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Development of *Copula sivickisi* (Stiasny, 1926) (Cnidaria: Cubozoa: Carybdeidae: Tripedaliidae) collected from the Ryukyu Archipelago, southern Japan

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Abstract: The small box jellyfish *Copula sivickisi* was collected from the Ryukyu Archipelago, southern Japan, in June 2011, in order to observe its early life history, including polyp formation and metamorphosis. Fertilization occurred internally. Fertilized eggs were packed in an embryo strand with nematocysts and released into the water from the female's manubrium. Blastulae developed into planulae bearing about 30 larval ocelli within two days, and then settled and metamorphosed into primary polyps. Primary polyps developed into adult polyps within 40 days, and both polyp stages could actively detach to change location by creeping on the central part of their body. Adult polyps formed cysts at temperatures below 20°C, which when favorable conditions retuned (above 28°C), excysted from the capsules within a week. Budding occurred in adult polyps, and buds were released two days after the commencement of budding. Metamorphosis of a whole polyp into a single medusa occurred within 10 days. Juvenile medusae were distinguished from those of other cubozoans by the pattern of nematocyst warts on the exumbrella and the adhesive pads on the apex. The developmental features of *C. sivickisi* resemble most closely those of *T. cystophora* among the cubozoans. The similarities in all early life stages of both species support recent molecular results.

Key words: asexual reproduction, Cubozoa, cyst, metamorphosis, polyp formation

Introduction

Copula sivickisi (Stiasny, 1926) is the smallest species in the class Cubozoa, with a maximum bell height of 12 mm and a maximum bell diameter of 14 mm (Stiasny 1926, Uchida 1970). The species displays sexual dimorphism and courtship behavior (Lewis and Long 2005, Lewis et al. 2008). *Copula sivickisi* has been studied under the name *Carybdea sivickisi* (Stiasny 1926, Uchida 1970, Hoverd 1985, Hartwick 1991, Matsumoto et al. 2002, Gershwin 2003, Lewis and Long 2005, Crow et al. 2006, Lewis et al. 2008). However, recent molecular phylogenetic analyses suggest that *C. sivickisi* is more closely related to *Tripedalia cystophora* Conant, 1897 than any species of the genus *Carybdea* (Collins 2002, Gershwin 2005, Collins et al. 2006, Bentlage et al. 2010). In addition, *C. sivickisi* and *T. cystophora* display sexual dimorphism of the gonads and produce spermatophores, and, at least the males, possess sub-gastric sacs/seminal vesicles (Bentlage et al. 2010). To retain monophyly of *Carybdea*, Bentlage et al. (2010) designated the new genus *Copula* to accommodate *Copula sivickisi*.

Copula sivickisi has been reported from a range of tropical, subtropical, and mild temperate localities in the Pacific and Atlantic, as well as one locality in the Indian Ocean, west of Sumatra (Lewis et al. 2008, Bennett et al. 2013). This species appears from spring to summer in Japanese waters (Uchida 1970, Lewis & Long 2005, Lewis et al. 2008). Only the embryological development of the life cycle of *C. sivickisi* is known (Hartwick 1991, Lewis & Long 2005, Lewis et al. 2008). The present paper describes its life history from fertilized eggs over polyp formation to metamorphosis into a medusa.

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Materials and Methods

Copula sivickisi medusae (Fig. 1) were collected using a lamp (GENTOS SuperFire X^3 SF-755X^3, Saint Gentleman, Japan) at Motobu Port, Okinawa Prefecture, southern Japan (Fig. 2), between 20 : 30 and 22 : 00 on June 30, 2011. Thirty individuals were taken by a ladle, 170 mm in diameter. Male and female medusae were kept in a deep petri-dish (diameter 149 mm, height 91 mm, water volume 1500 mL) with filtered seawater (1 μm) at about 26 to 28°C in the laboratory at the Sesoko Tropical Biosphere Research Center, University of the Ryukyus. The medusae were fed with *Artemia* nauplii on a daily basis. Culture water was replaced with fresh seawater about three hours after feeding.

Embryo strands released by females in the laboratory were incubated in petri-dishes (diameter 75 mm, height 45 mm) filled with filtered seawater (0.22 μm) at 28°C (temperature of the sampling site).

Primary polyps were transferred to petri-dishes (diameter 78 mm, height 24 mm) filled with filtered seawater (1 μm) and kept at 23 to 26°C. Chopped *Artemia* nauplii were fed directly to primary and secondary polyps using a fine needle, twice or thrice a week. Rearing water was completely replaced with filtered seawater (1 μm) about three hours after feeding.

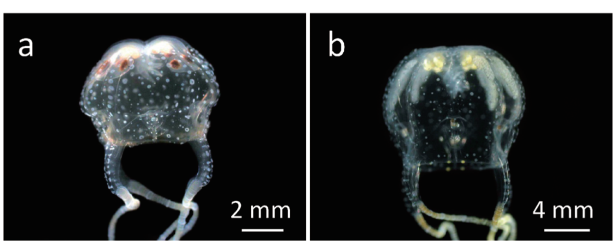

Fig. 1. *Copula sivickisi* (Stiasny, 1926), live, lateral view, in laboratory. a, mature female; b, mature male.

Metamorphosis was induced by raising the temperature to 28°C. During metamorphosis, the cultures were not fed nor was the water changed. Newly detached medusae were kept in a separate petri-dish (diameter 75 mm, height 45 mm) with filtered seawater (1 μm) at 28°C. *Artemia* nauplii were fed to the medusae on a daily basis. Culture water was replaced with fresh seawater about three hours after feeding.

For nematocyst identification in polyps and medusae, squash samples were prepared from fresh tissues and examined under an optical microscope (CX 21, Olympus, Japan). Nematocysts were identified according to Gershwin (2006) and Collins et al. (2011). For determination of the respective abundance of nematocyst types in polyps and medusae, at least 200 nematocysts were counted.

Results

Approximately 24 hours after medusae were collected, courtship behavior was observed in the laboratory. The following morning, gelatinous embryo strands (Fig. 3a) were observed in the petri-dish. The embryo strands were whitish to whitish-yellow, 1.5 mm in diameter and about 30 mm in length. Thousands of fertilized eggs (Fig. 3b) and nematocysts (sub-spherical euryteles) were packed inside the strand (Fig. 4a). The nematocysts were about 0.01 mm in diameter. Fertilized eggs and embryos were about 0.10 mm in diameter. Most of the eggs were at the blastula stage, and half of the blastulae were developing into planulae. Free-swimming planulae were seen within two days of egg strand release. Planulae (Fig. 3c, d) were about 0.10 mm in diameter and about 0.15 mm in length and had about 30 larval ocelli which are characteristic of most cubozoan planulae.

Two to three days after planulae formation, planulae settled on the bottom of petri-dishes. Within five days, planulae developed into primary polyps. The primary polyps

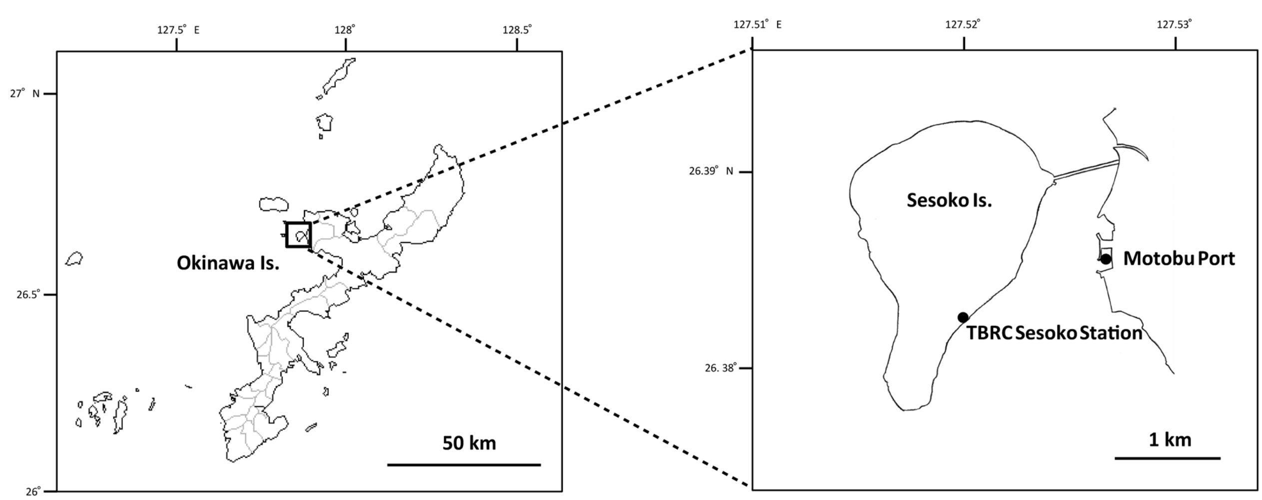

Fig. 2. Map of the sampling site, Motobu Port, Japan.

were either settled (Fig. 5a) or actively detached to start a creeping phase (Fig. 5b). The shape of the settled primary polyps resembled a pouch with a very short stalk, with one to four tentacles protruding from the ovoid calyx around the mouth cone. At a body length of about 0.10 mm the mouth disc diameter of the polyps was about 0.04 mm. The primary polyps in creeping phase had a long, worm-shaped body, and two to three outstretched tentacles. They glided with their oral pole leading and with the centre of their body and one tentacle in contact with the surface. They used this tentacle like a scanning antenna for assessing direction. At a body length of about 0.20 mm, the mouth disc diameter of the polyps was about 0.05 mm. Both settled and creeping primary polyps bore one to three nematocysts (American football-shaped *p*-rhopaloids) in the tip of their tentacles (Fig. 4b).

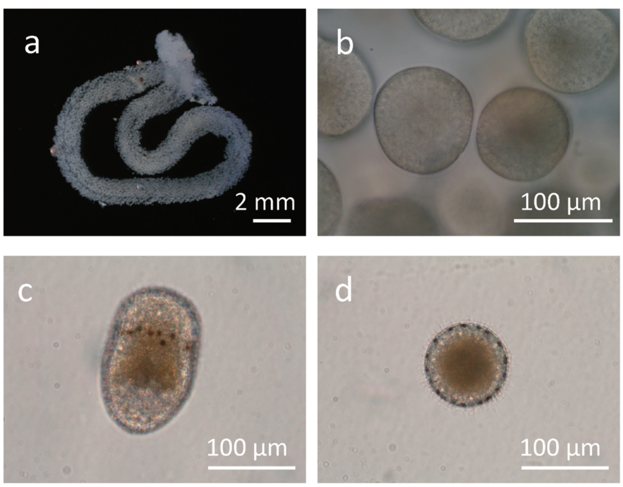

Fig. 3. Early embryogenesis of *Copula sivickisi*. a, embryo strand; b, fertilized eggs; c, planula, lateral view, note eye spots; d, planula, vertical view, note eye spots.

The primary polyps developed into adult polyps within 40 days. The settled adult polyps (Fig. 6a–c) were polyps able to actively detach, and creep (Fig. 6c) in order to change locations. When the polyps began to creep, a peris-

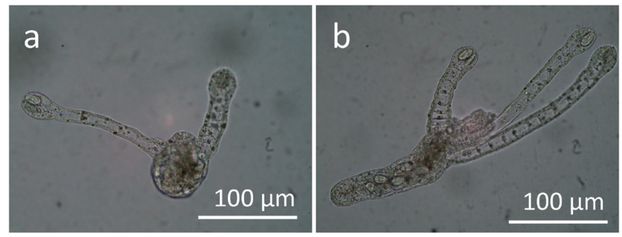

Fig. 5. Primary polyps of *Copula sivickisi*. a, settled, lateral view; b, creeping, lateral view.

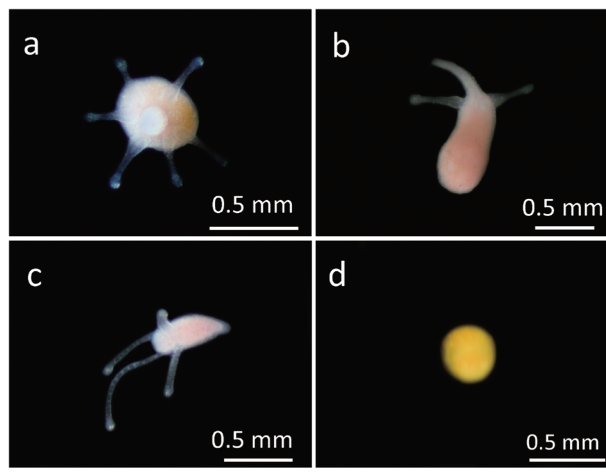

Fig. 6. Adult polyp of *Copula sivickisi*. a, settled, vertical view; b, settled, lateral view; c, creeping, lateral view; d, cyst.

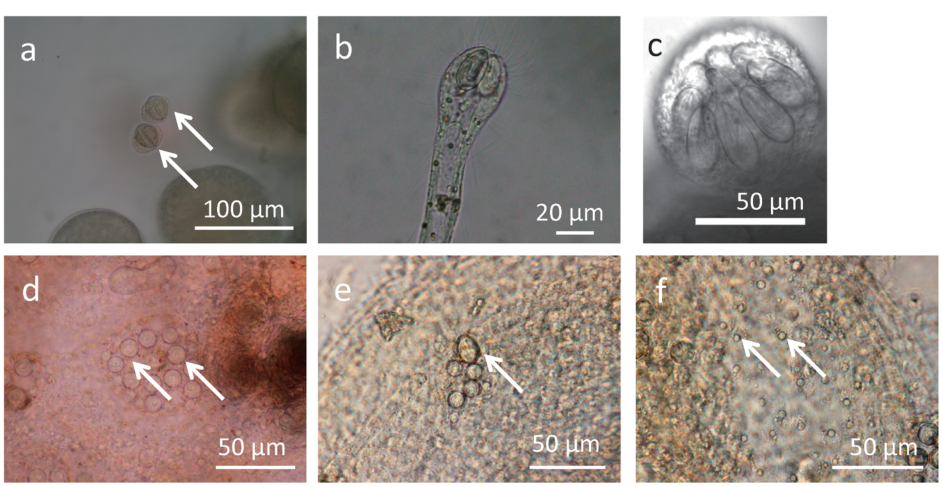

Fig. 4. Nematocysts of *Copula sivickisi*. Arrow indicates nematocyst. a, nematocysts of embryo strand; b, nematocysts of tentacle tip of primary polyp; c, nematocysts of tentacle tip of adult polyp; d, nematocysts of exumbrella warts, ovoid isorhizas; e, nematocysts of exumbrella warts, ovoid heterotrichous microbasic euryteles.

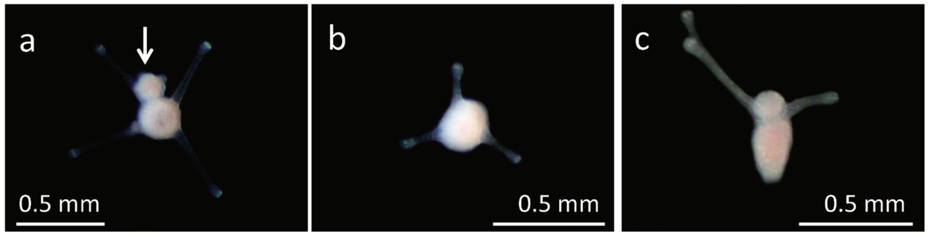

Fig. 7. Asexual reproduction of polyp. Arrow indicates bud. a, budding polyp, vertical view; b, settled polyp, vertical view; c, settled polyp, lateral view.

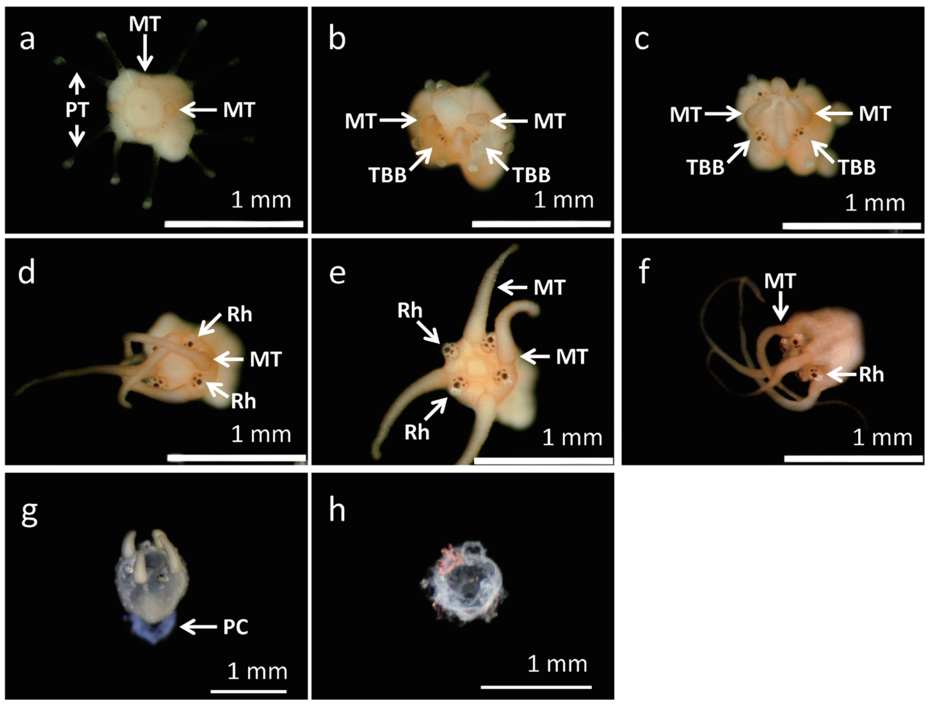

Fig. 8. The process of polyp metamorphosis. a, just after start of metamorphosis; b, 3 days later; c, 5 days later; d, 7 days later; e, 8 days later; f, 9 days later; g, 10 days later. MT=medusa tentacle; PC=peristomal cup; PT=polyp tentacle; TBB=tentacle base bulb; Rh=rhopalium.

tomal cup was left behind. The shape of the adult polyps was almost identical to the primary polyp stage, but they were larger (settled polyps: mean body length 0.50 mm, mean mouth disc diameter 0.17 mm; polyps in creeping phase: mean body length 0.61 mm, mean mouth disc diameter 0.11 mm). Both settled and creeping polyps had two to nine tentacles and each tentacle bore five to nine American football-shaped *p*-rhopaloids (Fig. 4c). Further developmental stages, i.e. budding and metamorphosis only took place in settled polyps.

Below temperatures of 20°C, adult polyps formed resting stages (Fig. 6d). The polyps contracted into a ball and were encapsulated by a soft layer, forming cysts. The cysts were yellowish, about 0.36 mm in diameter and fixed to the substrate. After the temperature was raised above 28°C, polyps regenerated within a week, excysting from the capsules.

Asexual reproduction was observed when the 2-tentacled stage was reached. Bud formation occurred on the middle part of the calyx (Fig. 7a). Two days after the start of bud formation, a secondary polyp (bud) was released from the parent polyp. The shape of newly released buds resembled the parent polyps. The buds were 0.24 mm in diameter and had three or four tentacles (Fig. 7b, c). Buds that settled at first detached later on and assumed a creeping phase like that observed in the adult polyp. The creeping phase lasted for two to three days until the creeping bud settled on the bottom of the petri dish and developed into an adult polyp.

One week after raising the temperature, metamorphosis from a single polyp into a medusa was observed. The first evidence of medusa development was the formation of four temporary vertical furrows in the hypostome of the polyp. The bases of different numbers (one to three) of the polyp

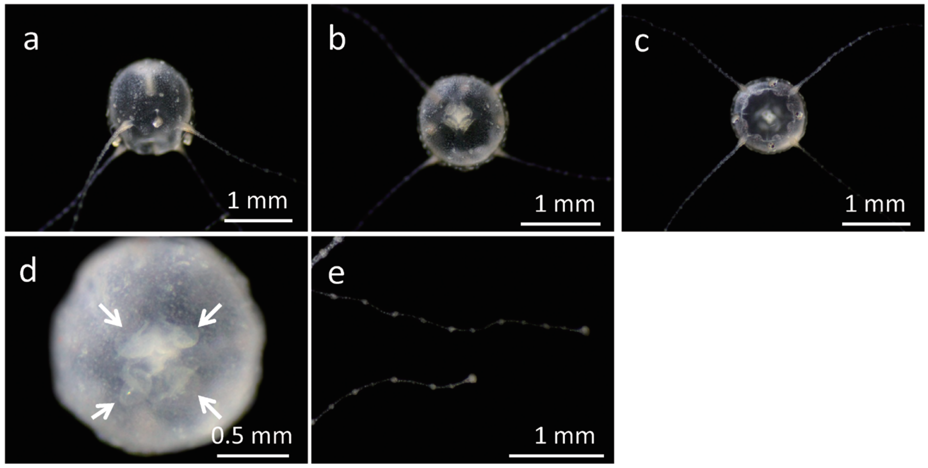

Fig. 9. Newly detached medusae of *Copula sivickisi*. a, lateral view; b, apical view; c, oral view; d, apical view, arrow indicates adhesive pad, gastric filaments located under adhesive pads; e, tentacle.

tentacles coalesced randomly on the four corners of the peristomal edge (Fig. 8a). One medusa tentacle formed between each rhopalium, for a total of four tentacles (Fig. 8a). Three days after the initiation of metamorphosis, the bases of the tentacles fused and thickened to bulbs and the free distal parts were absorbed (Fig. 8b). Pigmentation marks appeared on the hypostome-facing side of these tentacle base bulbs (Fig. 8b). Five days after the initiation of metamorphosis, the pigmented marks transformed into two lens eyes and four ocelli (Fig. 8c), while statocysts emerged on the opposite side of the bulbs. Seven days after the initiation of metamorphosis, transformation of the polyp tentacles into four rhopalia and the hypostome into a manubrium was complete (Fig. 8c, d). Eight days after the initiation of metamorphosis, the medusa opened its lips within the subumbrella and began to pulsate (Fig. 8e). Nine days after the initiation of metamorphosis, the medusa bell enlarged considerably and nematocyst clusters appeared on the exumbrella. The following day it detached (Fig. 8f) from the substrate, and transformed into a single medusa without leaving any regenerative remnants (Fig. 8g, h).

Newly detached medusae had a tetrameric, pyramid-like bell with a rounded top and were yellowish-brown to yellowish in color (Fig. 9a, b, c). They were about 1.2 mm in umbrella height, about 0.9 mm in umbrella width, and all had four tentacles. The exumbrella was sprinkled with very small (0.03–0.06 mm) round nematocyst clusters, consisting mainly of ovoid isorhizas (Fig. 4d) and ovoid heterotrichous microbasic euryteles (Fig. 4e)—two rows lined the edges of the radial furrows and two conspicuous nematocyst warts were located above the sense organs (rhopalia). The medusae bore adhesive pads on the apex (Fig. 9d). The manubrium had four lips and was about 40% of umbrella height. One to two gastric filaments per corner were visible through the apex of the umbrella (Fig. 9d). The sensory niches were shallow and still roofless. Tentacles appeared as a "string of pearls" (Fig. 9e) with about forty spherical nematocyst batteries in alternating colours of white and light brown, consisting of ovoid isorhizas and ovoid heterotrichous microbasic euryteles. When completely extended, the tentacles were up to 6 mm in length. Thirteen-day-old medusae were about 3.4 mm in height and about 2.7 mm in width (Fig. 10). Developmental events in *C. sivickisi* are summarized as Table 1.

Fig. 10. Thirteen day-old medusa of *Copula sivickisi*, lateral view.

Discussion

Polyps of *Copula sivickisi* can be distinguished from hitherto described polyps of other cubozoan polyps by their shape and size (Table 2). Polyps of other cubozoan species are flask-shaped in *Carybdea marsupialis* (Linnaeus, 1758) (Studebaker 1972, Straehler-Pohl & Jarms 2011) and *Alatina alata* (Reynaud, 1830) (Arneson & Cutress 1976 as *Carybdea alata*, Straehler-Pohl & Jarms 2011 as *Alatina mordens*), amphora-shaped in *Carybdea morandinii* Straehler-Pohl & Jarms, 2011 (Straehler-Pohl &

Table 1. Chronology of developmental events in *Carybdea sivickisi.*

Stage/Event	Cumulative interval (day)	Length or height (mm)	Diameter (mm)	No. of tentacles
Fertilization	0	—	—	—
Embryo strand released	1	30	1.5	—
Fertilized eggs		—	0.10	—
Planulae released	3	0.20	0.12	—
Planulae settled	6	—	0.12	—
Primary polyps	11	0.10	0.04	2–4
Bud formation	51	0.50	0.17	2–7
Adult polyps	53	0.50	0.17	2–7
Metamorphosis	85	0.50	0.17	6–7
Newly released medusae	95	1.2	0.9	4
13-day-old medusae	108	3.4	2.7	4

Jarms 2011), and tulip-shaped in *Morbakka virulenta* (Kishinouye, 1910) (Toshino et al. 2013). Polyps of *C. sivickisi* were pouch-shaped, as in its close relative *Tripedalia cystophora* (Werner 1975, 1983). Compared with *T. cystophora*, the numbers of tentacles and nematocysts at the tip of the tentacles were less (Werner 1975, Chapman 1978). The adult polyp of *C. sivickisi* is the smallest among known cubozoan polyps (Table 2).

The polyp of *C. sivickisi* had the ability for the polyps to alternately settle or creep. This flexibility has also been described for primary polyps of *Carybdea brevipedalia* Kishinouye, 1891 (Okada 1927 as *Carybdea rastonii*) and *Chironex yamaguchii* Lewis & Bentlage, 2009 (Iwanaga et al. 2005 as *Chiropsalmus quadrigatus*). The ability of adult polyps to creep has been referred to in the literature only once, by Werner (1975), which might suggest that this is a rare behavior in other cubozoans but was noted in this study as a usual behavior in *C. sivickisi*.

The asexual buds of *C. sivickisi* polyps were pouch-shaped with three or four tentacles. In contrast, other cubozoans have the form of worm-like creeping polyps (Werner 1975, 1983, Straehler-Pohl 2001, Straehler-Pohl & Jarms 2011) or ovoid-shaped swimming polyps from asexual buds (Toshino et al. 2013) (Table 2). Polyps in those studies moved on the substrate or swam in the water column until attaching to the substrate. The adult creeping polyps of *C. sivickisi* may play a role in assuring successful recruitment on adequate solid substrates in a manner similar to that observed in creeping or swimming buds of other cubozoans.

The polyps of *C. sivickisi* formed cysts in unfavourable conditions. Encystment of polyps has been observed in *T. cystophora* (Werner 1975, Straehler-Pohl 2001, 2009), *Chironex fleckeri* Southcott, 1956 (Hartwick 1991), *C. marsupialis* (Straehler-Pohl 2001, 2009), *A. alata* (Straehler-Pohl 2001, 2009 as *Alatina mordens*) and *Carybdea morandinii* (Straehler-Pohl & Jarms 2011). Conditions causing encystment were drastic salinity changes in *T. cystophora* and *C. fleckeri*, and water temperature changes above 27°C or below 15°C in *C. morandinii*. Under laboratory conditions in this study, polyps of *C. sivickisi* formed cysts at low temperature (20°C) and cysts excysted at a higher temperature (28°C). Therefore, the cyst stage of *C. sivickisi* may play an important role to enable survival at lower temperatures. The temperature range between encystment and excystment conditions in *C. sivickisi* is in agreement with that of the habitats in which they were collected for this study; for environmental data on Okinawan waters, see Japan Meteorological Agency (2013).

Metamorphosis in *C. sivickisi* was complete and without any residuum as in the majority of known cubozoan life cycles, with *C. marsupialis* and *M. virulenta* as exceptions (Straehler-Pohl & Jarms 2005, Toshino et al. in prep). Metamorphosis was induced by a return to warmer temperatures (23°C to 28°C). Around the coast of Okinawa, medusae of *C. sivickisi* are present between April and early August (Lewis & Long 2005), when temperatures rise from approximately 23 to over 30°C.

Newly detached medusae of *C. sivickisi* can be distinguished from hitherto-described medusae of other cubozoans by the pattern of nematocyst warts on the exumbrella and the adhesive pads on the top of the umbrella (Table 3). The shape of the medusae resemble more closely that of *T. cystophora* than *C. marsupialis* or *A. alata* (Straehler-Pohl & Jarms 2005, Straehler-Pohl & Jarms 2011). *Copula sivickisi* has a spherical umbrella with four long tentacles and nematocysts warts lining the radial furrows—very closely resembling the characters of *T. cystophora*, while *C. marsupialis* has only two tentacles and is pyramidal and *A. alata* has a pyramidal umbrella with four extremely short tentacles (Straehler-Pohl & Jarms 2011). Nematocyst types in *C. sivickisi* differ from those of *T. cystophora* and *C. marsupialis*; nematocyst clusters on the exumbrella of the latter two species are mainly comprised of atrichous and holotrichous isorhizas (Werner 1975, Stangl 1997). On the exumbrella of *A. alata*, clusters of microbasic euryteles and spherical holotrichous isorhizas are found (Straehler-Pohl & Jarms 2011). In nematocyst warts on the exumbrella of *C. morandinii*, ovoid heterotrichous microbasic euryteles are predominant and isorhizas are rare (Straehler-Pohl & Jarms 2011). Young medusae of *C. siv-*

Table 2. Size (mm) and morphology of cubozoan polyps in previous and the present studies. The proportion is the ratio of the mean length of that structure to the mean total body length.

	Total body length	Mouth disk diameter	Number of tentacles	Hypostome length proportion	Calyx length proportion	Stalk length proportion	Nematocyst types Number of nematocysts	Bud type Number of tentacles	Metamorphosis type	References
Copula sivickisi	0.28–0.97, mean: 0.50	0.15–0.20, mean: 0.17	2–7, mean: 4	0.09–0.14, mean: 0.12 35%	0.22–0.79, mean: 0.35 64%	0.02–0.05, mean: 0.04 1%	American football-shaped p-rhopaloids 5–9	settled polyp 3	complete metamorphosis	present study
Carybdea morandinii	0.83–1.80, mean: 1.28	0.34–0.74, mean: 0.51	9–18, mean: 13	0.16–0.36, mean: 0.26 21%	0.43–0.86, mean: 0.61 48%	0.22–0.54, mean: 0.39 31%	stenotele 1	creeping polyp 4–6	complete metamorphosis	Straehler-Pohl & Jarms 2011
Carybdea marsupialis	1.35–2.78, mean: 2.08	0.40–0.91, mean: 0.62	19–26, mean: 24	0.20–0.44, mean: 0.33 16%	0.72–1.50, mean: 1.12 54%	0.37–0.81, mean: 0.60 29%	stenotele 1	creeping polyp 4–6	complete metamorphosis strobilation	Straehler-Pohl & Jarms 2005
Tripedalia cystophora	0.52–1.02, mean: 0.83	0.17–0.31, mean: 0.26	7–13, mean: 9	0.12–0.22, mean: 0.18 22%	0.36–0.70, mean: 0.58 70%	0.04–0.08, mean: 0.07 8%	heterotrichous eurytele 20–40	creeping polyp 2–3	complete metamorphosis	Straehler-Pohl & Jarms 2011
Morbakka virulenta	2.25–4.66, mean: 3.50	0.49–1.08 mean: 0.65	8–17 mean: 13	0.27–0.62, mean: 0.48 14%	1.05–1.89, mean: 1.49 44%	0.60–2.71, mean: 1.52 41%	trirhopaloid, small spherical p-rhopaloid more than 30	swimming polyp 2	strobilation	Toshino et al. 2013, Toshino et al. (in prep.)
Alatina moseri	1.43–1.63, mean: 1.52	0.41–0.46, mean: 0.43	11–19, mean: 16	0.19–0.22, mean: 0.21 14%	0.87–0.94, mean: 0.91 60%	0.36–0.41, mean: 0.40 26%	stenotele 1	creeping polyp 4–6	complete metamorphosis	Straehler-Pohl & Jarms 2011
Chironex fleckeri	—	0.75	40–45	—	—	—	— 1	creeping polyp 4–8	complete metamorphosis	Yamaguchi & Hartwick 1980

Table 3. Size (mm) and morphology of cubozoan medusae in previous and the present studies. The proportion is the ratio of the mean length of the manubrium to the mean umbrella height. AI=atrichous isorhizas; soAI=small ovoid atrichous isorhizas; HI=holotrichous isorhizas; oHI=ovoid holotrichous isorhizas; rHI=round holotrichous isorhizas; sHI=spherical holotrichous isorhizas; sE=sub-spherical euryteles; ME=microbasic euryteles; oHME=ovoid heterotrichous microbasic euryteles; oI=ovoid isorhizas.

	Umbrella height	Umbrella diameter	Manubrium length proportion	No. of tentacles tentacle shape	Nematocyst type (tentacles)	Nematocyst warts (exumbrella)	Nematocyst types (exumbrella)	References
Copula sivickisi	1.0–1.3, mean: 1.15	0.7–1.0 mean: 0.90	0.37–0.48 mean: 0.43 40%	4 pearl string-like	ohME oI	very small (0.03–0.06 mm), round, alined two rows along radial furrow and two conspicuous nematocysts over sense organs	ohME sE oI	present study
Carybdea morandinii	0.6–0.9, mean: 0.7	0.4–0.6 mean: 0.5	0.18–0.27 mean: 0.21 30%	4 filiform	ohME (single) AI HI	very small (0.03–0.04 mm), circular shaped, irregularly scattered over whole exumbrella	oHME rHI	Straehler-Pohl & Jarms 2011
Carybdea marsupialis	1.0–1.4, mean: 1.2	0.9–1.2 mean: 1.2	0.30–0.40 mean: 0.36 30%	2 pearl string-like	ohME (two size classes)	large (0.14 mm × 0.06 mm), ovoid, ambilateral of the interradial furrow	oHI soAI	Straehler-Pohl & Jarms 2005
Tripedalia cystophora	1.0–1.4 mean: 1.2	1.3–1.6, mean: 1.5	0.50–0.70 mean: 0.60 50%	4 pearl string-like	ohME (two size classes)	medium large (0.06, 0.08 or 0.1 mm), round, alined along radial furrows	oHI	Straehler-Pohl & Jarms 2011
Alatina moseri	1.2–1.6 mean: 1.35	1.1–1.5 mean: 1.24	0.24–0.30, mean: 0.27 20%	4 pearl string-like	ohME	small (0.06 mm), innumerable, curcular shaped, scattered in a dense, regular pattern over the whole exumbrella	ME sHI	Straehler-Pohl & Jarms 2011

ickisi have four pearl string-like tentacles similar to those of *T. cystophora* and *A. moseri*. Two categories of nematocysts (ovoid isorhizas and ovoid heterotrichous microbasic euryteles) are apparent on the tentacles of *C. sivickisi*. Only euryteles are common to all young medusae of the cubozoans. The adhesive pads are characteristic of *C. sivickisi*. The obvious similarities in all early life stages of *T. cystophora* and *C. sivickisi* support the published molecular results (Collins 2002, Gershwin 2005, Bentlage et al. 2010).

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