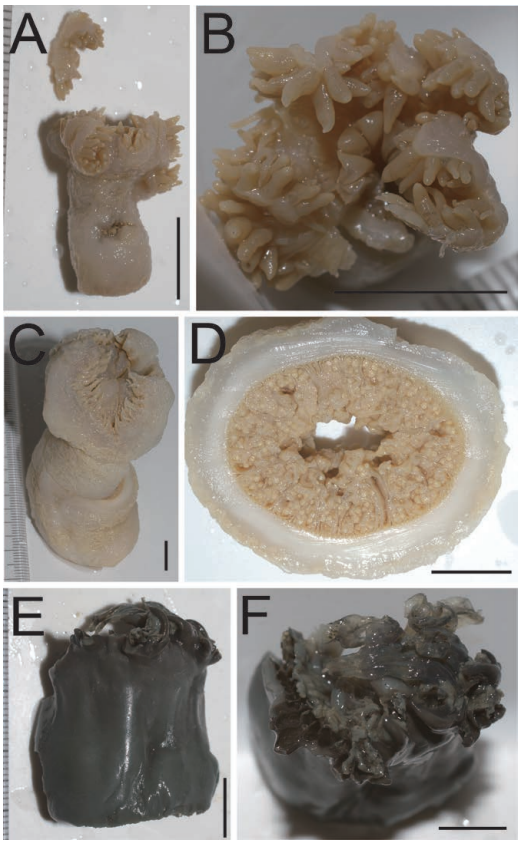

Fig. 6. The type specimens of *Synactinernus flavus* (**(A, B)**, UUZM232, holotype), *Isactinernus quadrilobatus* (**(C, D)**, UUZM 102a, one of the syntypes), and *Actinernus robustus* (**(E, F)**, BM 89-11-25-30, holotype). **(A)**, whole aspect; **(B)**, oral view. **(C)**, whole aspect; **(D)**, gross section of mesenteries of lower part; **(E)**, whole aspect; **(F)** oral view. All scale bars indicate 1 cm.

The number of macrocnemes is fixed to 36 in *S. flavus* (Fig. 3A), while over 100 in *I. quadrilobatus* (Fig. 6D; there are the same number of mesenteries next to the actinopharynx), but it is not a strong evidence to divide the species and genus because Fautin and den Hartog (2003) reported a range (18–40) in the number of mesenterial pairs among 81 specimens when the two species were synonymized. However, we confirmed that there are several differences between the two species other than the above-mentioned differences between the two genera; whether as mentioned in remarks of genus *Synactinernus*, thickening on the base of tentacle is only observed on *I. quadrilobatus*. At the oral disc, this species has apparently four large and four small lobes (Figs. 2B, C, 6A), but *I. quadrilobatus* has only four lobes; *S. flavus* fertilizes only the first and second cycle mesenteries, total 20 in number (Fig. 3E), while *I. quadrilobatus* has many fertile mesenteries (Fig. 6D). In addition, the size of *I. quadrilobatus* is approximately twice to three times that of *S. flavus* (Fig. 6A, C). These several morphological differences of both species are sufficient to discuss that they are independent groups, and our molecular phylogeny analysis strongly supports the division into two species based on the several morphological differences mentioned above; *S. flavus* and *I. quadrilobatus* are not closely related on the phylogenetic tree and the difference between them is considered to be at the genus level (Fig. 8; see Discussion for details). In conclusion, *S. flavus* is not a synonym of *I. quadrilobatus* and is different at the genus level both in morphology and molecular phylogeny. Therefore, the morphological features that Fautin and den Hartog (2003) described as intraspecific variations in *I. quadrilobatus* can also be used to distinguish *S. flavus* from *I. quadrilobatus*.

In the present study, we redescribed the species using our newly collected specimens with the addition of important features like cnidom of every part of the body (Fig. 5), which were lacking in Carlgren (1918), and also photographic information (Figs. 2, 3).

The locality of the holotype of *S. flavus*, UUZM 232, is described on the label as follows: “Goto Islands 28 miles N. 1/2 Ost fran Shirase fyr 128 50 O.L., 33 41 N.Br.” The locality, “Shirase”, would mean “Shiroze”, an isolated, uninhabited island north of the Goto Islands (33°11′00″N, 128°48′14″E). In Japanese, the Kanji-character of “Shiroze” is sometimes pronounced as “Shirase”. Swedish “fyr” means a lighthouse. The lighthouse on Shiroze was built in December 1904, and was obviously present in 1914, when the holotype of *S. flavus* was collected. The point, 1/2 sea mile east off Shiroze, is approximately 40–45 km north from Fukue Island (the main island of Goto Islands). These distances and directions completely match “Goto Islands 28 miles N” on the label. The depth, 110 m written on the label, is also a match. Considering the above information, the exact type locality should be here. The latitude and longitude written on the label is far away from “Shiroze”, so it may be mistaken. Our specimens would be the first topotype specimens after the collected type specimen.

Ecological remarks. It was observed that *Synactinernus flavus* reproduced several times by transverse fission in the tank at Okinawa Churaumi Aquarium. When the bodies were mechanically or thermally stimulated, for example when their basal disc was detached from the substrate, it cracked on the surface of the column, and transverse fission of the body occurred soon thereafter (Fig. 2D). Both halves became independent full-grown individuals. This is the first observation of transverse fission in Actinernoidea, while fission from the basal disc has been observed in Halcuriidae of Actinernoidea (Uchida, 2004).

Synactinernus churaumi sp. nov. Izumi and Fujii, 2019
(New Japanese name: Churaumi-kawari-ginchaku)
(Figs. 2, 5, 7; Table 2)

Material examined. Holotype: NSMT-Co 1661: specimen dissected, tissues embedded in paraffin, histological sections (20 slides) prepared, nematocysts prepared (four slides); originally collected on February 22, 2004, off northwestern Ishigaki Island, Okinawa Prefecture, at a depth of 281–312 m, using LEO, ROV of Okinawa Churaumi Aquarium, by Takuo Higashiji, and fixed by Takuma Fujii and Hiroko Takaoka from the aquarium tank on 25 January 2013. Paratype: CMNH-ZG 09733: originally collected on 5 September 2018, off Onna Village, Okinawa Island, Okinawa Prefecture (26°34.22′N, 127°47.96′E), at a depth of 320 m, using ROV of the Okinawa Churaumi Aquarium by Takuo Higashiji, and fixed by Takato Izumi from the aquarium tank on 10 September 2018 (Fig. 1).

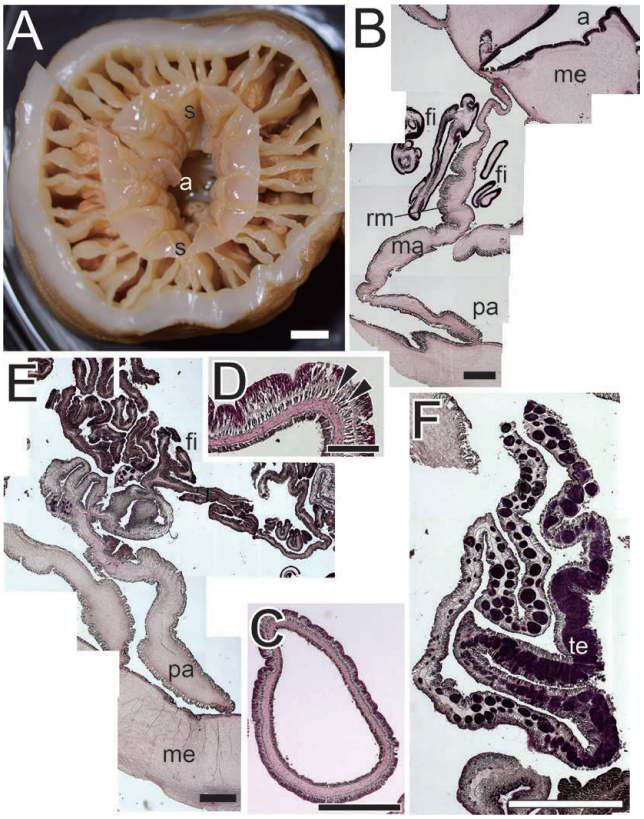

Fig. 7. Internal morphology of *Synactinernus churaumi* sp. nov. (NSMT-Co 1661). **(A)** Transverse section of actinopharynx of the gross specimen. **(B)** Transverse section of a macrocneme; the dark purple tissues around the macrocneme are fragments of filaments. **(C)** Transverse section of the tentacle. **(D)** Enlarged view of transverse section of tentacle. Arrowheads indicate tentacular longitudinal muscle. **(E)** Transverse section of filaments and gonads. **(F)** Enlarged view of the gonad. Abbreviations: a, actinopharynx; fi, filament; ma, macrocneme; me, mesoglea; pa, parietal muscle; rm, retractor muscle; s, siphonoglyph; te, testis. Scale bars indicate 5 mm in **(A)**; 1 mm in **(B, C, E, F)**; and 200 μm in **(D)**.

Description. *External anatomy.* Body cylindrical (Fig. 2E), up to 20–25 cm in length and 15 cm in width when alive. Regarding specimens, 12 cm in length and 10 cm in width on holotype, and 13 cm in length and 9 cm in width on paratype. Column surface smooth, without tenaculi, pale surface with orange or yellow ectoderm layer. Ectoderm of column without nematocyst batteries and nematocysts very sparsely distributed but contain numerous spirocysts. Upper part of column widely expanded and thrown into eight lobes of the same size (Fig. 2F, G). Upper margin of the lobes same as column in color (Fig. 2E). Tentacles simple, all marginal, 5–20 mm in length, no thickenings including their aboral base, pale white in color, and ca. 350–500 in number on oral disk; inner and outer ones alternatingly bared (Fig. 2F). The tip of tentacles pointed. Pedal disk in aboral end, opaque and mesenterial insertion invisible. Oral disk diameter ca. 12–20 cm in living specimen, same color as tentacles or pale orange, Mouth at center of oral disk, exceedingly swollen, lip-like, pale white to yellow (Fig. 2F).

Internal anatomy. 36 (18 pairs of) macrocnemes (Figs. 4, 7A) on actinopharynx; 12, including four directives, in the first cycle; eight in the second cycle; and 16 in the third cycle. Macrocnemes in the second cycle born in the endocoel of the first cycle mesenteries; their arrangement obeying the rule of mesenterial arrangement of Actinernidae (Fig. 4). Mesenteries in younger than the fourth cycle microcnemes, and judged by the numbers of tentacles, mesenteries of *Synactinernus churaumi* sp. nov. in the first–seventh cycles. All mesenteries perfect near the mouth, and each tentacle between either exo- or endocoelic. Tentacular longitudinal muscle ectodermal (Fig. 7C, D); tentacular circular muscle too weak to observe in histological section; retractor muscles comparatively weak and restricted on the middle of mesenteries (Fig. 7B); or diffused and integrated to parietal muscle (Fig. 7E). Muscle processes short, mostly simple or slightly branched, around 60–90 in each muscle pennon. Parietal muscles of macrocnemes weak, distinct with approximately 10 muscle fibers in upper part near actinopharynx (Fig. 7B) but integrated to retractor muscle near filaments (Fig. 7E). Mesoglea thickest in body wall and actinopharynx (Fig. 7A, B), reaching to 8–10 mm in thickness, and far thicker than ectoderm and endoderm. However, mesoglea thinner in mesenteries (Fig. 7B, E) and thinnest in tentacle (Fig. 7C, D). Actinopharynx, with siphonoglyphs on dorsal and ventral sides (Figs. 7A, 4), always connected to actinopharynx, and with 10 longitudinal grooves as deep as siphonoglyphs. Sphincter muscle absent. On the aboral end, basilar muscle absent. Dioecious, matured testes in NSMT-1661; a variety of stages of maturation in the same testis (Fig. 7F). Mesenteries in the first and second cycles only fertile. The release of eggs and sperm by *S. churaumi* was observed in the tank at the Okinawa Churaumi Aquarium.

Cnidom. Basitrichs, spirocysts, and microbasic *p*-mastigophores. See Table 2 and Fig. 5 for size and distribution

Etymology. The species epithet “churaumi” is named after Okinawa Churaumi Aquarium, an aquarium in Okinawa, Japan, which provided us the specimens of this species. “Chura” means beautiful in Okinawan language, and “umi” means sea in Japanese.

Derivation of new Japanese name. Same as the species epithet.

Taxonomic remarks. *Synactinernus churaumi* sp. nov. is the second species of this genus; this species is clearly distinguished from *S. flavus*. Comparing the two species in adult individuals which have matured gametes (Figs. 3E, 5E), *S. churaumi* is approximately 3–5 times larger than *S. flavus* in body length (*S. churaumi* is one of the largest species of Actinernidae), and *S. churaumi* has over 350 tentacles while *S. flavus* has around half the number. The margin of the oral disk of *S. churaumi* develops into eight same sized lobes, while that of *S. flavus* has four larger and four smaller lobes. The molecular phylogenetic tree (Fig. 8) indicated that these two species formed apparently separate clades. These evidences indicate that *S. churaumi* is not conspecific with *S. flavus* but is an independent species. In addition, there is possibly a difference in the presence of asexual fission (see Ecological remarks).

The eight-lobed oral disk of *S. churaumi* sp. nov. resembles that of *Actinernus* Verrill, 1879. However, *S. churaumi* does not correspond to the diagnosis of *Actinernus* of

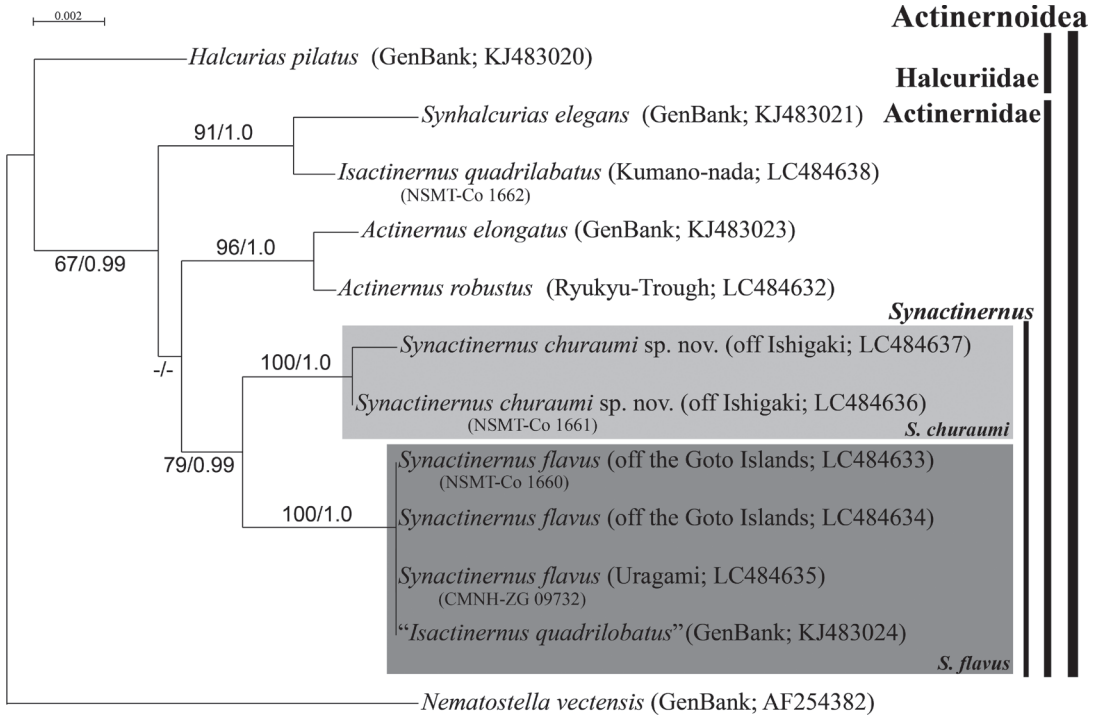

Fig. 8. Maximum-likelihood tree of nuclear 18S rDNA for Actinernidae, including *Synactinernus flavus* and *Synactinernus churaumi* sp. nov. Numbers above or below branches indicate ML bootstrap support values followed by BI posterior probabilities of each node (values less than 50 of bootstrap support or 0.90 of posterior probability are indicated by "–").

Actinernidae Stephenson, 1922. *Actinernus* species have mesogleal thickening at the tentacle base, and we confirmed this in both the holotype of *Actinernus robustus* Hertwig, 1882, the only *Actinernus* species from the North Pacific, (Natural History Museum, London, BM 89-11-25-30; Fig. 6E, F) and our specimens (CMNH-ZG 09735). However, tentacles of *S. churaumi* were not thickened at any part. Moreover, mesenterial arrangement of *Actinernus* is bilateral, and the fourth cycle mesenteries are born in the endocoel of the third cycle (Carlgren, 1918), and Uchida (2007) confirmed this for *A. robustus*. The mesenterial arrangement of *S. churaumi* is completely different from that of *Actinernus* (Fig. 4).

Ecological remarks. *Synactinernus churaumi* sp. nov. is distributed around depths of 320–350 m off Okinawa Island. This species forms small aggregations on the top of hill-like submarine topography as recorded by the ROV (Fig. 2H). Incidentally, no asexual fission of *S. churaumi* in the tank at the Okinawa Churaumi Aquarium was observed for 15 years, while *S. flavus* has self-divided several times over seven years (Fig. 2D).

Phylogenetic analyses

Regarding the phylogenetic tree of 18S rDNA (1623 bp), all *Synactinernus* specimens significantly formed a clade (ML boot strap value = 79; BI posterior probability = 0.99), which was separated from the other genera of Actinernidae (Fig. 8). The *Synactinernus* clade was separated into two clades at high support (ML bootstrap value = 100; BI posterior probability = 1.0 for both clades), and they corresponded to respectively *S. flavus and S. churaumi* specimens. All three sequences of *S. flavus* were completely identical, and *S. churaumi* showed only a 2-base-pair difference between the two sequences (this small difference may be intraspecific variation because nuclear 18S marker has higher base pair substitution late than mitochondrial ones in Actiniaria [Daly et al., 2010]). In addition, the sequence of *I. quadrilobatus* deposited in GenBank (KJ483024) was also identical with *S. flavus*. On the other hand, our specimen of *I. quadrilobatus* (NSMT-Co 1662) was nested with *S. elegans* (KJ483021), the most basal clade in Actinernidae, and completely separated from "*I. quadrilobatus*" in GenBank. The relationship between *Actinernus* and *Synactinernus* was unsolved with unreliable node (ML bootstrap value = 43; BI posterior probability = 0.64).

Regarding 16S (581 bp), there were no differences in the sequences between *S. flavus* and *S. churaumi* at all. It has been shown that 16S is less useful than 18S in the phylogenetic analyses of sea anemone for phylogeny in species level in previous research (Daly et al., 2010), and the results of this research support that assertion.

DISCUSSION

Taxonomic interpretation of molecular phylogeny

The phylogenetic tree (Fig. 8) certifies several taxonomic suggestions as below. *Synactinernus flavus* and *S. churaumi* sp. nov. are distinct species, but they belong to the same genus. That the specimens from Uragami are conspecific with *S. flavus* from Goto is clearly shown by the molecular data.

Isactinernus quadrilobatus was completely separated from *S. flavus* on the 18S phylogenetic tree (Fig. 8) suggesting these two species are different, contrary to their synonymization by Fautin and Hartog (2003). The 18S sequence of "*Isactinernus quadrilobatus*" deposited in GenBank

(KJ483024), which was used in recent phylogenetic analyses (Rodríguez et al., 2014), is in fact the sequence of *S. flavus* specimen, which was mistakenly identified as *I. quadrilobatus.* Rodríguez et al. (2014) showed other sequences of four markers, 12S, 16S, 28S, and COXIII, as well as an 18S sequence from the same specimen for their phylogenetic analyses. The sequences of these markers should be inspected using our *I. quadrilobatus* specimen (NSMT-Co 1662) in the future. The locality of "*I. quadrilobatus*" (PNG 9032) is Papua New Guinea, so it is possible that the distribution of *Synactinernus flavus* is far broader than we realize.

Higher diversity of Japanese Actinernidae

Yanagi (2006) and Uchida (2007) reported three valid species of Actinernidae from Japan: *Actinernus robustus*, *Isactinernus quadrilobatus,* and *Synhalcurias elegans*. The present study adds two *Synactinernus* species; thus, five species of Actinernidae, covering all four genera of the family, are distributed in Japanese waters. Of the four genera, *Synhalcurias* Carlgren, 1914 and *Synactinernus* are endemic to Japan (Fautin, 2013). The key to the species currently found in Japan modified from Uchida (2007) is shown below.

A1. Margin of the oral disk not developing into any lobes ..... *Synhalcurias elegans* (Wassilieff, 1908)
A2. Margin of the oral disk developing four or eight lobes ..... B
B1. Aboral side of the tentacles not thickening C
C1. Four larger and four smaller lobes on the margin of oral disk. Tentacle number ca. 150–200. Body size around 3–5 cm .... *Synactinernus flavus* Carlgren, 1918
C2. Eight lobes of almost the same size on the margin of oral disk. Tentacle number over 350. Body size over 10 cm .... *Synactinernus churaumi* sp. nov.
B2. Aboral side of the tentacles apparently thickening ..... D
D1. Four lobes on the margin of oral disk ....*Isactinernus quadrilobatus* Carlgren, 1918
D2. Eight lobes on the margin of oral disk ....*Actinernus robustus* (Hertwig, 1882)

CONCLUSIONS

1. *Synactinernus* Carlgren, 1918 is a valid genus which includes two species: *S. flavus* Carlgren, 1918 and *S. churaumi* sp. nov.

2. *Iscactinernus* Carlgren, 1918 is independent and less closely related genus from *Synactinernus*; *Isactinernus quadrilobatus* Carlgren, 1918 is distinguished from *S. flavus* by several features.

3. This is the first report of the transversal fission of *S. flavus*, and of the aggregations of *S. churaumi* sp. nov. in situ for actinernid sea anemones.

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