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Studies on Japanese Botryllid Ascidians. IV. A New Species of the Genus *Botryllus* with a Unique Colony Shape, from the Vicinity of Shimoda

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ABSTRACT—The morphology and life history of a strange and unidentified botryllid ascidian were investigated. This ascidian was first collected from the stony shore of Ebisu Island in Shimoda, a city on Izu peninsula in central Japan. Unlike other botryllid ascidians, whose colonies are flat and smooth, this ascidian's colonies are rugged. In each colony, zooids are arranged into several oval systems, each of which has a thick part containing zooids and very thin parts that do not. The arrangement of ovary and testis in this species is the same as in other species of the genus *Botryllus*; the ovary is situated anterior to the testis. The embryo of this ascidian develops in the peribranchial cavity of its mother zooid without any brooding organs, as is the case with *Botryllus scalaris* and *Botryllus puniceus*. Meanwhile, the results of cut colony assay experiments did not show the existence of colony specificity in this ascidian. Even when two syngeneic colonies were brought into contact at their growing edges, none fused together. On the other hand, when two colonies were brought into contact with each other at their cut surfaces, they always fused into a single colony, regardless of their origin. Therefore, this species may be the only species that lacks colony specificity among the botryllids studied so far.

Key words: ascidian, new species, *Botryllus*, taxonomy, colony specificity

INTRODUCTION

Botryllid ascidians are relatively common compound ascidians found on the stony or rocky shores of Japan. In 1981, we reported three new species of botryllid ascidians from the coast near Shimoda Marine Research Center (SMRC), University of Tsukuba, Shizuoka Prefecture in central Japan, and redescribed *Botrylloides violaceus* Oka 1927 in two papers (Saito *et al*., 1981a, b). In those papers, we pointed out that there was some confusion in the taxonomy of Japanese botryllid ascidians, and proposed a detailed examination of their life histories to resolve that confusion. Morphological data is not always sufficient for precise classification among botryllids, because sometimes the morphologies of their blastozooids and colonies differ from each other only slightly, except for their color.

After those two studies appeared, many colonies of botryllid ascidians were collected from the coast around Shimoda, a city near the end of the Izu Peninsula. They were cultured in a cove (Nabeta Bay) near SMRC all through the year, and their life histories and morphologies were observed. Those observations revealed many undescribed species of botryllid ascidians. Thus far, six of these have been reported as new species (Saito and Watanabe, 1985; Okuyama and Saito, 2001a, 2002; Saito and Nagasawa, 2003). On the other hand, a study of colony specificity in botryllid ascidians has revealed several variations among them in their manner of allogeneic rejection (Saito *et al*., 1994). This finding suggests that the manner of allogeneic rejection is a good characteristic by which to classify botryllid ascidians. Variations among botryllids were found also in the manner of sexual reproduction (Mukai, 1977; Saito and Watanabe, 1985; Okuyama and Saito, 2001b). That is, a species is either ovoviviparous or viviparous, it either forms brooding organs for its embryos or not, and, if it does have brooding organs, they are formed from either the peribranchial epithelium or the epithelium of the branchial sac. Besides revealing such taxonomically useful reproductive differences, those investigations pointed out that the commonly accepted definition of the genera in the family Botryllidae should be amended (Saito *et al*., 2001).

The present species was one of the undescribed botryllids collected from the coast around Shimoda. We report here on the morphology of this botryllidís colonies and zooids, and describe its life history in detail. We also report the results of investigations into the existence of colony speci-

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ficity and brooding organs in this species. We then compare the features of this species with those of other known botryllid ascidians, and discuss the phylogeny of botryllids in general.

MATERIALS AND METHODS

Animals

To observe the morphology and life history of this species, several colonies of it were collected from the stony shores of Ebisu Island in Shimoda (Shizuoka prefecture, Japan) and a cove called Nabeta Bay in front of SMRC. The distance between these collecting fields is about two linear kilometers. The collected colonies were fastened to glass slides with cotton thread and cultured in a box immersed in seawater (13–25°C) in Nabeta Bay, a natural and undisturbed environment. Colonies were cleaned every two weeks by removing other sessile organisms and mud. The morphology of each colony and its zooids was observed under a binocular stereomicroscope (Nikon SMZ-10, Japan).

Observations on morphology

Living and fixed specimens of whole colonies, larvae, and oozooids were observed under the binocular stereomicroscope. For fixation, we anesthetized the living colonies, larvae, and oozooids by immersing them in 0.32 M $MgCl_2$ for about 15 min and then transferred them to 10% formalin in filtered seawater.

For histological study, pieces of sexually mature colonies were fixed in Bouin's solution containing filtered seawater (seawater saturated with picric acid, formalin, and acetic acid at the ratios of 15:5:1). The fixed materials were dehydrated in a graded ethanol-*n*-butanol series and embedded in paraplast (Oxford Labware, USA). They were sectioned to 7 μm thick and stained with Delafield's hematoxylin and eosin gelblich (Merck, Germany). The sections were observed under a light microscope (Nikon Optiphoto, Japan).

Colony specificity

Colony specificity was examined by means of the cut colony assay (Oka and Watanabe, 1957). A small piece was dissected from each of two colonies, and the pieces were juxtaposed on a glass slide so that they were in contact with each other at either their growing edges or their cut surfaces. After incubation for 30–40 min in a moisture chamber, the slide was transferred to a laboratory tank with continuous running seawater (about 20°C). Observations of the colony specificity reaction were made every 2 hr using a binocular stereomicroscope.

The holotype and paratypes have been deposited at University of Tsukuba (TKB), Ibaraki Prefecture, Japan.

RESULTS

Botryllus horridus Saito and Okuyama n. sp.

Type-series: HOLOTYPE: a colony (TKB-anim-2364); 2.5×5.0 cm; Y. Saito; Sept. 20, 1982. PARATYPES: a colony (TKB-anim 2365); 2.5×5.0 cm; Y. Saito; Sept. 20, 1982, and larvae (TKB-anim 2366); Y. Saito; Jul. 14, 1982. The colonies of the type specimens had been cultured in Nabeta Bay in front of the center. The larvae of the paratype were released from one of the cultured colonies.

Type locality: Shimoda, Shizuoka Prefecture, Japan.

Description

In September 1980, colonies of a strange and rugged botryllid were first collected in the lower intertidal zone at the stony shore of Ebisu Island in Shimoda, a city on the end of the Izu Peninsula in Shizuoka Prefecture, located in the middle of Japan. Later, several other colonies were found in the lower intertidal zone and in the subtidal zone (0–1 m in depth) at the stony shore of Nabeta Bay, near SMRC. However, colonies of this species were not so common at the shore around Shimoda.

Colonies usually become encrusted on the surfaces of stones in shallow water at a stony shore. Though they measure less than 3–4 cm across in natural conditions, they can grow to 10 cm across in culture. The part of a colony where zooids (blastozooids) form a system is 2.5–3.5 mm thick, but parts without zooids, such as the periphery of the colony and the space between systems, are very thin, only 0.5–1.0 mm. Therefore, the surface of the colony is rugged (Figs. 1and 2), and sometimes many grains of sand settle in the gaps between systems but are not deposited on the surface of a system. Each system has oval-shaped formations of 5-18 zooids, with a common cloacal aperture in the center of the system. A colony is usually composed of many zooids, and all zooids in a colony are connected to one another by a common vascular network. The periphery of the growing part of a colony is fringed with sausage-shaped large vascular ampullae of about 500–800 μm in length and 200–250 μm in width; on the other hand, the peripheries of other parts of the colony are composed of either very small ampullae or none (Fig. 2). The tunic, covering all of the zooids and the vascular system, is gelatinous, thin and a little translucent. Colonies are dark brown when alive, and each zooid has yellow pigment cells deposited around its branchial siphon as well as on the atrial languet and along the endostyle.

Blastozooids (Fig. 3a) are 2.5–3.0 mm in length and stand almost vertically in a colony. Four large and four small branchial tentacles regularly alternate with each other, in addition, each interspace has one minute tentacle. Zooids in a mature colony have 8–11 rows of stigmata on the left side and 9–11 on the right. Usually the right side has one more stigmatal row than the left. On either side, the second stigmatal row is perfect and reaches the dorso-median line. Around the middle of the branchial sac, stigmata are arranged between the three inner longitudinal vessels as follows: dorsal lamina 4–5.2–3.2–3.4–5 endostyle (periods represent longitudinal vessels; Fig. 3a). Many blood cells are deposited along each side of the endostyle from the second to seventh rows. The anterior edge of the intestinal loop extends to the level of the eighth or ninth stigmatal row, and the anus opens at the level of the fifth to seventh rows. Most of the stomach is exposed posteriorly to the rear end of the branchial sac. The stomach is orange in fresh specimens and is furnished with nine longitudinal plications and a large curved pyloric caecum, which is connected to the circumintestinal gland region by a thick duct. The testis is situated anteriorly to the intestinal loop on the left side of the body,

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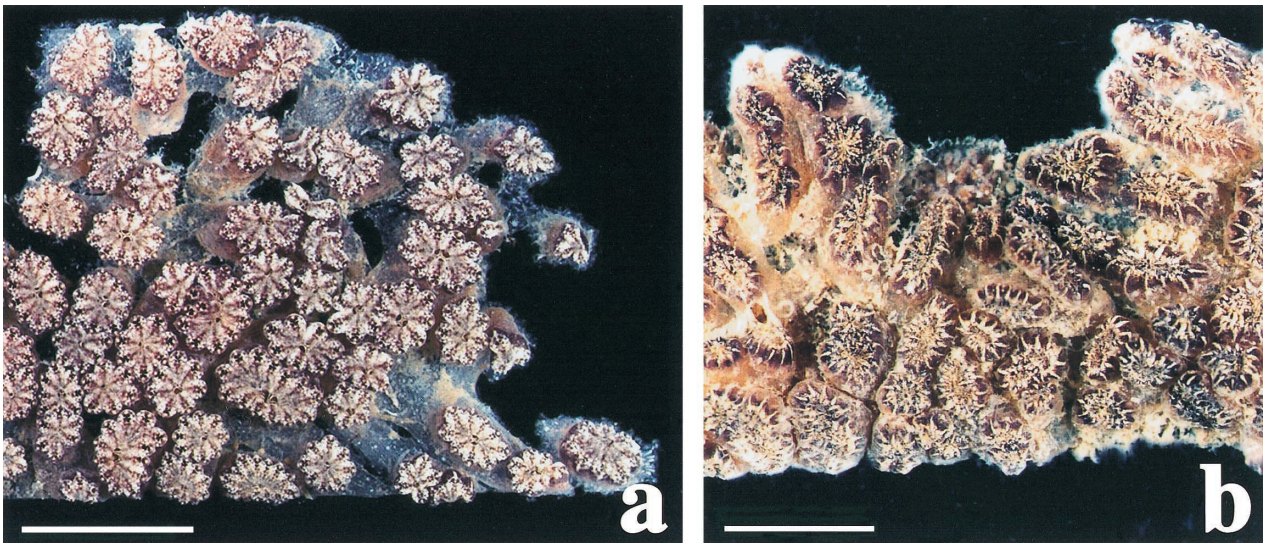


Fig. 1. Photographs of living colonies of *Botryllus horridus* n. sp. (a) A colony grown on a glass plate. (b) A colony packed with systems. In a crowded colony with systems, elongated oval systems tend to increase in number. Scale bar is 1 cm.

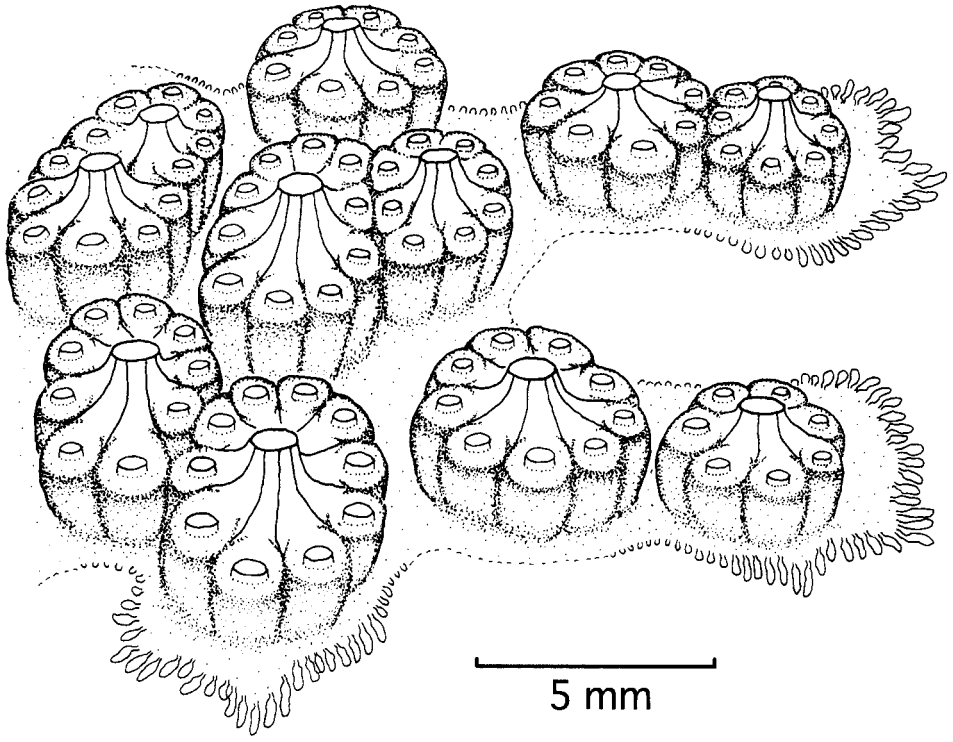


Fig. 2. *Botryllus horridus* n. sp. Peripheral region of a colony.

and at the level of the sixth to eighth stigmatal rows on the right side. It consists of 6-10 round lobes gathered into a rosette, and is grayish in fresh specimens. The ovaries are situated anteriorly to the testis, one ovary on each side. Usually a single egg matures on each side of the body, though sometimes there are two eggs on a side. Mature eggs are yellowish orange and measure 250–260 μm in diameter just before ovulation.

The larva (Fig. 3b) is about 1.7–1.8 mm in total length and yellowish orange when alive. The trunk has an oval outline about 400–430 μm long and 280–290 μm wide, and it includes a single photolith, which is typical among botryllids. Three attachment processes are arranged in a triangle at the anterior edge of the trunk, and eight ampullae surround the anterior half of the trunk. The primordial branchial and atrial siphons are not recognizable in the trunk at this stage.

Asexual peribranchial budding can be observed throughout the year. Usually a single bud is produced on

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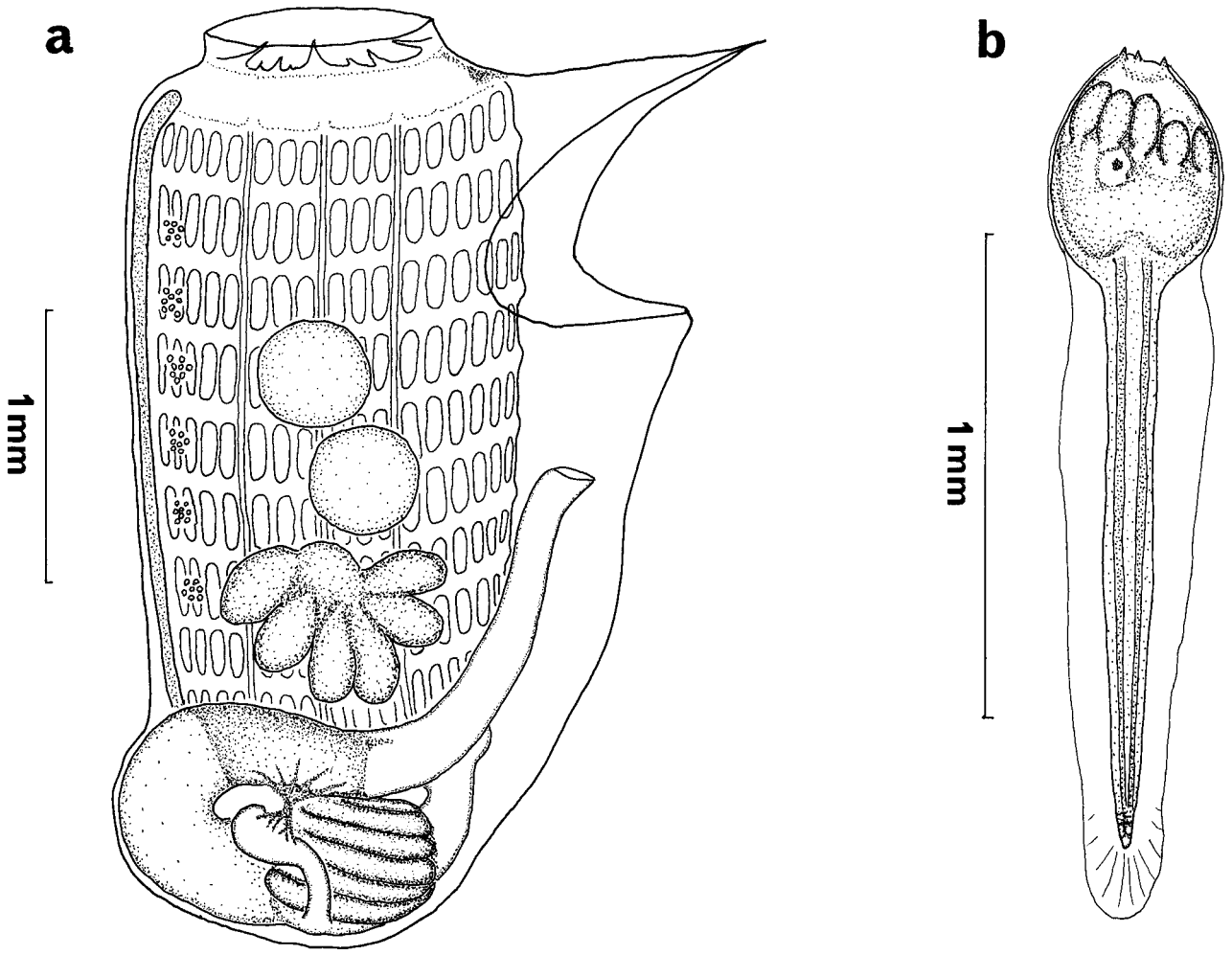


Fig. 3. *Botryllus horridus* n. sp. (a) A zooid from the left side. (b) A larva from the right side.

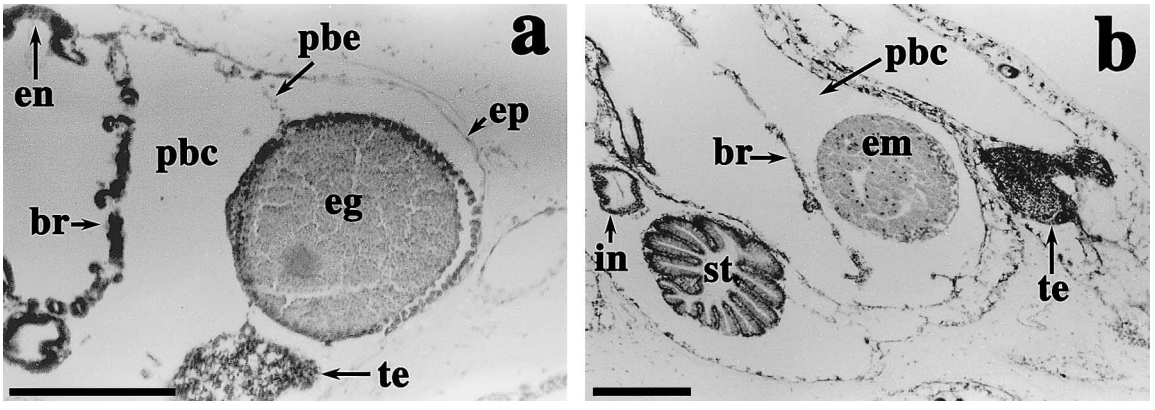


Fig. 4. Cross-sections of a bud and a functional zooid of *Botryllus horridus* n. sp. (a) A mature egg before ovulation in an ovary of a bud. Neither the peribranchial epithelium nor the branchial sac forms any brooding organs. (b) An embryo developing in the peribranchial cavity of a zooid. There is no brooding organ around the developing embryo. br, branchial sac; eg, egg; em, embryo; en, endostyle; in, intestine; pbc, peribranchial cavity; pbe, peribranchial epithelium; st, stomach; te, testis. Scale bar is 200 μm.

each side of the body, and rarely two. All functional zooids in a colony are replaced synchronously by asexually derived new blastozooids of the next generation. The period of alternation of generations is about 10 days. Sexual reproduction occurs in the summer, from July to September (22–25°C). The ovarian egg in a bud will ovulate after the bud has grown into an adult zooid, to take the place of its degenerated parent zooids. The egg at this time is about 250–260

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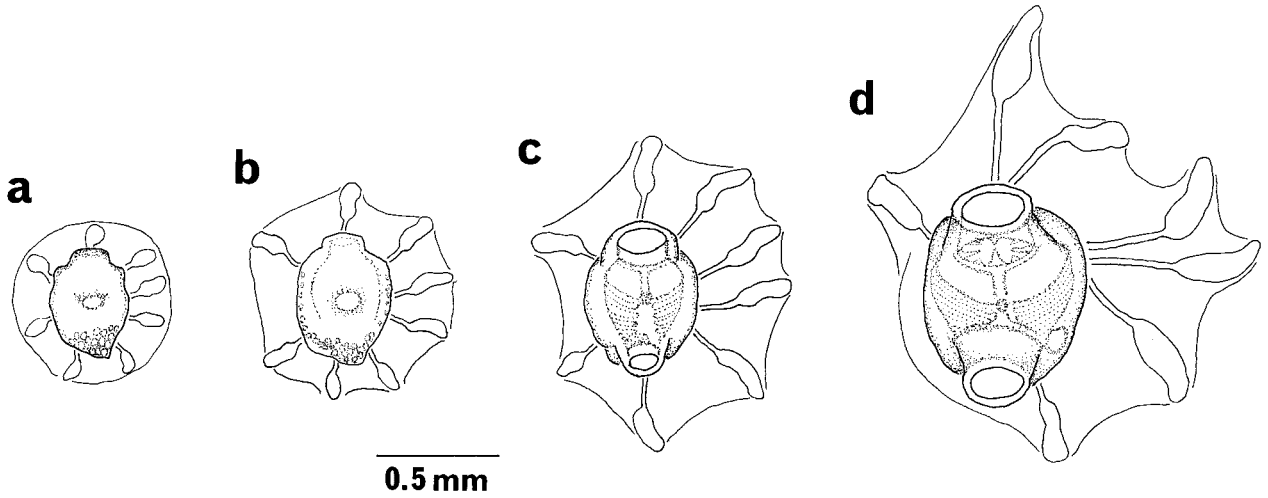


Fig. 5. *Botryllus horridus* n. sp. Stages in the metamorphosis from larval attachment to a functional oozooid. (a) An oozooid, three hr after larval attachment. Tail absorption is finished and the primordial branchial and atrial siphons are recognizable. (b) The same, six hr after attachment. Morphogenesis of the organs is proceeding, and the branchial sac has become visible through the tunic. (c) The same, 20 hr after attachment. Metamorphosis is almost complete. (d) The same, 44 hr after attachment. Both branchial and atrial siphons open, and on the right side of the branchial sac the first pallial bud is formed.

μm in diameter. The egg ovulates in the peribranchial cavity and is fertilized there. In this species the embryo is free in the peribranchial cavity; that is, there are no brooding organs to protect it (Fig. 4). About 8 days after ovulation the embryo has become a fully developed larva with a diameter of about 350 μm.

Fully developed larvae synchronously swim out from the colony in the morning, from 11 a.m. to noon, before the degeneration of their parent zooids. Two to three hr after liberation, the larva becomes attached to the substratum by three attachment processes, and then extends its eight ampullae to complete the attachment, after which its metamorphosis into a primary zooid begins (oozooid; Fig. 5a–d). Soon after the larval attachment, the absorption of the larval tail begins. About an hour after attachment, primordial siphons become recognizable. The morphogenesis of the oozooid is completed about 20 hr after the larval attachment. Both branchial and atrial siphons open about one day after attachment, and 30–36 hr after attachment a small vesicle, which is the first bud, appears on the right side of the heart. The oozooid has five protostigmata on each side of the branchial sac. The oozooid takes its place with the first blastozooids about 9–10 days after its siphons open.

Colony specificity in the present species

Five colonies of different origin were used. In preparation for the cut colony assay, each colony was cut into several pieces, which were then reared in the bay to prepare several clones from each colony. The cut colony assay in this botryllid was very difficult, because at the colony margin its tunic was very thin and was damaged easily by peeling from a glass plate. Therefore, repetitive trials could not be performed well. Of 15 combinations, 11 (3 syngeneic and 8 allogeneic) were used to study contact between growing edges, and the other 4 (2 syngeneic and 2 allogeneic) were used to study contact between cut edges. When two allogeneic colonies came into contact with each other at their growing edges, neither fusion nor rejection occurred between them in any of the trials. In the cross-sections of the contact area, destruction of both tunic cells and infiltrated blood cells in the subcuticular region of the tunic, which is the characteristic of the subcuticular rejection (Hirose *et al*., 1988), was not recognized (data not shown). Even when two syngeneic colonies were brought into contact at their growing edges, none fused together. On the other hand, when two colonies were brought into contact with each other at their cut surfaces, they always fused into a single colony, regardless of their origin.

Remarks

The colony of the present species is rugged, whereas colonies of most botryllid ascidians are flat and smooth. This colony form is a unique feature of the present species. Only two species reported thus far have a similar appearance: *Botryllus compositus* (Tokioka, 1967) and *Myxobotrus japonicus* (Oka, 1931). In *B. compositus*, a colony consists of a number of cormidia, and each cormidium is 14–15 mm in thickness and composed of several circular systems of zooids. The surface of a colony of *B. compositus* is encrusted with fine sand grains. However, in the present species a cormidium is 2.5–3.5 mm in thickness and formed by usually one and sometimes two systems of zooids, and sand grains are found only in the hollows between systems. Furthermore, the vascular system of *B. compositus* does not develop, though it develops well in the present species. The colony shape of *M. japonicus* also resembles that of the present species, but in *M. japonicus* zooids do not form systems of blastozooids, and neither the vascular network nor

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the ampullae are formed in the colony.

In a zooid of the present species, the second row of stigmata is perfect and reaches the dorso-median line. However, the second stigmatal row never reaches the dorso-median line in most Japanese botryllids, whose zooids have more than five stigmatal rows, except in *Botryllus magnicoecus* (cf. Nishikawa, 1991). In the two species having only four rows of stigmata, *Botryllus primigenus* (Oka, 1928) and

Table 1. Characteristics in morphology and life history in twelve botryllid ascidians with more than five stigumatal rows

	Botryllus						
	B. horridus	*B. scalaris*	*B. puniceus*	*B. sexiens*	*B. delicatus*	*B. schlosseri*	*B. promiscuus*
Colony							
Shape	rugged	flat	flat	flat	flat	flat	flat
Thickness (mm)	2.5–3.5	1.0–1.5	0.8–2.2	2.5–3.0	2.5–4.0	1.0–1.5	1.5–2.0
Arrangement of zooids	orval	ladder	ladder	ladder	ladder	orval	ladder
Tunic	a little translucent, gelatinous	transparent, gelatinous, soft,	transparent, gelatinous, soft,	transparent, gelatinous, extremely soft	transparent, gelatinous, extremely soft	transparent, gelatinous, soft,	transparent, gelatinous, soft,
Zooid							
Length (mm)	2.5–3.0	1.5	2.8–3.8	2.5–3.0	2.5–3.0	2.0–2.8	1.5–2.0
Number of tentacles (Large), (Small), (Minute)	L4, S4, M8	L4, S4	L4, S4	L6, S6	L4, S4	L4, S4	L4, S4
Number of stigmatal rows	R9-11, L8-11	8	8–11	9–10	9–10	8	12–13
Second row of stigmata	complete	incomplete	incomplete	incomplete	incomplete	incomplete	incomplete
Number of longitudinal vessels	3	3	3	3	3	3	3
Number of stomach plication	9	8–9	9–10	11	8–9	8	8–9
Pyloric caecum	large	large	large	large	large	large	large
Asexual reproduction							
Peribranchial budding	all seasons	all seasons	all seasons	all seasons	all seasons	all seasons	all seasons
Number of buds in each zooid	R1-2, L1-2	R1, L1	R1-2, L1	R1, L1	R1, L1	R1-2, L 1-2	R1, L1
Vascular budding	infrequently	infrequently	sometimes	infrequently	sometimes	infrequently	infrequently
Cycle of takeover	10 days	5–6 days	about one week	5–6 days	about one week	about one week	about one week
Sexual reproduction							
Breeding season	Jul-Sep	Jun-Nov	Apr-Jun	Jul-Dec	Jul-Dec	Mar-Nov	Jul-Dec
Arrangement of gonads (position of ovary)	anterior to the testis	anterior to the testis	anterior to the testis	anterior to the testis	anterior to the testis	anterior to the testis	anterior to the testis
Testis shape & color	rosette, grayish	rosette, grayish	rosette, milky-white	rosette, grayish	rosette, grayish	rosette, white opalescent	rosette, grayish
Color of eggs	yellowish orange	orange	orange	yellow	yellow	yellowish orange	orange
Production of larvae	ovoviviparous	ovoviviparous	ovoviviparous	ovoviviparous	ovoviviparous	ovoviviparous	ovoviviparous
Mature egg diameter (μm)	250–260	220	240	150	250	230–250	230–250
Number of mature eggs	R1-2, L1-2	R1-2, L1-2	R1-5, L1-5	R2, L2	R1-2, L1-2	L &R: 1-3	R1, L1
Brooding organ	no brooding organ	no brooding organ	no brooding organ	pouch-like organ formed from branchial sac	pouch-like organ formed from branchial sac	oviducal cup formed from atrial wall	pouch formed from atrial wall
Embryo size just before hatching (μm)	350	–	–	–	–	280–300	–
Development to larvae	in 8 days	in 4–5 days	in one week	in 4-5 days	in one week	in one week	in one week
Release of larvae	shortly before degeneration of parent zooid	shortly before degeneration of parent zooids	shortly before degeneration of parent zooids	shortly before degeneration of parent zooids	shortly before degeneration of parent zooids	shortly before degeneration of parent zooids	shortly before degeneration of parent zooids
Larva							
Length (mm)	1.7–1.8	1.5	1.5	1.5–1.8	1.5	1.6	1.5–1.8
Length of larval trunk (μm)	400–430	400	350	440	400	400	400
Number of larval ampullae	8	8	8	6	8	8	8
Oozooid							
Length (μm)	520	–	500	–	750	500	900
Number of tentacles	L4	–	L4	–	L4. S4	–	L4. S4
Number of protostigmata	5	–	4	–	8–10	L4, R4-5	9–10
Number of stomach plication	4–5	–	4–5	–	5	5	5
Number of buds	R1	R1	R1	R1	R1	R1	R1
References	Present work	Saito et al., 1981a	Saito & Nagasawa, 2003	Saito et al., 1981a	Okuyama & Saito, 2001a	Boyd et al.,1990	Okuyama & Saito, 2002

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Botryllus communis (Oka, 1927), all stigmatal rows are perfect and reach the dorso-median line. In *B. magnicoecus*, the second stigmatal row is also complete, but the tunic of its colony is tough and leathery and the colony is not rugged, unlike the present species. Thus, this feature of a complete second row seems to be uncommon in Japanese botryllid ascidians, and it is a good taxonomic marker of the present species.

In the present species zooids do not form any brooding organs for their developing embryos. It has already been shown that in *Botryllus scalaris* (Saito *et al*., 1981a) and *Botryllus puniceus* (Saito and Nagasawa, 2003) zooids never form any brooding organs for their embryos. However, the colonies of these two species are thin, flat and smooth. Furthermore, all botryllids studied so far have shown colony specificity, fusion or rejection, when two allogeneic colonies came into contact with each other at their growing edges, whereas the present species did not show colony specificity. In the case of growing-edge contact, fusion of the tunics never occurred between two allogeneic colonies; fusion never occurred between two syngeneic colonies, either. On the other hand, in the case of cut surface contact, fusion always occurred, whether between two allogeneic colonies or two syngeneic colonies. Those findings may show a typical case of the absence of colony specificity in colonial ascidians.

It is likely that the present species may safely be defined as a new species. It may also be that none of the species reported thus far are related to the present species. Thus, we classified this new ascidian as a member of *Botryllus* because of its arrangement of gonads and named it after the rugged appearance of its colonies—*Botryllus horridus* Saito and Okuyama n. sp.

Etymology

The specific name, *horridus*, is derived from the Latin for rough, referring to the appearance of the species' colonies.

Botrylloides				
B. simodensis	*B. lentus*	*B. fuscus*	*B. lenis*	*B. violaceus*
flat	flat	flat	flat	flat
2.0–3.0	3.0–3.5	2.5–3.0	1.7–2.0	2.0-3.0
ladder	ladder	ladder	ladder	ladder
transparent, gelatinous, soft,	translucent, gelatinous, soft	translucent, gelatinous, soft	tranparent, gelatinous, very soft	transparent, gelatinous, soft,
2.0–2.5	3.1–3.5	2.2–2.7	2.0–2.3	2.5-3.0
L4, S4, M1-2	L4, S4, M1-2	L4, S4	L4, S4	L4, S4, M2-3
11	18	9	R9, L8	10-11
incomplete	incomplete	incomplete	incomplete	incomplete
3	3	3	3	3
9	9	8	8	9
small	small	small	small	small
all seasons	all seasons	all seasons	all seasons	all seasons
R1, L1	R1, L1	R1, L1	R1, L1	R2, L2
infrequently	infrequently	infrequently	sometimes	infrequently
5–6 days	7–9 days	7–9 days	6–7 days	5-6 days
Jul–Sep	Jul–Aug	Jul–Sep	Jul–Oct	May–Aug
posterior to the testis	posterior to the testis	posterior to the testis	posterior to the testis	posterior to the testis
rosette, grayish	rosette, grayish	rosette, grayish	rosette, grayish	rosette, grayish
yellowish orange	orange	orange	pinkish or orange	yellow
ovoviviparous	viviparous	viviparous	viviparous	viviparous
250	300-330	160-200	90-100	<100
R1, L1	R1, L1	R1, L1	R1, L1	R1, L1
pouch formed from atrial wall	pouch formed from atrial wall	pouch formed from atrial wall	pouch formed from atrial wall	pouch formed from atrial wall
300	1000-1100	800-1000	750-800	1000-1200
in 4–5 days	in 10–12 days	in 12–14 days	in 20 days	more than one month
shortly before degeneration of parent zooids	shortly before degeneration of parent zooids	5 days after degeneration of parent zooids	2 weeks after degeneration of parent zooids	after 6-7 times of takeover of blastozooids
1.7–2.0	2.5–2.7	2.6–2.7	2.2–2.4	3
500	800	900	700	1000
8	8	8	14–24	24-34
–	–	–	–	–
–	–	–	–	–
–	–	–	8	–
–	–	–	–	–
R1	R1, L1	R1, L1	R1	R2, L1
Saito et al., 1981b	Saito & Watanabe, 1985	Saito & Watanabe, 1985	Saito & Watanabe, 1985	Saito et al., 1981b

DISCUSSION

The present species has very unique features. The colony is rugged, whereas most botryllid ascidians around Shimoda are flat and smooth when cultured on glass plates. Furthermore, the second stigmatal row is complete in this species. This feature is uncommon; among botryllid ascidians around Shimoda, it is found in only two of the species that have four stigmatal rows, *Botryllus primigenus* and *Botryllus communis*. In addition, the most impressive feature of the present species is the absence of colony specificity. In the present species, fusion never occurred even when two colony pieces prepared from the same colony came into contact with each other at their growing edges. In the case of cut surface contact, on the other hand, colonies always fused even when both colonies were allogeneic. In colonial sessile animals, it is believed that fusion between two syngeneic colonies at their growing edges is the essential condition for the possession of colony specificity. Although a sufficient number of experimental trials have yet to be performed, it appears that the present species lacks colony

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specificity. Colony specificity is shown in *Symplegma reptans* of the family Styelidae, which is considered to be most closely related to botryllid ascidians (Mukai and Watanabe, 1974; Shirae *et al*., 1999). Colony specificity is also found in other groups of colonial ascidians, such as Perophoridae (Freeman, 1970; Koyama and Watanabe, 1981; 1982) of the suborder Phlebobranchia, Polyclinidae (Freeman, 1970; Watanabe and Taneda, 1982) and Didemnidae (Mukai and Watanabe, 1974) of the suborder Aplousobranchia. Each of these groups contains species with colony specificity and species without it. Therefore, the acquisition of colony specificity might have occurred in each of those groups during the evolution of colonial ascidians. Among botryllid ascidians, all species examined thus far have shown colony specificity, except for the present species. This suggests the possibility that colony specificity in botryllid ascidians was acquired after the progenitor of botryllid ascidians separated from other compound styelid ascidians and the present species without colony specificity is the most primitive species of botryllid ascidians. This also suggests the other possibility that the progenitor of botryllid ascidians possessed colony specificity and its colony specificity was lost during the evolution into the present species.

Many botryllid colonies have been collected from the vicinity of Shimoda and cultured in the cove near SMRC. We have observed their morphologies and life histories, and examined them for colony specificity. These studies have allowed us to find many unknown species of botryllid ascidians. The results of these studies have shown that many species of botryllid ascidians are living around Shimoda, including the following 14: *Botryllus communis*, *Botryllus primigenus*, *Botryllus horridus* (the present species), *Botryllus puniceus*, *Botryllus scalaris*, *Botryllus sexiens*, *Botryllus delicatus*, *Botryllus schlosseri*, *Botryllus promiscuus*, *Botrylloides simodensis*, *Botrylloides lentus*, *Botrylloides fuscus*, *Botrylloides lenis*, and *Botrylloides violaceus*. From these studies we have accumulated a good deal of data on the species, life histories, on their morphologies (colonies, zooids, larvae, and oozooids), on their manners of sexual reproduction, and on their colony specificity, as well as on the local ecology. Those data demonstrated that the definitions of the two genera proposed by the predecessors of the botryllid taxonomy are not applicable to all botryllid ascidians. According to the definitions of the two genera by Van Name (1945), in the genus *Botryllus* the ovary is situated anterior to the testis and there is no brooding organ, whereas in the genus *Botrylloides* the ovary is situated posterior to the testis and the embryo develops in an incubatory pouch formed from the atrial wall. His definitions of the two genera have been used for the classification of botryllid ascidians for a long time, but his definition cannot be applied to *B. primigenus* (Mukai, 1977), *B. sexiens* (Okuyama and Saito, 2001b), *B. delicatus* (Okuyama and Saito, 2001a, 2001b) or *B. promiscuus* (Okuyama and Saito, 2002). Kott (1985) also defined these two genera according to the place of embryogenesis. From her definition, in *Botryllus* embryos develop in the peribranchial cavity of the parent zooid, and in *Botrylloides* the embryo develops in the protruded incubatory pouch from the atrial wall. She also referred to the arrangement of gonads, but in her definition the ovary of *Botrylloides* is anterior to the testis, as it is in *Botryllus*. Therefore, neither the genus *Botryllus* nor the genus *Botrylloides* is applied to many species of *Botrylloides*, in which the ovary is situated posterior to the testis as in *B. simodensis* (Saito *et al*., 1981a), *B. lentus* (Saito and Watanabe, 1985), *B. fuscus* (Saito and Watanabe, 1985), *B. lenis* (Saito and Watanabe, 1985) and *B. violaceus* (Saito *et al*., 1981a). Monniot and Monniot (1987) proposed that the genus *Botrylloides* should be united with the genus *Botryllus* under the name of the latter. According to them, the characteristics used by Van Name and Kott to define these two genera are not so important, and thus there is no reason to divide botryllid ascidians into two genera. However, we cannot accept their opinion unconditionally, because their report did not clearly explain, on the basis of scientific fact, why the characteristics they cited were unimportant. Therefore, we have temporarily used a part of Van Name's definition of the two genera (the arrangement of gonads) to classify botryllid ascidians found around Shimoda into two genera *Botryllus* and *Botrylloides*.

The accumulated biological data on the botryllid ascidians around Shimoda made it possible to guess the phylogenies of botryllid ascidians. The group of botryllid ascidians with only four stigmatal rows, *B. primigenus* and *B. communis*, should be separated from the group of botryllid ascidians that have more than five rows since, as mentioned before, there are many big differences between these two groups (Mukai, personal communication; Saito *et al*., 2001). The latter group of botryllid ascidians includes both *Botryllus* species and *Botrylloides* species. The characteristics in morphology and life history of the twelve species of this group living around Shimoda are summarized in Table1. From this table, it is difficult to find any good reasons for dividing this group into two groups, the group of *Botryllus* species and the group of *Botrylloides* species, because the clear difference between them is only the arrangement of gonads. It may be possible to divide this group into smaller groups on the basis of other characteristics, but in this case the taxonomic class between these small groups and the group of botryllid ascidians with four stigmatal rows is not the same. Therefore, the group of botryllid ascidians with more than five stigmatal rows should be dealt with as a single group. This group includes *Botryllus schlosseri,* the type species of the genus *Botryllus*, and the genus name *Botryllus* is older than *Botrylloides*, so the group would be named the genus *Botryllus*. Then, the group of botryllid ascidians with four stigmatal rows would need a new genus name. These two groups might have separated from each other in the early stage of the evolution of botryllid ascidians. The present species belongs to the group of botryllid ascidians with more than five stigmatal rows, and the present species may be one of the most primitive species because it does

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not form any brooding organs. Therefore, the reason why the present species lacks colony specificity is very important to build the botryllid phylogeny. As mentioned above, there are two views about that reason, but nobody knows which view is better. The molecular phylogeny may give us a clue to solve that question. However, the present information on the molecular features of rDNA, mtDNA or isozymes is not enough to build the molecular phylogeny of botryllid ascidians. We therefore cannot say conclusively whether or not the botryllid phylogeny built from the biological data accumulated in Shimoda is appropriate.

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