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Studies on Japanese Botryllid Ascidians. I. A New Species of the Genus *Botryllus* from the Izu Islands

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ABSTRACT—An investigation was made of the morphology and life history of an unidentified botryllid ascidian that was first collected from the stony shore of Shikine Island of the Izu Islands in Japan. This species has a very soft and transparent tunic, which is different from that of other botryllids. The arrangement of ovary and testis in this species is the same as those of other species of *Botryllus*. The embryo develops in the sac-like brooding organ formed in the peribranchial cavity of a zooid. Some colonial parts of significant size that include only the vascular system (i.e., without zooids) often extend from the colony margins, and in these parts vascular budding often occurs. Then, the processes and features of the allorecognition reaction of this ascidian were observed. Allorejection occurred after fusion of the vascular systems between two incompatible colonies. This feature is also observed in *Botryllus scalaris*, but it takes this ascidian much longer to initiate the allorejection reaction than *B. scalaris* after fusion of blood vessels between two incompatible colonies. It was concluded, on the basis of this study, that this ascidian should be designated as a new species belonging to the genus *Botryllus*.

INTRODUCTION

Botryllid ascidians are relatively common compound ascidians at stony or rocky shores in Japan. They belong to the family Botryllidae, which is composed of two genera, *Botryllus* and *Botrylloides*. Many species of botryllids live at the coast near Shimoda Marine Research Center (SMRC), University of Tsukuba, and six new species of botryllids were reported at this location (Saito *et al*., 1981a, b; Saito and Watanabe, 1985). However, some botryllids are still not identified and their features have not been studied in detail. As pointed out by Saito *et al*. (1981a, b), to classify botryllids precisely, it is necessary to study their life history as well as morphology, because sometimes there are only slight differences in the morphology of their blastozooids and colonies. Therefore, we have cultured several colonies of unknown species in the bay near SMRC and observed their morphology and life history throughout the year.

It is known that some botryllid ascidians exhibit colony specificity, which is an allorecognition reaction observed in many colonial forms of animals. When a colony comes into contact with another colony of the same species at their growing edges, the two colonies either fuse (fusion), or do not fuse (rejection). The processes and features of allorecognition reactions have been studied in six botryllid ascidians, *Botryllus scalaris* (Saito and Watanabe, 1982; Shirae *et al*., 1999), *Botryllus primigenus* (Tanaka and Watanabe, 1973), *Botryllus schlosseri* (Boyd *et al*., 1990), *Botrylloides simodensis* (Hirose *et al*., 1997), *Botrylloides fuscus* (Hirose *et al*., 1997) and *Botrylloides violaceus* (Hirose *et al*., 1988). The processes of fusion are the same in all botryllids examined thus far, but there are some differences in the processes of rejection reactions among botryllids. Therefore, variations in the allorejection process might become a good feature for the classification of botryllids (Boyd *et al*., 1990).

In the present study, we observed the morphology and life history of an unknown species. We also examined the process of allorejection in colony specificity of this species. Then, we compared the results of this study with the features of other known botryllid ascidians.

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MATERIALS AND METHODS

Animals

For the observations on morphology, life history, and colony specificity, several colonies of this species were collected from the stony shore in the lower intertidal zone in Shimoda (Shizuoka prefecture, Japan) and Shikine Island (Izu Islands, Japan). Collected colonies were fastened to glass slides with cotton thread, and cultured in a box immersed in Nabeta Bay near SMRC (13–25°C). Colonies were cleaned every two weeks, and their morphology was observed under the stereomicroscope.

Observations on morphology

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

For histological study, pieces of sexually mature colonies were fixed in Bouin's solution made in filtered seawater (seawater saturated with picric acid, formalin, and acetic acid at the ratio 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 at 7 μm and stained with Delafield's hematoxylin and eosin G. The sections were observed under a light microscope.

Colony specificity

Colony specificity was examined by means of the cut colony assay (Oka and Watanabe, 1957a). A small piece was dissected from each of two colonies, and then the two colony pieces were placed in juxtaposition on a glass slide to allow them to contact each other at their growing edges. After incubation for 30–40 min in a moisture chamber, the slide was transferred to a laboratory tank with continuous running seawater (about 15°C). Observations of the colony specificity reaction were made every 2 hr using a binocular stereomicroscope. The timing and details of tunic fusion, ampullar fusion or deterioration, and blood cell behavior were recorded as the two colonies underwent fusion or nonfusion.

The holotype and paratypes are deposited in SMRC.

RESULTS

Botryllus delicatus *Okuyama and Saito n. sp.*

Type series: HOLOTYPE: a colony (TKB-anim.1001); 7.5 by 8.0 centimeters; Y. Saito; 7 Jun. 1983. PARATYPES: colonies (TKB-anim.1002); Y. Saito; 20 Oct. 1983, larvae (TKB-anim.1003); Y. Saito; 31 Oct. 1983, and oozooids (TKB-anim.1004); Y. Saito; 2 Nov. 1983. Each colony of the type specimens (HOLOTYPE and PARATYPE) was one of offsprings of the colonies collected from the surfaces of stones in the lower intertidal zone of Shikine Island of the Izu Islands (1978) and had been cultured in a box immersed in Nabeta Bay near SMRC. The larvae and oozooids were derived from those offspring colonies.

Type locality: Shikine Island of the Izu Islands, Japan.

Other locations: Nabeta Bay in Shimoda and Habu Harbor of Oshima Island of the Izu Islands, Japan.

Biology and Morphology: Some small colonies of this new ascidian were first collected in 1978 at Kama-no-shita, the stony shore of Shikine Island of the Izu Islands. Colonies of this ascidian are usually found encrusting the surface of stones and rocks in the lower intertidal zone. They often compete with colonies of *Botryllus scalaris*, *Botryllus primigenus*, and *Botrylloides simodensis* in that habitat. Colony size varies from a small disc of a few millimeters in diameter to a large sheet about 10 cm across. Colony thickness is usually 2.5–3.0 mm and sometimes attains 4.0 mm. The colony surface is generally flat and free of foreign matter. The gelatinous tunic is extremely soft, transparent, and colorless. The color of live colonies is basically yellow. In addition, orange, white, or purple pigment cells (a type of blood cells) are sometimes deposited around the branchial siphon and on the atrial languet of respective zooids (Fig. 1a). A colony is composed of many zooids, called blastozooids, which are arranged in ladder systems with several common cloacal apertures in the common tunic. They are always connected with one another by a common vascular system. The periphery of the colony is fringed with sausage-shaped vascular ampullae about 1500 μm in length and 300 μm in width (Fig. 1a, e). Some colony parts of significant size that include only the vascular system often extend out from the colony margin (Fig. 1b, f). In that extending part, the blood vessels are very crowded, and the internal vascular network is rather obscure. Vascular budding (noted by Oka and Watanabe, 1957b, 1959) often occurs in this part of the colony (Fig. 1c, g). When colonies are exposed to unfavorable conditions, such as high (>25°C) or low (<13°C) temperature of seawater, their blastozooids degenerate and only the vascular system remains (Fig. 1d, h). These colonies consisting only of vascular systems can live for more than one month, and they begin to recover by vascular budding if the surrounding environmental conditions improve sufficiently.

Zooids (Fig. 2a) are 2.5–3.0 mm in length and situated more or less vertically, although obliquely in the periphery. The branchial tentacles of each zooid consist of four large and four small, regularly alternating. The ciliated groove forms a small round opening. There are 9 to 10 rows of stigmata on each side; the second row never reaches the dorso-median line. Around the middle of the branchial sac, stigmata are arranged between the three inner longitudinal bars as follows: dorsal 4.2–3.3.3 endostyle. The anterior edge of the intestinal loop attains anteriorly the level of the eighth transverse vessel, and the anus opens at the level of eighth transverse vessel. Many blood cells are deposited along each side of the endostyle in the range from the second to the eighth or ninth stigmatal row. Most of the stomach is exposed posterior to the rear end of the branchial sac. The stomach is orange in fresh specimens and has eight or nine longitudinal plications and a very elongate pyloric caecum.

Asexual reproduction occurs throughout the year. In a colony, bud development is synchronized. Usually, a single bud is produced on each side of a zooid. The cycle of the alternation of generations (known as takeover) is about one week. Sexual reproduction can be observed from July to December, with a peak in August (16–25°C). The testis is situated along the anterior edge of the circum-intestinal gland area on the left side and at the level of the eighth row of stigmata on the right side, and posterior to the ovary. It consists of several (5–8) lobes forming a rosette. Eggs mature in the ovary of a bud during bud development and reach a maximum size of 250 μm just before takeover. Usually, a single egg (sometimes two) matures on each side of the body. Fully mature eggs are yellow, and are ovulated when new blastozooids open their branchial siphons. The release of sperm occurs about two days after ovulation in the same zooids. Ovulation and sperm release occur synchronously in all zooids of a colony. The eggs are ovulated into the brooding organs formed with the expansion of the branchial sac to the ovary and peribranchial epithelium (Fig. 3) and are fertilized there. In that brooding organ, the fertilized egg develops into a tadpole larva for about one week and the larva swims out of the parent colony before degeneration of the parent zooid. Larvae usually swim out from their parent colony between 9 a.m. and noon. The larva (Fig. 2b) is about 1.5 mm in total

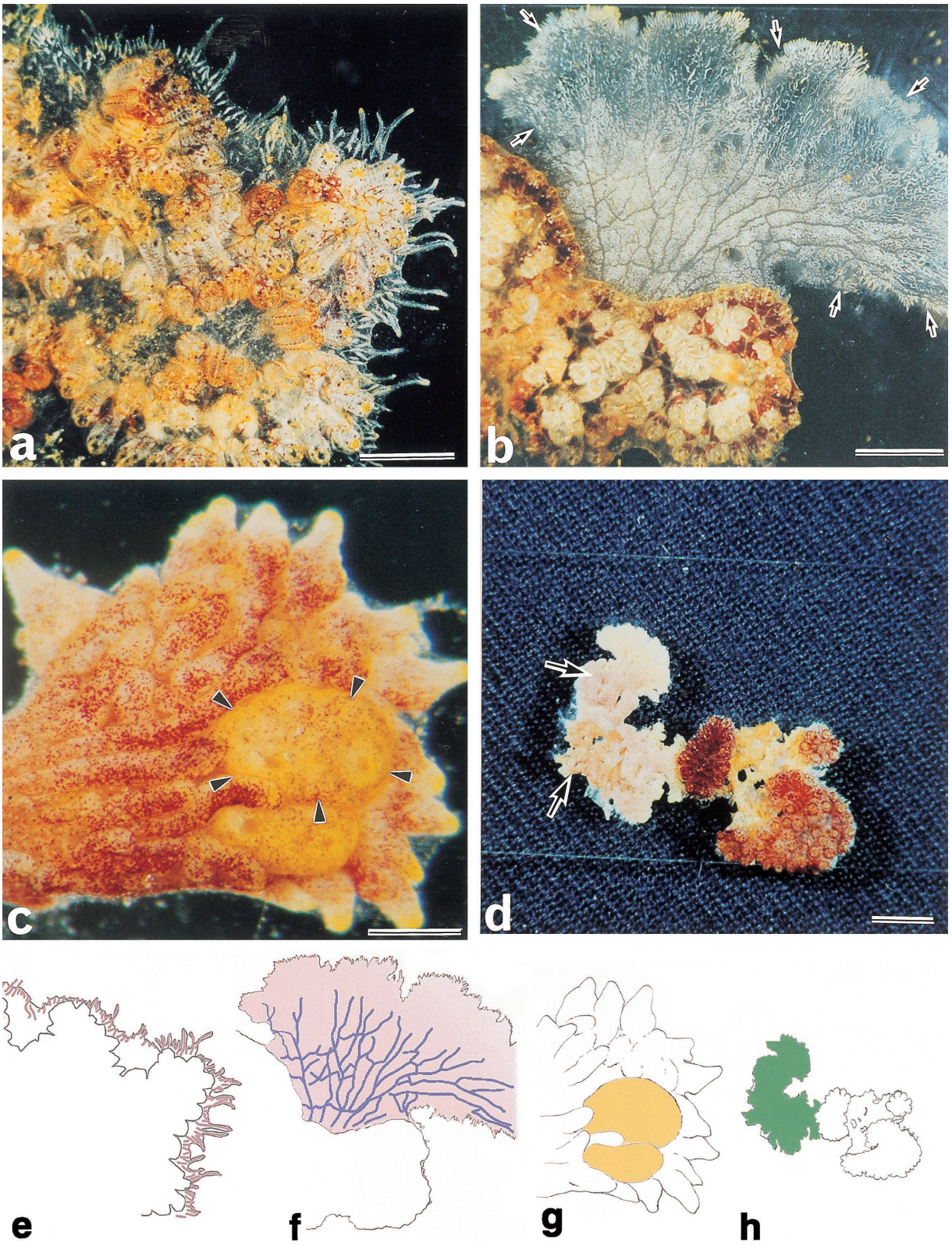

Fig. 1. Photographs of living colonies of *Botryllus delicatus* n. sp. (a)–(d), different colonies. (e)–(h), traced drawings of (a)–(d). (a) Colony. The periphery of the colony is fringed with sausage-shaped vascular ampullae. (b) Vascular system extending from the colony margin (arrows indicate the extending vascular system). This part was crowded with blood vessels. (c) Vascular buds in the margin of the extending vascular system (arrowheads indicate a bud). (d) Degenerating colony. Densely packed vascular system remains after the degeneration of zooids (arrows). Ampullae of the colony margin become obscure. (e) Tracing of (a). Vascular ampullae are shown in brown. (f) Tracing of (b). Main blood vessels are shown in blue line. Vascular system is shown in pink. (g) Tracing of (c). Vascular buds are shown in yellow. (h) Tracing of (d). Degenerating area is shown in green. Scale bars are 5 mm in (a), (b), and (d) and 1 mm in (c).

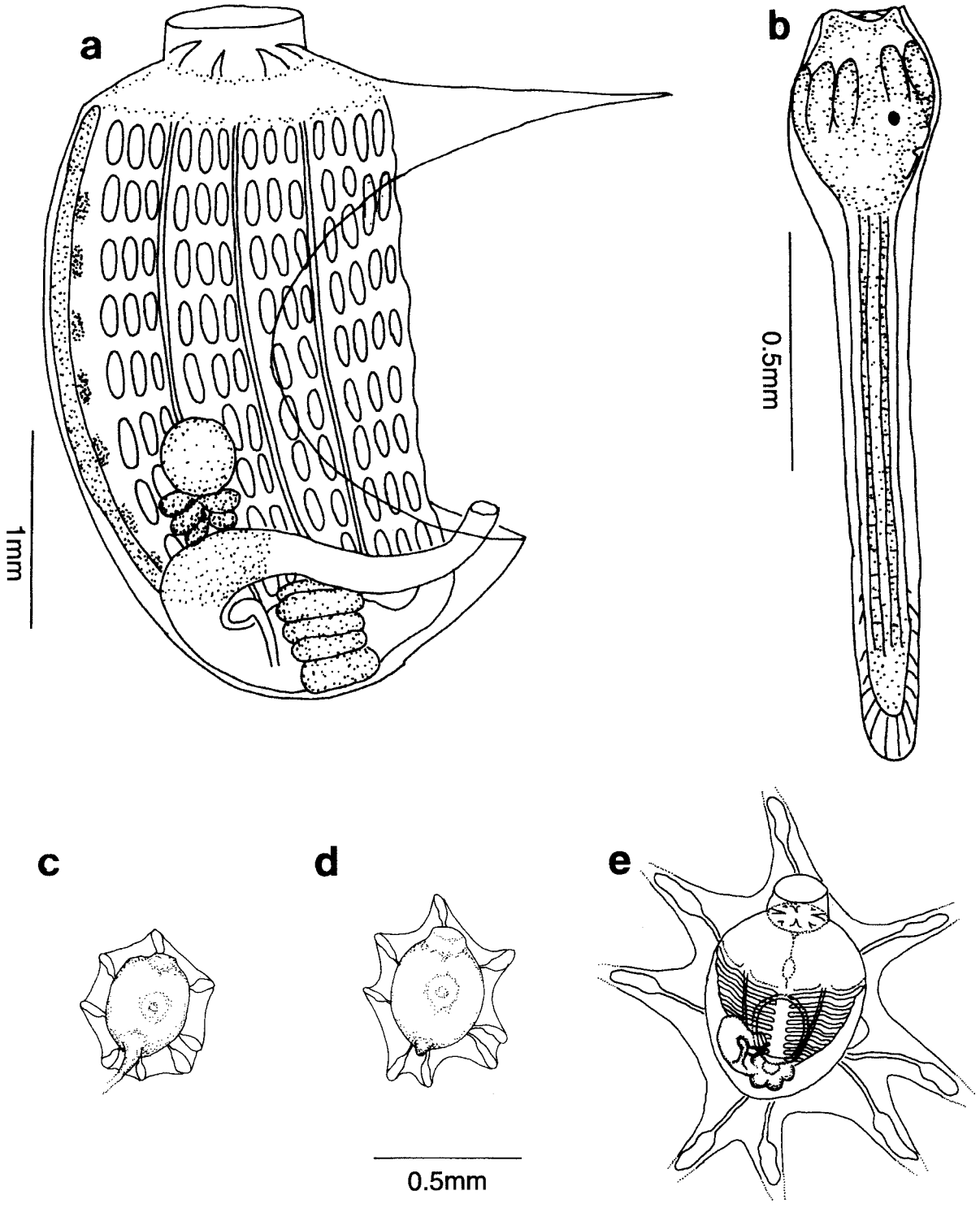

Fig. 2. *Botryllus delicatus n.* sp. a, Holotype (TKB-anim.1001); b, Paratype (TKB-anim.1003); e, Paratype (TKB-anim.1004). (a) A zooid, from left side. (b) A larva, from left side. (c) An oozooid, 2 hr after larval attachment. Tail absorption is in progress. (d) The same, 6 hr after larval attachment. Tail absorption is almost completed. (e) The same, 2 days after larval attachment. Both branchial and atrial siphons open, and the first bud appears.

length and light yellow in color when alive. The trunk is about 400 μm, oval in outline, and has a single photolith, as is typical of botryllids. The primordial siphons are formed on the posterior half of the trunk. Three adhesive papillae are arranged in a triangle on the anterior end of the trunk and eight ampullae form a circular ampullar band surrounding the anterior half of the trunk. One or two hr after liberation, the larvae attach to a suitable substratum using the adhesive

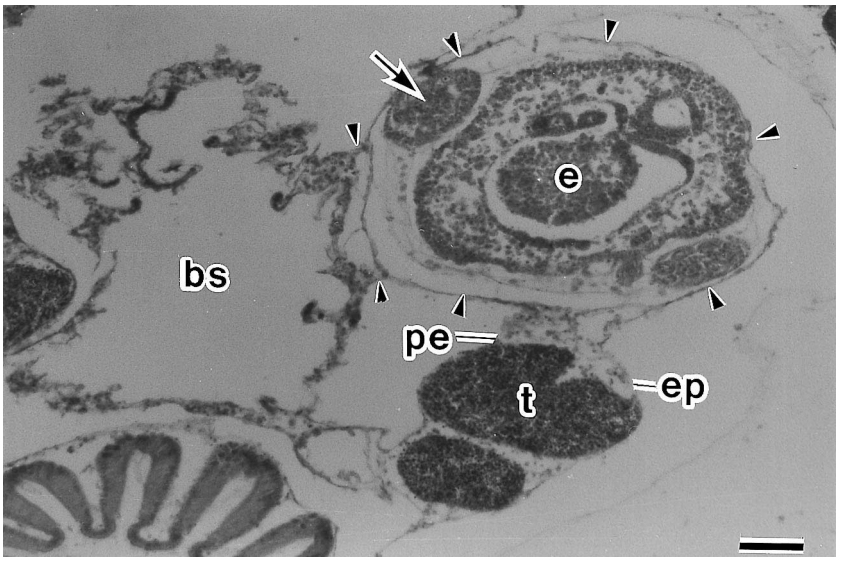

Fig. 3. A developing embryo in the brooding organ that was formed from both the branchial sac and peribranchial epithelia. This embryo has already formed a larval tail (arrow). Arrowheads indicate the brooding organ. bs, branchial sac; e, embryo; ep, epidermis; pe, peribranchial epithelium; t, testis. Scale bar is 50 μm.

papillae. Each larva extends its eight ampullae to complete attachment and begins metamorphosis into a primary zooid (oozooid; Fig. 2c, d). The larva becomes a functional oozooid by opening its siphons and beginning to feed about two days after attachment. An oozooid (Fig. 2e) is about 750 μm long and 500 μm wide and has 8–10 long transverse stigmata (protostigmata) on both sides of the branchial sac. There is one inner longitudinal blood vessel on each side of the branchial sac. The branchial tentacles of an oozooid consist of four large and four small, regularly alternating. The stomach has five longitudinal plications and a long pyloric caecum. The anus opens at the level of the sixth or seventh protostigma. About two days after attachment, the first pallial bud is formed on the right side of the body of the oozooid.

Colony Specificity in This New Ascidian

When two colonies were brought into contact with each other at their growing edges, their blood vessels fused with each other such that a single colony was formed, or they rejected each other. The processes of the allorecognition reaction of this new ascidian are illustrated schematically in Fig. 4.

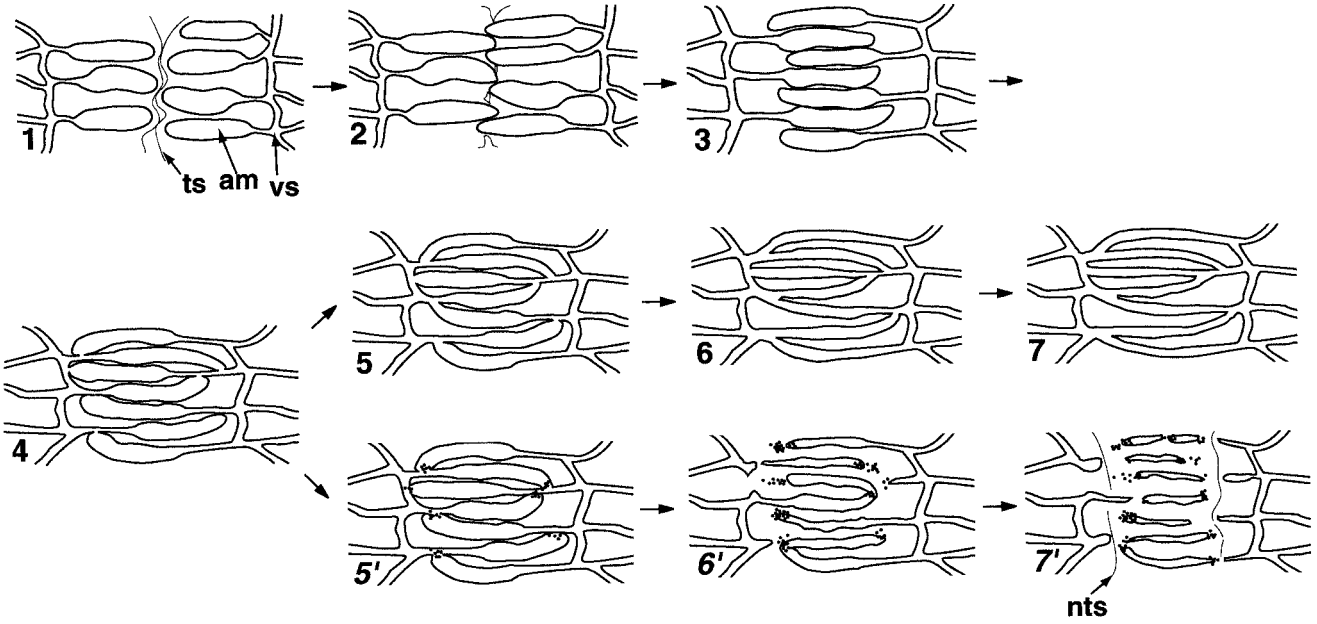

Fig. 4. Scheme showing the processes of fusion (Stages 1–7) and nonfusion (Stages 1–7') in *Botryllus delicatus* n. sp. am, ampulla (terminus of vascular system); nts, new tunic surface; ts, tunic surface; vs, vascular system.

In the case of fusion, the process was the same as that observed in other botryllids. The tunic surfaces of the two compatible colonies contacted each other (Stage 1). Four to five hr after contact, the cuticle layers of both tunic surfaces began to dissolve and tunic fusion was established between these two colonies. Then, ampullae, which were the tips of the vascular systems, of each colony extended into the tunic matrix of the facing colony (Stage 2). Fourteen to eighteen hours after contact, the tips of these ampullae came into contact with the proximal part of the ampullae of the facing colony (Stage 3). About one day after contact, the ampullae of the two colonies began to fuse at the contact points to form a single vascular system (Stage 4). Subsequently, the number of fused ampullae increased, and those ampullae became thin like the blood vessels in the center of the colony (Stages 5–6). Finally, the two colonies became a single unit (Stage 7).

In the case of rejection, fusion of both tunic and ampullae occurred in the same manner as that observed in the case of fusion (Stages 1–4). That is, even between incompatible colonies, the vascular systems of these two colonies became connected with each other, and blood cells of both colonies were exchanged through the fused ampullae. However, after a while, the blood flow gradually became slower, and blood cells began to aggregate at the fused points of ampullae (Stage 5'). Three to four hr after ampullar fusion, the blood exchange was completely stopped by the clusters of blood cells. Then, the fused ampullae became thin and were amputated near the fused points (Stage 6'). About one day after the ampullar fusion, the vascular systems of the two colonies were separated from each other. Typical necrosis seldom occurred at the fused area, and fusion of the tunic matrices remained unaffected for several days until the blood vessels of the individual colonies withdrew from the area. Finally, the tunics of the contact areas were broken down, and new tunic surfaces were made (Stage 7').

With regard to the features of the morphology, life history, and colony specificity of this species, there was no difference among colonies collected from different locations.

DISCUSSION

The external appearance of this new ascidian bears some resemblance to a known species, *Botryllus sexiens*. Both have very soft tunic matrices that are much softer than those of other botryllids. However, in *B. sexiens*, the number of the first ampullae seen on the larval trunk and the number of tentacles in the branchial siphon is six or a multiple of six (Saito *et al*., 1981a), whereas the number of the first ampullae of this ascidian is eight and that of branchial tentacles is a multiple of four, as seen in most other known species of botryllids.

Because the ovary is situated anterior to the testis, this species should be a member of the genus *Botryllus* (Van Name, 1945). However, an embryo develops in the sac-like brooding organ as do embryos in the genus *Botrylloides*. From our detailed observations on the brooding organ, it became clear that this organ was formed from the expanded epithelium of the branchial sac and a part of the peribranchial epithelium (Okuyama and Saito, in preparation). It is already known that in *B. sexiens* the epithelium of the branchial sac expands to form the brooding organ, which holds embryos in the peribranchial cavity (Mukai, 1986). Therefore, this ascidian is the second example among botryllids that forms a brooding organ from the branchial sac. However, the brooding organ of this ascidian has a more complicated structure than that of *B. sexiens* (Okuyama and Saito, in preparation).

In this ascidian, a colony part composed of only vascular system within the tunic often extends from the colony margin. In this part, sometimes vascular budding occurs. Vascular budding is a form of asexual reproduction that was first reported in *Botryllus primigenus* (Oka and Watanabe, 1957b). This process involves the formation of a new bud from blood cells gathered at the base of an ampulla in the periphery of a colony. This bud develops in a manner similar to that of a pallial bud. In *B. primigenus*, vascular budding can occur at the ampullae of the entire colony margin, but in this ascidian vascular budding is limited to these unique colony parts. The same feature was also reported in *Botrylloides lenis* (Saito and Watanabe, 1985), but not in other botryllids. That is, this ascidian and *B. lenis*, as well as *B. primigenus*, can usually extend their colonies not only by pallial budding, but also by vascular budding.

In this study, we examined the processes of allorecognition in this ascidian. It showed a rejection reaction like that of *B. scalaris* (Saito and Watanabe, 1982; Shirae *et al*., 1999). In both, the allorejection reaction begins after blood is exchanged through the fused ampullae. However, it takes a longer time for this ascidian to begin the allorejection reaction than *B. scalaris*. In *B. scalaris*, hemocytes aggregate in the fused ampullae and blood flow is stopped soon after the blood exchange occurs (Shirae *et al*., 1999), but in this ascidian, blood flow does not stop for a few hours, thereby allowing more blood to be exchanged between the two incompatible colonies. Therefore, this allorejection reaction may be different from that of *B. scalaris* and may be unique among botryllids.

This new ascidian is classified into the genus *Botryllus* according to the definition of Van Name (1945) and closely resembles *B. sexiens* in its morphological features. However, as mentioned above, we believed this ascidian is different from *B. sexiens* and have named this new species after its extremely soft tunic—*Botryllus delicatus* Okuyama and Saito n. sp.

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