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## Studies on Japanese Botryllid Ascidians. II. A New Species of the Genus *Botryllus* from the Vicinity of Shimoda

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**ABSTRACT**—Morphology and life history of a new species of the genus *Botryllus* belonging to the family Botryllidae were described in detail. This ascidian was collected from the stony shore in the vicinity of Shimoda (Shizuoka prefecture, Japan). The arrangement of ovary and testis in this ascidian was the same as that in other species of the genus *Botryllus*, while the embryo developed in a brood pouch formed from the invagination of peribranchial epithelium, as in the other genus *Botrylloides*. The processes and features of the allorecognition reaction of this ascidian were observed. The reaction showed the same processes as that in the species of the genus *Botrylloides*. Therefore, this ascidian has both features of the two genera of the family Botryllidae, which strongly suggests the necessity of reconsidering on the classificatory criteria of botryllid ascidians.

**Key words:** compound ascidian, botryllid, *Botryllus*, new species

### INTRODUCTION

Botryllids are relatively common compound ascidians at stony or rocky shores in Japan. Van Name (1945) defined the differences between two genera of the botryllids, *Botryllus* and *Botrylloides*, in terms of the features of sexual reproduction. According to the definition, the ovaries of mature zooids in the genus *Botryllus* are situated anterior to the testes, and zooids do not have any brood pouch. In the genus *Botrylloides*, the ovaries of mature zooids are situated posterior to the testes, and zooids have the brood pouches. Many species of botryllids live at the coast near Shimoda Marine Research Center (SMRC), University of Tsukuba; seven species of botryllids have been reported at this location (Saito *et al.*, 1981a, b; Saito and Watanabe, 1985; Okuyama and Saito, 2001a). In SMRC, a great deal of research on botryllids has been performed over many years, mainly to study their allorecognition reaction known as “colony specificity” (Oka and Watanabe, 1957). However, some botryllid ascidians remain undescribed.

As stated above, 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 eight botryllid ascidians, *Botryllus scalaris* (Saito and Watanabe, 1982; Shirae *et al.*, 1999), *Botryllus primigenus* (Tanaka and Watanabe, 1973), *Botryllus schlosseri* (Boyd *et al.*, 1990), *Botryllus delicatus* (Okuyama and Saito, 2001a), *Botrylloides simodensis* (Hirose *et al.*, 1997), *Botrylloides fuscus* (Hirose *et al.*, 1997), *Botrylloides leachi* (Zaniolo and Ballarin, 2001) 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 allorejection among botryllids. Therefore, variations in the allorejection process might be 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 undescribed ascidian found at Noroshi Point near SMRC. We also examined the process of allorejection in colony specificity of this ascidian. Based on the observations of its morphology and life history, it has become clear that this ascidian is a unique species with features of both genera, *Botryllus* and *Botrylloides*, that is, although the ovary is positioned anterior to the testis, this ascidian has a brooding organ like the brood pouch. No ascidian having such features has ever been found until now. In a previous study, we pointed out that the definition of the two genera of the family Botryllidae should be revised (Okuyama and Saito, 2001b), because there were some species that were not consistent with the definition. The find-

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ing of this ascidian supports this idea strongly.

## MATERIALS AND METHODS

### Animals

For the observations on morphology and life history, several colonies of this ascidian were collected in the tide pool at a depth of around 1 meter at Noroshi Point in Shimoda (Shizuoka prefecture, Japan). Collected colonies were fastened to glass slides with cotton thread and cultured in a box immersed in Nabeta Bay near SMRC (13–26°C). To keep the colonies clean, dirt, hydrozoans and bryozoans on the colony surface were removed every week, and their morphology was observed under a binocular stereomicroscope.

### Observations on morphology

Living and fixed specimens of whole colonies, larvae, and oozoids were observed under a binocular stereomicroscope. For fixation, living colonies, larvae, and oozoids were immersed in 0.32 M MgCl<sub>2</sub> for about 15 min to anesthetize them, and then were transferred to 10% formalin in filtered seawater.

Sexually mature colonies were cut and fixed in Bouin's solution, which was made in filtered seawater, for histological study. The fixed materials were dehydrated and embedded in paraplast (Oxford Labware, USA). They were sectioned at 7 µm and stained with Delafield's hematoxylin and eosin G.

### 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 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 filled with continuous running seawater (about 24.5°C). Observations of the colony specificity reactions were made every 2 hr using a binocular stereomicroscope. The timing and details of tunic fusion, fusion or deterioration of ampullae (terminus of the common vascular system at the periphery of the colony), and blood cell behavior were recorded as the two colonies underwent fusion or rejection.

The holotype and paratypes are deposited at the University of Tsukuba (TKB).

## DESCRIPTION

### *Botryllus promiscuus* Okuyama and Saito n. sp.

*Type series*: HOLOTYPE: a colony (TKB-anim. 2355); 2.0 by 3.5 centimeters; Y. Saito; 20 Sep. 1982. PARATYPES: colonies (TKB-anim.2356); M. Okuyama; 20 Oct. 2000, larvae (TKB-anim.2357); Y. Saito; 19 Aug. 1982, and oozoids (TKB-anim.2358); M. Okuyama; 4 Nov. 2000.

*Type locality*: Shimoda, Japan.

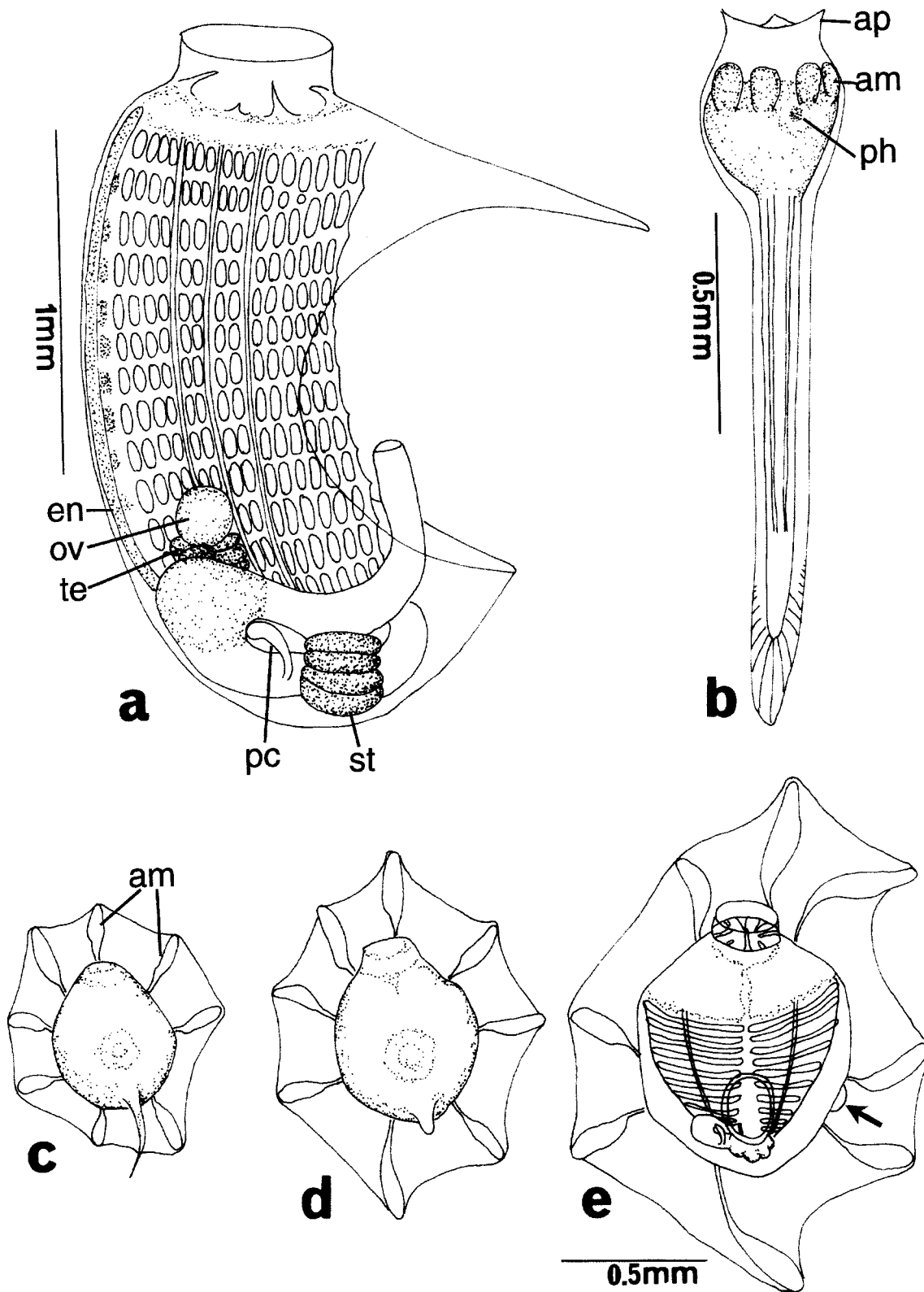
### *Diagnosis*

Colonies of this ascidian are found only in the tidepool at Noroshi Point near SMRC. They encrust the surfaces of rocks at a depth of around 1 meter. They often compete with colonies of *Botrylloides lentus* in that habitat. Colony size varies from a disc of a few millimeters in diameter to about 10 cm across. Colony thickness is usually 1.5–2.0 mm. The colony surface is generally flat and free of foreign matter. The gelatinous tunic is soft, transparent, and colorless. The color of live colonies is basically pink. In addition, orange

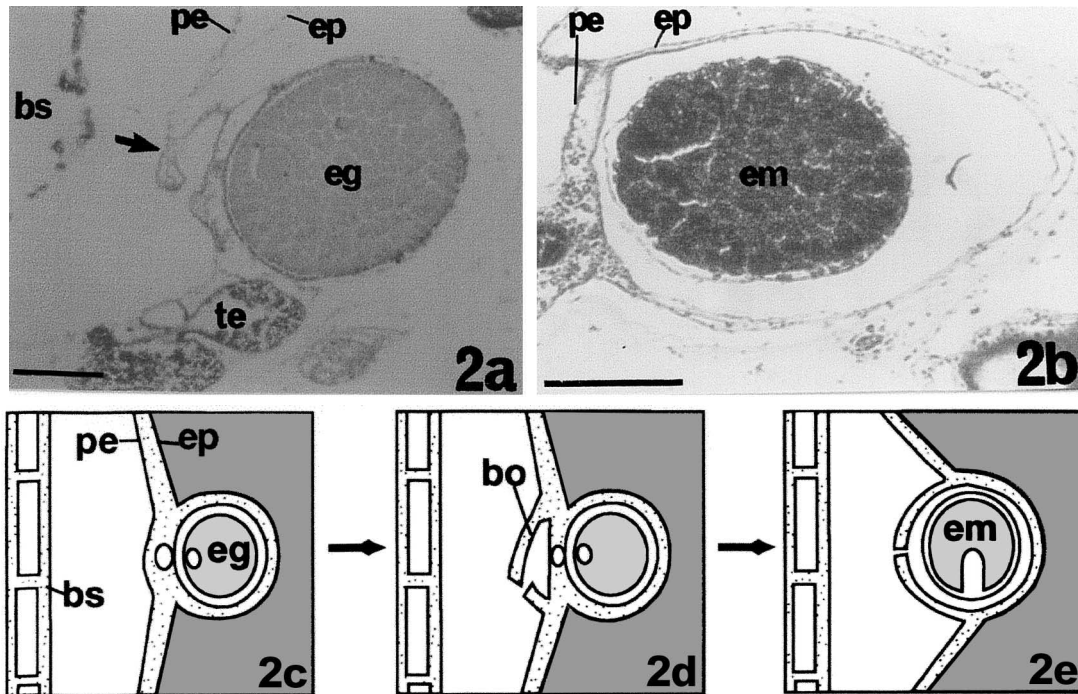
and white pigment cells (a type of blood cells) are sometimes deposited around the branchial siphon and on the atrial languet of respective zooids. A colony is composed of many zooids, called blastozooids, which are arranged in ladder systems with several common cloacal apertures in the common tunic. They are always connected with one another by common vascular system. The periphery of the colony is fringed with sausage-shaped vascular ampullae about 800 µm in length and 200 µm in width.

Zooids (Fig. 1a) are 1.5–2.0 mm in length and situated more or less obliquely. There are four large and four small branchial tentacles on each zooid that alternate regularly. The ciliated groove forms a small round opening. There are 12–13 rows of stigmata on each side. Around the middle of the branchial sac, stigmata are arranged between the three inner longitudinal bars as follows: dorsal 6. 2–3. 2–3. 4 endostyle. The anterior edge of the intestinal loop attains anteriorly the level of the eleventh transverse vessel, and the anus opens at the level of the ninth transverse vessel. Many blood cells are deposited along each side of the endostyle in the range from the second to the tenth or eleventh stigmatal row. Most of the stomach is exposed posterior to the rear end of the branchial sac. The stomach is pink in fresh specimens and has eight or nine longitudinal plications and a large pyloric caecum.

Asexual reproduction occurs throughout the year. Usually, a single bud is produced on each side of a zooid. The cycle of the alternation of generations (known as takeover; Watanabe, 1953) is about one week. Sexual reproduction can be observed from July to December (16–25°C), with a peak in August. The testis is situated along the anterior edge of the circum-intestinal gland area on the left side and at the level of the tenth 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 and reach a maximum size of 230–250 µm just before ovulation. A single egg matures on each side of the body of the new blastozooid, and is ovulated when the blastozooid opens its branchial and atrial siphons. The release of sperm occurs about two days after ovulation in the same zooids. When the egg matures sufficiently, the peribranchial epithelium in front of the ovary thickens. The thickening part then begins to invaginate into the mesenchymal space. This invagination grows large gradually, and an enclosed space is formed in front of the ovary (Fig. 2a, c, d). The mature egg is ovulated into this space, and fertilization occurs there (Fig. 2b, e). Therefore, the invagination that was formed from the peribranchial epithelium functions as a brooding organ in this ascidian. Ovulation takes place synchronously in all zooids of a colony. Usually, only a single embryo develops in the 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 larvae (Fig. 1b) are about 1.5–1.8 mm in total length and light yellow in color when alive. The trunk is



**Fig. 1.** *Botryllus promiscuus* n. sp. (a) A zooid, from the left side. Holotype (TKB-anim.1005). (b) A larva, from the left side. Paratype (TKB-anim.1007). (c) An oozoid, 2 hours after larval attachment. Tail absorption is in progress. (d) The same, 8 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 (arrow) appears on the right side of the body. Paratype (TKB-anim.1008). am, ampulla; ap, adhesive papillae; en, endostyle; ov, ovary; pc, pyloric caecum; ph, photolith; st, stomach; te, testis.



**Fig. 2.** The brooding organ in *Botryllus promiscuus* n. sp. (a) The brooding organ being formed by the invagination of the peribranchial epithelium (pe) (arrow). (b) An embryo (em) developing in the brooding organ. (c)–(e) Scheme showing the process of formation of the brooding organ. (c) A part of the peribranchial epithelium becoming thick. (d) The brooding organ almost completed. (e) An embryo developing in the brood pouch. bs, branchial sac; eg, egg; ep, epidermis; te, testis. Scale bars are 100  $\mu$ m.

about 400  $\mu$ m, oval in outline, and has a single photolith, as is typical of botryllids. 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 hours after liberation, the larvae attach to suitable substrata using their adhesive papillae. Each larva extends its eight ampullae to complete the attachment and begins its metamorphosis into a primary zooid (oozooid; Fig. 1c–d). The larva becomes a functional oozooid by opening its siphons and beginning to feed about two days after the attachment. An oozooid (Fig. 1e) is about 900  $\mu$ m long and 750  $\mu$ m wide and has 9–10 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 and are regularly alternating. The stomach has five longitudinal plications and a long pyloric caecum. The anus opens at the level of the ninth or tenth protostigma. About two days after attachment, the first pallial bud is formed on the right side of the body of the oozooid.

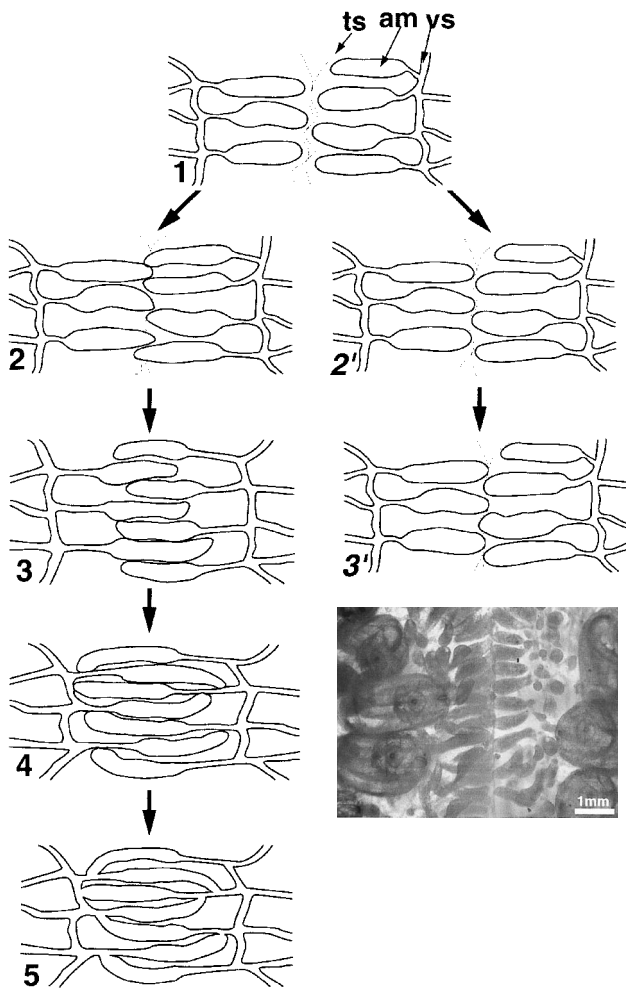
#### *Colony Specificity in This New Botryllid*

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 botryllid are illustrated schematically in Fig. 3.

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). Two to four hours after the contact, the cuticle layers of both tunic surfaces began to dissolve, and tunic fusion was established between these two colonies (Stage 2). The ampullae, which are the termini of the vascular systems of each colony, then extended into the tunic matrix of the facing colony (Stage 3). Twelve to fourteen hours after contact, the tips of these ampullae came into contact with the proximal part of the ampullae of the facing colony (Stage 4). 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 5). Subsequently, the number of fused ampullae increased, and those ampullae became thin, like the blood vessels in the center of the colony. Finally, the two colonies became a single unit.

In the case of rejection, the allojection of this ascidian was similar to that of the species of the genus *Botrylloides*. Two to four hours after contact at the tunic surfaces (Stage 1), the ampullae of both colonies pushed each other, but never penetrated into the tunic of the opposite colony (Stage 2'–3'). Although obvious rejection reactions were rarely found under a binocular stereomicroscope, the occurrence of the rejection reaction was clearly demonstrated in the histological observations. Along the tunic cuticle of the two facing colonies, some parts of the tunic cuticle fused (Fig. 4a), and we occasionally found some blood cells passing through the ampullar epithelium into the tunic. Those infiltrated blood cells aggregated and disintegrated at the fused



**Fig. 3.** Processes of the allorecognition reaction in *Botryllus promiscuus* n. sp. The process of fusion runs from Stages 1 to 5. The process of nonfusion runs Stages 1 to 3'. The photograph shows Stage 3'. am, ampulla (terminus of vascular system); ts, tunic surface; vs, vascular system.

area (Fig. 4b, c). The formation of the rejection lesion was limited in the subcuticular areas. Most of the infiltrated cells were morula cells that contained eosinophilic materials in their vacuoles.

#### Remarks

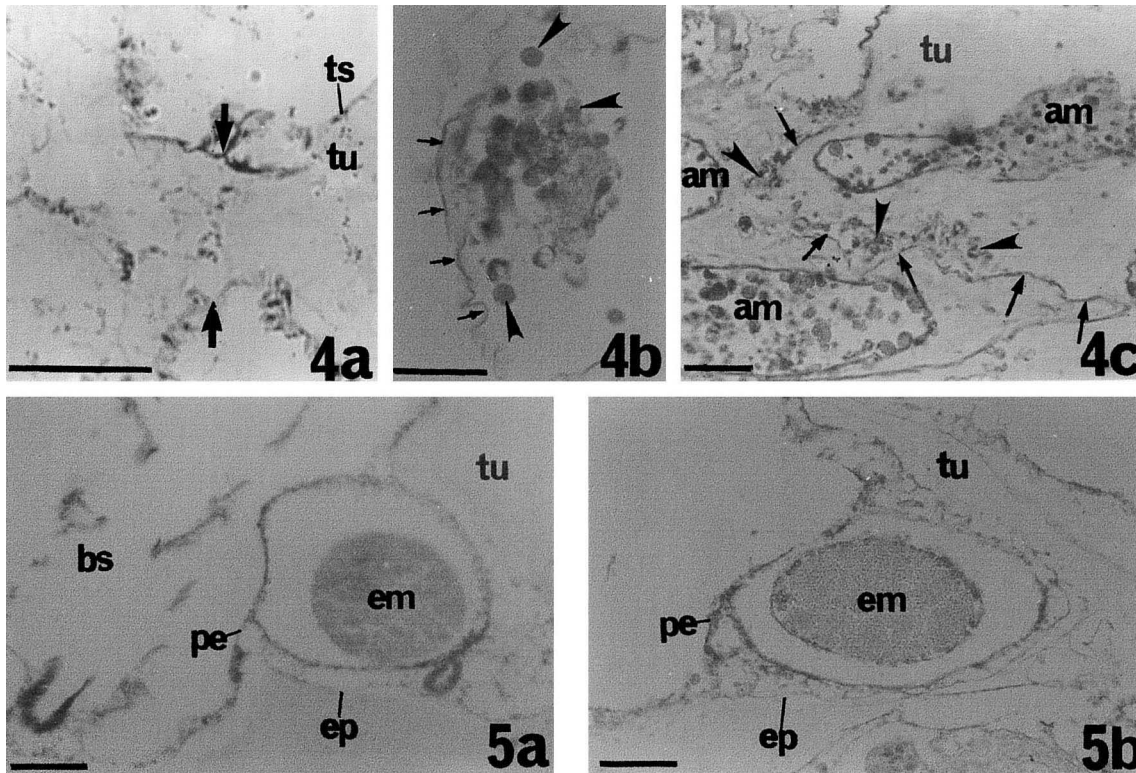
This botryllid ascidian revealed some interesting features; the arrangement of gonads was identical to that of the genus *Botryllus*, but the structure of the brooding organ was the same as the brood pouch of the genus *Botrylloides*. These features of this ascidian are not consistent with either the definition of *Botryllus* or that of *Botrylloides*. There are other botryllid ascidians that also do not coincide with either of these definitions, namely *Botryllus primigenus* (Mukai, 1977), *Botryllus sexiens* and *Botryllus delicatus* (Okuyama and Saito, 2001b). The arrangement of gonads in these three species is identical to that of *Botryllus*, but the appearance of the brooding organ with a developing embryo resembles the brood pouch of *Botrylloides*. However, in the

latter two species, the brooding organs were formed with an extended epithelium of the branchial sac to surround the embryo. The brooding organ of the former species, *B. primigenus*, is then formed by the invagination of the peribranchial epithelium and envelops the embryo perfectly (Fig. 5a). The forming process and the appearance of the brooding organ of *B. primigenus* is quite the same as the brood pouch of the *Botrylloides* species (Fig. 5b). In this study, it became clear that the brooding organ of this ascidian is formed by the same process as in *B. primigenus*, and its brooding organ was to all appearances the same as the "brood pouch" of the *Botrylloides* species.

Although this ascidian and *B. primigenus* have brooding organs with the same structure, their other features are quite different from each other. The zooid of *B. primigenus* has only 4 stigmatal rows, does not have a lobed testis, and performs ovulation and sperm release at the same time. In botryllid ascidians, each bud is connected to the mother zooid by a peduncle, and in most species, the peduncle functions as a connective vessel to the vascular system of the colony after the disintegration of the mother zooid. However, in *B. primigenus*, the peduncle disappears and is replaced by a newly formed connective vessel (Mukai *et al.*, 1987). *B. primigenus* can be thought of as an exceptional species of botryllid ascidians because of the features mentioned above.

In this study, we examined the processes of allorecognition in this ascidian. The ascidian showed the same rejection reaction as that of the species belonging to the genus *Botrylloides* (Hirose *et al.*, 1988; Hirose *et al.*, 1997). The allorecognition reaction occurred only at the subcuticular area. The ampullae never penetrated into the tunic of the opposite colony. However, infiltration of the blood cells was induced at the contact area. Therefore, this ascidian might recognize the allogeneic colony at the tunic of the colony. In most species of the genus *Botryllus*, the allorecognition reaction occurs after the penetration of the ampullae into the opposite colony (Saito *et al.*, 1994). In *B. primigenus*, the sign of the allorecognition reaction appears after the penetration of the ampullae into the facing colony. In addition, *B. scalaris* and *B. delicatus* recognize the allogeneic colony after the fusion of the vascular system. *B. schlosseri* can recognize it earlier than the former three species can, since the fusion of the tunic and the penetrating of the ampullae into the facing colony are restricted to the only small areas. However, the allorecognition of this ascidian occurs much earlier than in those *Botryllus* species.

It should be concluded that the arrangement of the gonads of this ascidian is the same as that of the genus *Botryllus*, but its brood pouch and allorecognition reaction are identical to those of the genus *Botrylloides*. This ascidian has the features of both genera of the family Botryllidae. In an earlier report, we reported a species *B. delicatus* as a member of *Botryllus* (Okuyama and Saito, 2001a) because of the arrangement of its gonads, although it had a brooding organ like the brood pouch of the *Botrylloides* species.



**Fig. 4.** Histological sections of allogeneic rejection. (a) Contact area between the incompatible colonies. Partial fusion of the tunic has occurred (between arrows). (b) Mass of infiltrated cells (arrowheads). Arrows point to the plane of contact. (c) Infiltrated blood cells (arrowheads) in the tunic. Arrows point to the plane of contact. am, ampulla; ts, tunic surface; tu, tunic. Scale bars are 50 μm.

**Fig. 5.** Brood pouches incubating embryos of two botryllids. (a) *Botryllus primigenus*. (b) *Botrylloides simodensis*. The structure of the brooding organ of *B. promiscuus* is the same as in these two species. bs, branchial sac; em, embryo; ep, epidermis; pe, peribranchial epithelium; tu, tunic. Scale bars are 100 μm.

Therefore, we classified this new ascidian as a member of *Botryllus* because of its arrangement of gonads and named this new species after the intermingled features of both genera---*Botryllus promiscuus* Okuyama and Saito n. sp.

#### Etymology

The specific name, *promiscuus*, is derived from the Latin for intermingling, referring to the presence of the features of the two genera in a species.

## DISCUSSION

The arrangement of the gonads of this new species was identical to that of the genus *Botryllus*, but the structure of the brooding organ was the same as the brood pouch of the genus *Botrylloides*. We classified this species as a member of the genus *Botryllus* because of the arrangement of the gonads. On the basis of the arrangement of the gonads, which was a part of the definition of the botryllid genera as defined by Van Name (1945), botryllids can be classified into only two groups, *Botryllus* and *Botrylloides*, without exception. This is why we classified this new species and previously described another species as the members of the genus *Botryllus* (Okuyama and Saito, 2001a).

However, we do not think that only the arrangement of the gonads reflects the phylogeny of botryllids. We have

proposed that sexual reproduction, the formation of brooding organ, the formation of the vascular network, and the allojection reaction also reflect the phylogeny, and we are trying to make new classificatory criteria for botryllids on the basis of those features (Saito *et al.*, 2001). Based on our study, botryllids would be classified into five groups as follows, according to the existence and origin of the brooding organ and the arrangement of the gonads. A) No brooding organ, and ovaries are positioned anterior to testes (e.g. *B. scalaris*). B) Brooding organs are formed from the branchial sac, and ovaries are positioned anterior to testes (e.g. *B. sexiensi* and *B. delicates*). C) Brooding organs are formed from the peribranchial epithelium, and ovaries are positioned anterior to testes (e.g. *B. schlosseri*, and the botryllid studied here). D) Brooding organs are formed from the peribranchial epithelium, and ovaries are positioned anterior to testes. However, zooids have hemispherical testes and only four stigmatal rows, and they make a vascular network in a unique manner, as mentioned above (e.g. *B. primigenus*). E) Brooding organs are formed from the peribranchial epithelium, and ovaries are positioned posterior to testes (e.g. all *Botrylloides* species). Since the embryos are free in the peribranchial cavity (Saito *et al.*, 1981) in the species of Group A without any brooding organ, such as *B. scalaris*, this group is considered to be the most primitive of the botryllids.

With respect to the features of the brooding organ, the evolutionary current of botryllids might be divided into two streams (Saito *et al.*, 2001, Okuyama and Saito, 2001b). One stream includes the species that make the brooding organ from the branchial sac (Group B), and other stream includes the species that make the brooding organ from the peribranchial epithelium (Groups C, D, and E). However, the species of Group D with the four stigmatal rows might have branched off from other groups in the early stages of the evolution, because this group shows many different features from other groups (Saito *et al.*, 2001). *Botrylloides* species (Group E) are thought to be derived from the *Botryllus* of Group C on the basis of the origin of the brooding organs and the existence of viviparous species. This grouping is consistent with the grouping divided by the mode of allorecognition reaction, for the most part. The former two groups (A and B) recognize the allogeneic colony after the vascular fusion, and the latter three groups (C, D, and E) recognize it before the vascular fusion (Saito *et al.*, 1994). The differences in these five groups might have to be reflected in the classificatory criteria of botryllid ascidians. To verify the phylogenetic relationship of these groups, the information from the molecular phylogenetic analysis should be very useful. Using 18SrDNA, Cohen *et al.* (1998) showed the monophyly of botryllids, and also showed that *B. scalaris* was placed as the basal botryllid. Other species of the genus *Botryllus* fell into one group, and species belonging to the genus *Botrylloides* were also confirmed to be a monophyletic group. However, the phylogenetic relationship of each species of the genus *Botryllus* was not discussed sufficiently, because it dealt with only three species, *B. scalaris*, *B. primigenus*, and *B. sexiensi*. In order to make new classificatory criteria for botryllids, much more information regarding 18SrDNA and other genes is needed.

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