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Source: Zoological Science, 18(3) : 397-404

Published By: Zoological Society of Japan

URL: <https://doi.org/10.2108/zsj.18.397>

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Studies on the Structure of the Brooding Organs of Two Botryllid Ascidians, *Botryllus delicatus* and *Botryllus sexiens*

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ABSTRACT—The formative processes of the brooding organs of two botryllid ascidians, *Botryllus delicatus* and *Botryllus sexiens*, were observed. The structure of these organs was also studied in detail histologically. The brooding organs of these two species were formed from the branchial sac, in contrast to the brooding organs of other botryllids, which are formed from the peribranchial wall. The structures of the brooding organs of *B. delicatus* and *B. sexiens* were different, but the processes of their formation were almost the same. As the maturation of the oocytes occurred, a part of the epithelium of the branchial sac extended toward the peribranchial epithelium covering the ovary. The extension of the branchial epithelium looked like a bowl and made a surrounding space with the peribranchial epithelium in a peribranchial cavity. The oocyte was ovulated into this space and fertilized there. After ovulation, the extension of the branchial epithelium formed a sac-like brooding organ in combination with the peribranchial wall and held the embryo tightly, like the incubatory pouch of the genus *Botrylloides*. The unusual characteristics of the brooding organs in these two species lead to reconsideration of phylogenetic relationships and taxonomic criteria in botryllids.

INTRODUCTION

Botryllids are compound ascidians belonging to the family Botryllidae (Stolidobranchia). All of the zooids and buds in a colony are connected to one another by a common vascular system. Botryllids are hermaphrodites, and the gonads develop in the body wall on either side of the body. Botryllids are classified into two major genera, *Botryllus* and *Botrylloides*. According to the definitions of these genera by Van Name (1945), which generally have been accepted, the zooid of *Botryllus* has one or several ovaries situated anterior or dorsal to the testis, no brood pouch is formed, and embryos develop in the peribranchial cavity of the zooid. On the other hand, the zooid of *Botrylloides* has one ovary posterior to the testis on each side of the body, and the ovulated and fertilized egg develops in a sac-like brooding organ formed as an outgrowth of the body wall near the ovary. Therefore, the features of sexual reproduction must be considered for proper classification of botryllids.

The mode of sexual reproduction has been observed in some species of botryllids: *Botryllus scalaris* (Saito *et al.*, 1981a), *Botryllus schlosseri* (Berrill, 1941; Mukai, 1977),

Botryllus primigenus (Mukai, 1977), *Botrylloides leachi* (Mukai, 1977), *Botrylloides simodensis* (Mukai, 1977; cf. Saito *et al.*, 1981b), and *Botrylloides violaceus* (Mukai *et al.*, 1987; Manni *et al.*, 1995; Zaniolo *et al.*, 1998). From these observations, it was found that there is a species that does not coincide with either definition of the genus in botryllids. The position of the gonads of *B. primigenus* was the same as that of *Botryllus*, but it made a brood pouch from the peribranchial epithelium, like *Botrylloides*. In some botryllids, the mode of sexual reproduction has not been studied yet. If it is discovered that other species do not fit either of the two definitions of botryllid genera on the basis of mode of sexual reproduction, those definitions may not be useful.

In the present work, the mode of sexual reproduction in *Botryllus delicatus* (Okuyama and Saito, 2001) and *Botryllus sexiens* (Saito *et al.*, 1981a) was examined in detail. Thus far, the structure and formative process of the brooding organs of these two species have not been studied, except for a brief note on the brooding organ of *B. sexiens* (Mukai, 1986). Our work shows that these two botryllids form brooding organs in a different way from that of other botryllids. These results strongly suggest that the past classification criteria for the two genera of botryllids are defective. Classification criteria and the phylogenetic relationship among botryllids are discussed.

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MATERIALS AND METHODS

Animals

Colonies of *Botryllus delicatus* were collected at the stony shore of Shikine Island of the Izu Islands (Tokyo Prefecture, Japan). Colonies of *Botryllus sexiens* were collected at the stony shore near Shimoda Marine Research Center (SMRC), University of Tsukuba (Shizuoka Prefecture, Japan). They were tied to glass slides with cotton thread and reared in culture boxes immersed in Nabeta Bay near SMRC. After the colonies grew well and attached to the glass slides by themselves, the cotton thread was removed. The colonies were periodically observed and cleaned under a stereomicroscope.

Development of all buds proceeds synchronously in colonies of *B. delicatus* and *B. sexiens*, as well as in other botryllids. In *Botryllus primigenus*, the development of the bud was well studied, and, as shown in Fig. 1, the process was divided into 11 stages (Watanabe, 1953; cf. Berrill, 1941). These developmental stages can be applied to other botryllid species as we have done in the present study. The bud first appears as a small, disk-like thickening of the peribranchial epithelium on each side of the body, immediately anterior to the gonads (Stage 1). The disk expands to its maximal size (Stage 2). Then, it curves into a hemisphere and eventually into a closed sphere (Stage 3). As the anterior arc continues to expand, two vertical folds appear and finally divide the vesicle into two lateral (atrial) chambers and one median (pharyngeal) chamber (Stage 4). Next, three evaginations are formed, representing the heart, the neural mass, and the intestine (Stage 5). Gill slits are about to become perforated, and the Stage 1 bud of the next generation appears (Stage 6). The stigmata become perforated (Stage 7). Then the heart begins to beat (Stage 8). Subsequently, the zooid enters its most active phase, with the two siphons fully functioning (Stages 9 and 10). In the breeding season, maturation of gametes is attained only in Stage 9. When the bud reaches its full size, the zooid of Stage 9 commences involution and eventually disappears within the common vascular system (Stage 11).

Light microscopy

Sexually mature colonies were cut and fixed in Bouin's solution, made in filtered seawater, for histological study. Various stages of the buds and zooids were fixed. Their stages were determined by observation under a stereomicroscope. The fixed materials were dehydrated and embedded in paraplast (Oxford Labware, USA). They were sectioned at 7 μ m and stained with Delafield's hematoxylin and eosin G.

Scanning electron microscopy

To observe the details of internal structure using scanning electron microscopy (SEM), some paraplast-embedded specimens of the two botryllids, including buds forming brooding organs, were cut with a microtome blade to expose the surfaces that were to be studied. Then, the paraplast was removed from the specimens with xylene (1 hr, three changes). Next, the specimens were dehydrated with acetone, and the acetone in the specimens was replaced with isoamyl acetate. After being dried at a critical point and sputter-coated with gold-palladium, the specimens were examined in a Hitachi S-570 scanning electron microscope at 8.0 kV.

RESULTS

Development of the brooding organ of *Botryllus delicatus*

Botryllid ascidians reproduce sexually and asexually. Asexual reproduction in *Botryllus delicatus* was carried out by peribranchial budding throughout the year. Sexual reproduction was observed from July to December, with a peak in August.

The brooding organ of *B. delicatus* was made of both the extension of the branchial sac and the peribranchial wall (Fig. 2a). From the observation of a series of histological sections, it became clear that the structure of the brooding organ formed from the branchial sac looked like a bowl. Observation of the internal structure of the bowl using SEM showed that the main part of this organ was made of two layers of the branchial epithelium, and the inside of its basal part was not filled with anything, that is, a cavity had formed (Fig. 3).

In the early stages of the developing bud (Stages 1–4), there were no organs in the bud. At Stage 5, some organs were being formed, such as the branchial sac, heart, neural mass, and intestine, but there was no brooding organ within the bud. At this stage, gonads first appeared and developed in the mesenchymal space between the epidermis and the peribranchial epithelium of the bud. Only one or two oocytes were observed in the ovary. At Stages 6 to 7 (Fig. 4a), formation of the brooding organ was not observed yet, although

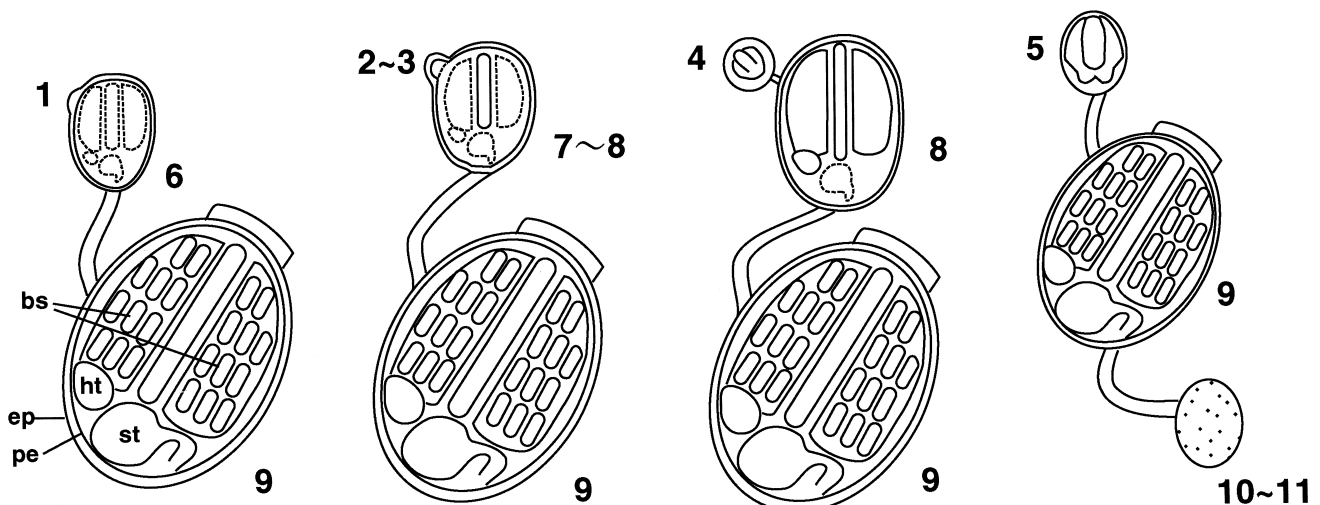


Fig. 1. Schematic representation of bud development in botryllid ascidians modified from Watanabe (1953). The numbers represent stages in development. bs, branchial sac; ep, epidermis; ht, heart; pe, peribranchial epithelium; st, stomach.

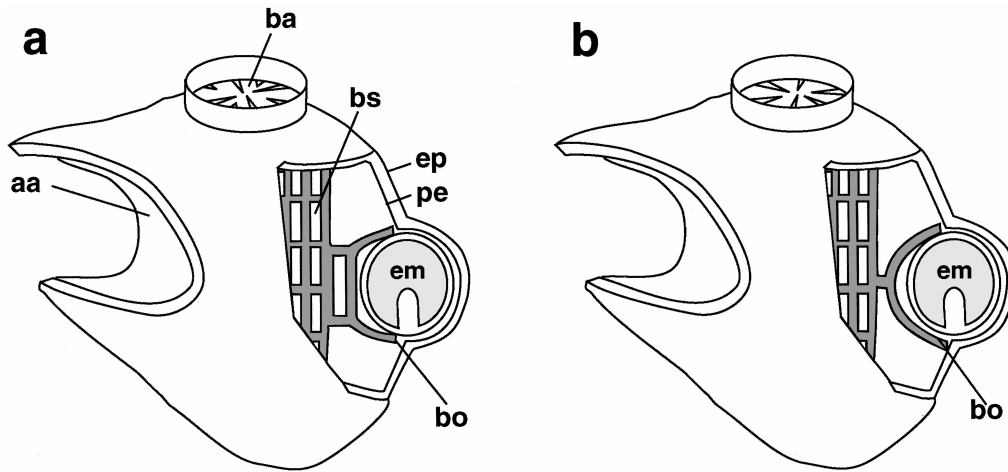


Fig. 2. Scheme showing zooid after ovulation. (a) Zooid of *B. delicatus*. (b) Zooid of *B. sexiens*. aa, atrial aperture; ba, branchial aperture; bo, brooding organ; bs, branchial sac; em, embryo; ep, epidermis; pe, peribranchial epithelium.

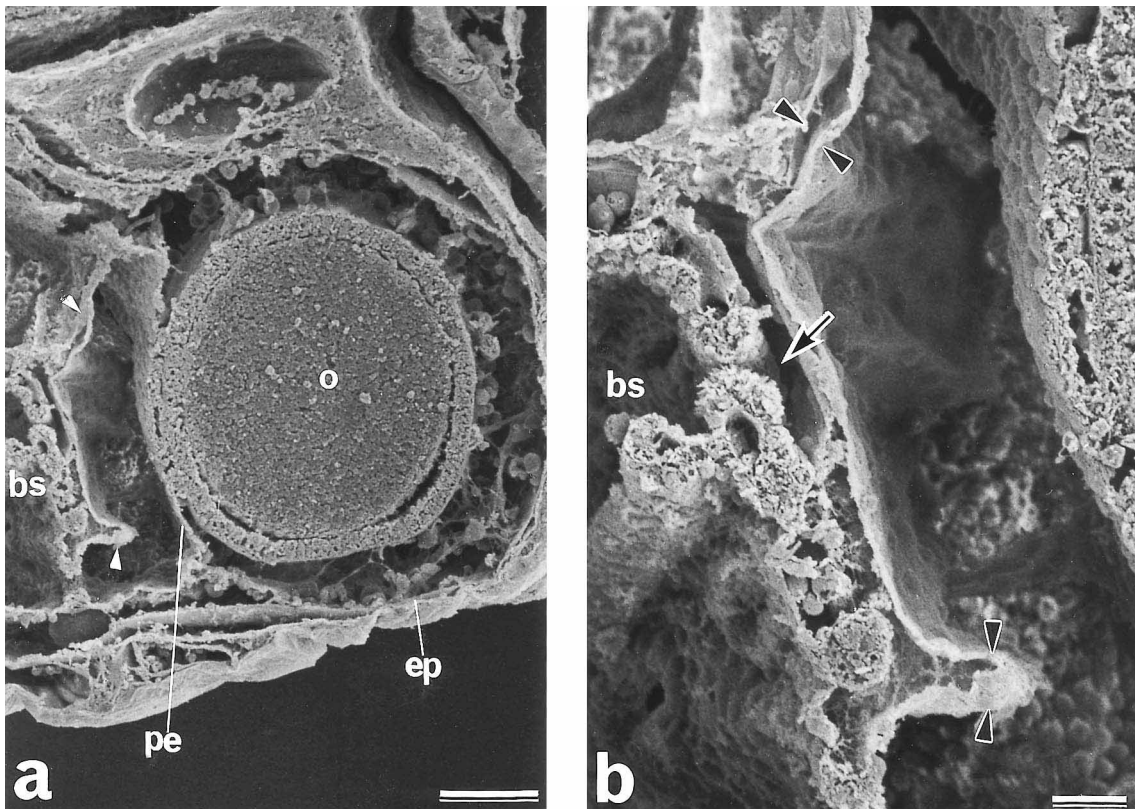


Fig. 3. Scanning electron micrograph of a bud at Stage 8 in *B. delicatus*. Arrowheads indicate the brooding organs (a) Branchial sac (bs) and ovary. A part of the branchial sac extends toward the peribranchial epithelium (pe) near the ovary to form the brooding organ. (b) Magnified image around branchial sac. The stem of the brooding organ appears to be hollow (arrow). ep, epidermis; o, oocyte. Scale bars are 50 μm in (a) and 10 μm in (b).

morphogenesis of other organs, such as the branchial sac, intestine, and heart, was almost finished. In the ovary, two layers of cells, the outer and the inner follicle cells, surrounded the oocyte, and the so-called test cells lay embedded in a peripheral layer of oocyte cytoplasm. The oocyte became larger and almost mature at late Stage 7. The mature oocyte was heavily yolked, and yolk granules were extremely eosi-

nophilic. At Stage 8, when the oocyte matured sufficiently, the branchial epithelium began to extend out at two parts of the branchial sac (Fig. 4b). Those two extensions fused with each other and then began to expand toward the peribranchial epithelium near the ovary (Fig. 4c). At late Stage 8, the extension of the branchial sac touched and adhered to the peribranchial epithelium (Fig. 4d). As a result, a space was

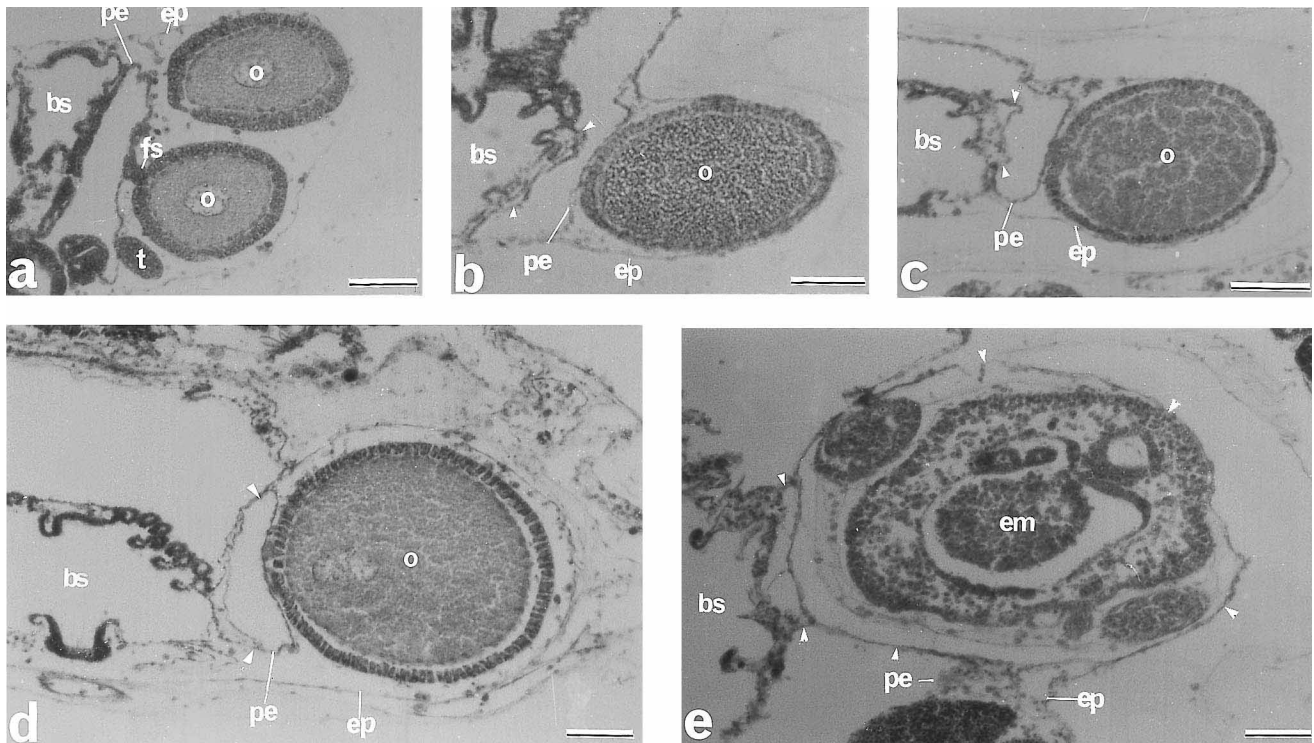


Fig. 4. Formation of the brooding organ in *B. delicatus*. Arrowheads indicate the brooding organ. (a) Early stage (Stage 6) of the developing bud. The oocyte (o) is surrounded by an outer follicle and an inner follicle. (b) Early Stage 8. Two parts of the branchial sac (bs) are extended outward. (c) Middle Stage 8. An extended part of the branchial sac spreads toward the peribranchial epithelium (pe) near the ovary. (d) Late Stage 8. The fully developed brooding organ formed from the extension of the branchial sac and the peribranchial epithelium. A space is made between them. (e) Late Stage 9. An embryo (em) is in the brooding organ. The brooding organ becomes a sac-like structure in the peribranchial cavity. ep, epidermis; fs, follicle stalk; t, testis. Scale bars are 50 μ m.

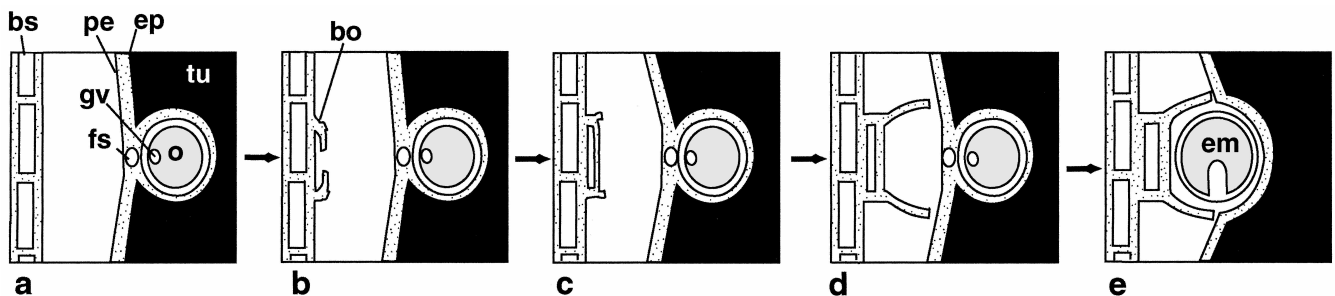


Fig. 5. Scheme showing the process of formation of the brooding organ (bo) in *B. delicatus*. (a) Early stage of the developing bud. (b) Early Stage 8. Two parts of branchial sac (bs) begin to extend toward the peribranchial epithelium (pe). (c) Middle Stage 8. Two extensions fuse with each other, and the margin of the fused extension continues to expand. (d) Late Stage 8. The brooding organ is almost complete. (e) Late Stage 9. An embryo (em) is developing in the sac-like brooding organ. ep, epidermis; fs, follicle stalk; gv, germinal vesicle; o, oocyte; tu, tunic.

made between the extension of the branchial sac and the peribranchial epithelium. When the bud became a functional zooid (Stage 9), the mature egg was ovulated into that space via the very short oviduct called the follicle stalk, and fertilization and embryogenesis occurred there (Fig. 4e). Thus, in *B. delicatus*, the brooding organ was made of both the extension of the branchial sac and the peribranchial wall.

This brooding organ was only formed in front of an ovary that contained a mature oocyte. In a bud without a mature oocyte, no brooding organ was formed. The brooding organ enveloped the embryo tightly and had a sac-like structure like

a brood pouch in the peribranchial cavity. The tadpole larva that developed in the brooding organ left its mother colony through a common cloacal aperture just before degeneration of the functional zooid (late Stage 9). A part of the brooding organ remained open so that the tadpole larva could escape from that opening. Soon after the release of a larva, its mother zooid began to degenerate and was finally resorbed into the common vascular system. The process of formation of the brooding organ is represented schematically in Fig. 5.

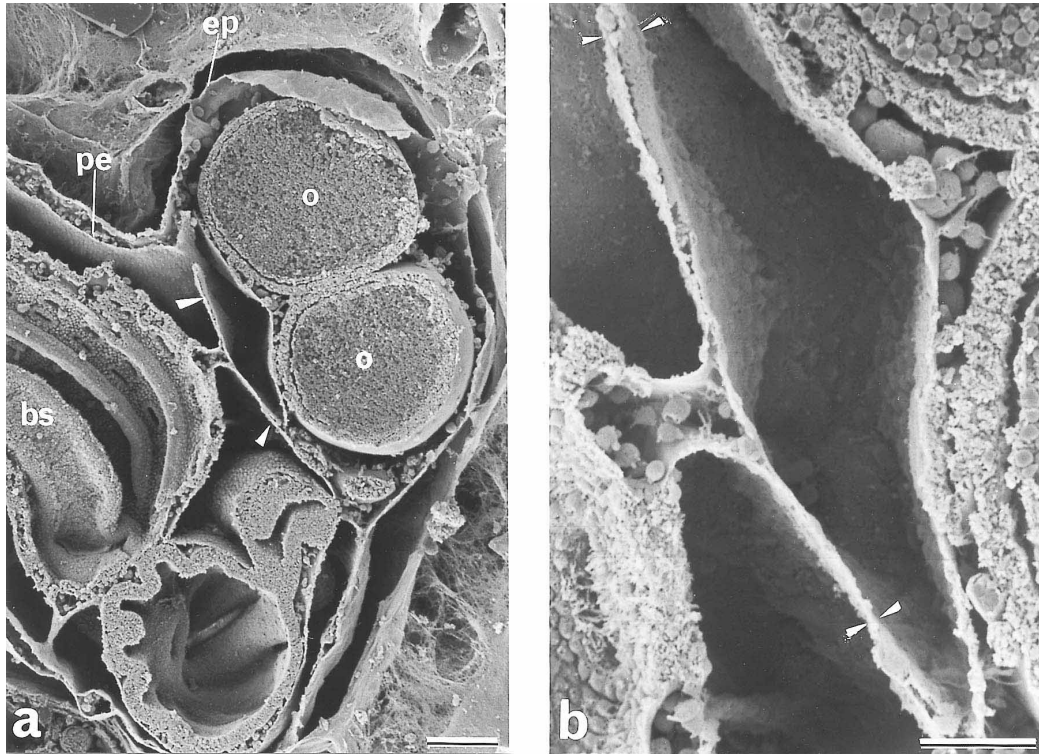


Fig. 6. Scanning electron micrograph of a bud in *B. sexiens*. Arrowheads indicate the brooding organ. (a) Branchial sac (bs) and ovary. A part of the branchial sac extending toward the peribranchial epithelium (pe) comes close to the ovary to form the brooding organ. (b) Magnified image around branchial sac. ep, epidermis; o, oocyte. Scale bars are 50 μ m in (a) and 25 μ m in (b).

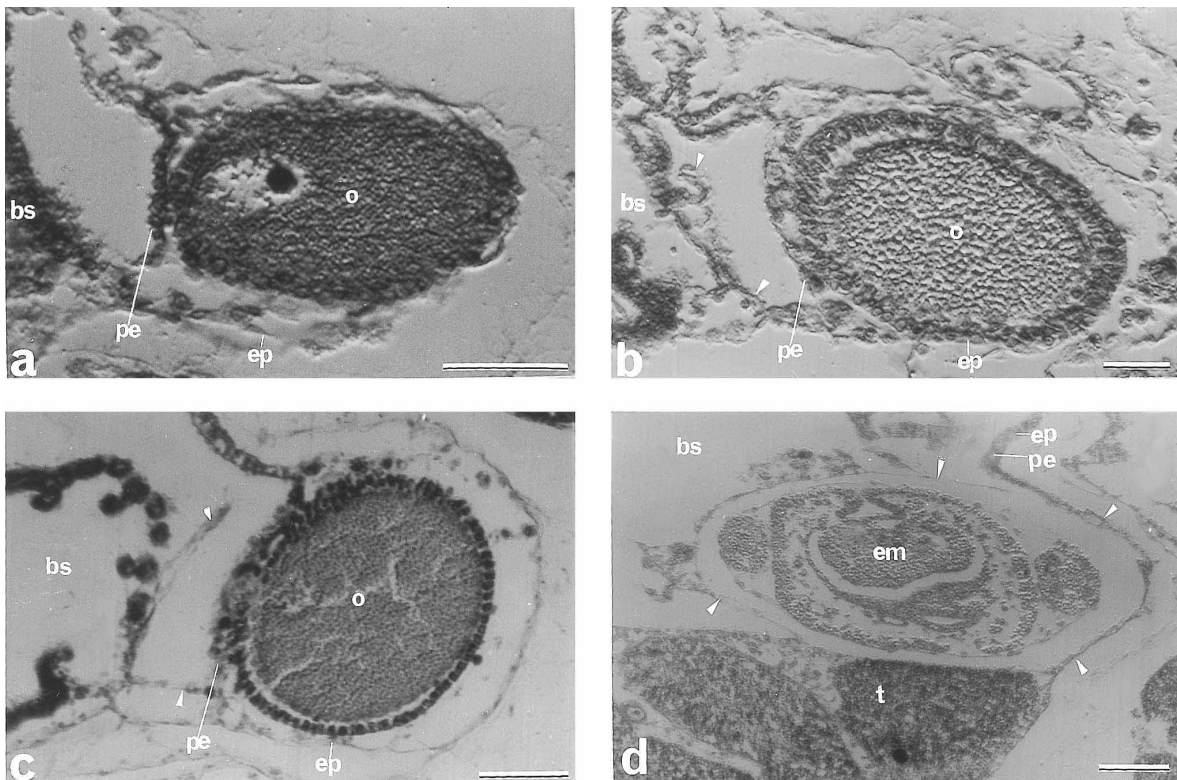


Fig. 7. Formation of the brooding organ in *B. sexiens*. Arrowheads indicate the brooding organs. (a) Early stage (Stage 6) of the developing bud. There is no brooding organ. (b) Middle Stage 8. A part of the branchial sac (bs) next to the ovary extends toward the peribranchial epithelium (pe). (c) Late Stage 8. The brooding organ is fully developed. A space is made in the peribranchial cavity. (d) Late Stage 9. An embryo (em) is developing in the brooding organ. ep, epidermis; o, oocyte; t, testis. Scale bars are 50 μ m.

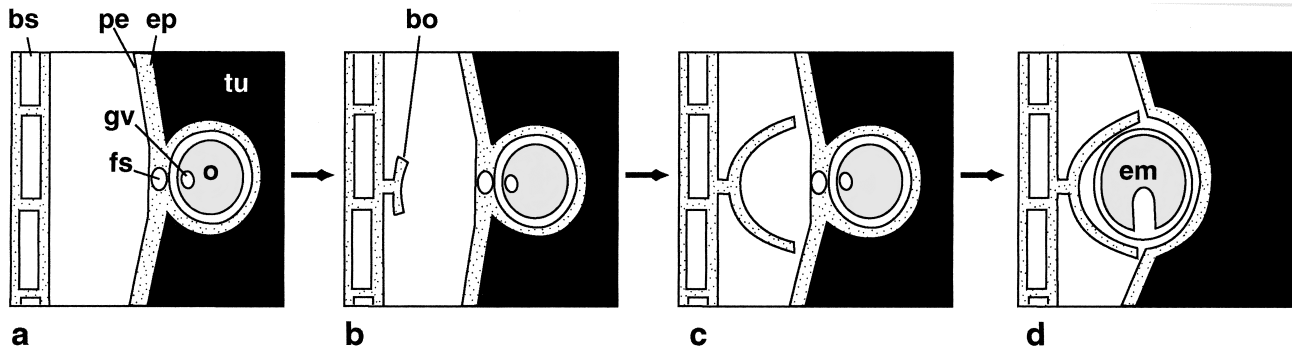


Fig. 8. Scheme showing the process of formation of the brooding organ (bo) in *B. sexiens*. (a) Early stage of the developing bud. (b) Middle Stage 8. A part of the branchial sac (bs) begins to extend toward the peribranchial epithelium (pe). (c) Late Stage 8. The brooding organ, in the shape of a bowl, is almost complete. (d) Late Stage 9. An embryo (em) is developing in the sac-like brooding organ. ep, epidermis; fs, follicle stalk; gv, germinal vesicle; o, oocyte; tu, tunic.

Development of the brooding organ of *Botryllus sexiens*

In *B. sexiens*, sexual reproduction was observed from July to December, with a peak in August (Saito *et al.*, 1981a). The brooding organ in this species was made of both the extension of the branchial sac and the peribranchial wall as well as in *B. delicatus* (Fig. 2b). From observations by SEM, the extension of the branchial sac had a thin stalk, which was different from that of *B. delicatus* (Fig. 6a). The main part of this organ consisted of two layers of the branchial epithelium (Fig. 6b). After ovulation, as in *B. delicatus*, the brooding organ with a developing embryo looked like a brood pouch in appearance.

The formative process of the brooding organ of *B. sexiens* resembled that of *B. delicatus*. The formation of the brooding organ was not observed in the early stages of the bud (Fig. 7a). At Stage 8, a small part of the branchial epithelium began to extend out toward the peribranchial wall. Then, the tip of the extension expanded widely toward the peribranchial epithelium in front of the ovary that contained mature oocytes (Fig. 7b). The extension of the branchial epithelium adhered to the peribranchial epithelium, and a space was made between the extension and the peribranchial epithelium (Fig. 7c). The extension of the branchial epithelium looked like a bowl with a short stalk. When the zooid became functional at Stage 9, the mature egg was released from the ovary into that space through the follicle stalk, and fertilization occurred there (Fig. 7d). The fertilized egg developed into a tadpole larva. Thus, the extension of the branchial sac functioned as the brooding organ in this species. The embryo was held tightly by the brooding organ in the peribranchial cavity during its development into a tadpole larva. At the end of Stage 9, the larva left its mother colony through a common cloacal aperture. Then the mother zooid degenerated and was resorbed into the common vascular system. The process of formation of the brooding organ is represented schematically in Fig. 8.

DISCUSSION

Van Name (1945) defined the differences between the two genera of the family Botryllidae, *Botryllus* and *Botrylloides*.

A zooid of *Botryllus* has one or several ovaries situated anterior or dorsal to the testis on each side of the body, without any brood pouch, and embryos develop in the peribranchial cavity. On the other hand, a zooid of *Botrylloides* has one ovary posterior to the testis on each side of the body and the embryo develops in a sac-like brood pouch formed next to the ovary as an outgrowth of the body wall. As mentioned before, it has already been shown that *Botryllus primigenus* does not fit either of these two definitions (Mukai, 1977). In this study, we showed the existence of other species that do not conform to these definitions. In the sexually mature zooid of *B. delicatus* and *B. sexiens*, the ovary is anterior to the testis, as seen in other species of *Botryllus*, but the zooid of these two species has a sac-like brooding organ that resembles the brood pouch of *Botrylloides*. As stated above, the sac-like brooding organ is typical of the genus *Botrylloides*. Therefore, these two species seem to have characteristics of both genera. However, these two species form sac-like brooding organs from both branchial and peribranchial epithelium, whereas the brood pouch of *Botrylloides* is formed only from peribranchial epithelium. Furthermore, the brooding organ in these two species is situated in front of the ovary in the peribranchial cavity, whereas in *Botrylloides* the brooding organ is formed posterior to the ovary in the body wall. These differences in the origin and position of the brooding organs suggest that these two species should belong to a different group from the genus *Botrylloides*. The findings in these two species and in *B. primigenus* lead us to reconsider the classification criteria in botryllids. At present, to describe a new species, we use the following simple criteria: In a zooid of *Botryllus* the ovary is situated anterior or dorsal to the testis, whereas in a zooid of *Botrylloides* the ovary is situated posterior to the testis. However, according to that definition, *Botryllus* should be polyphyletic, although *Botrylloides* is monophyletic. The study on the 18S rDNA sequence also supports the idea that the genus *Botryllus* is polyphyletic (Cohen *et al.*, 1998). Therefore, as pointed out by Monniot and Monniot (1987), it might be difficult to find definitive characteristics to divide the group of botryllid ascidians into two or three subgroups.

The discovery of brooding organs formed from the bran-

chial sac brings a new point of view to the phylogeny of botryllids. The characteristics of brooding organs among botryllids are summarized in Fig. 9. In *Botrylloides*, although there are ovoviviparous and viviparous species in this genus, their brood pouches have the same origin and structure. All species of *Botrylloides* make the brood pouches by invagination of peribranchial epithelium near the ovary. On the other hand, all species of *Botryllus* are ovoviviparous, but the structure of their brooding organs are different from each other. Zooids of *Botryllus scalaris* do not have any brooding organs, and the embryos are free in the peribranchial cavity (Saito *et al.*, 1981a); thus this species is considered to be the most primitive species among the botryllids. In *Botryllus schlosseri*, a brooding organ termed the “brood cup” is made of peribranchial epithelium for each embryo (Mukai, 1977). In *Botryllus primigenus*, a brood pouch is made of peribranchial epithelium, like those in *Botrylloides* (Mukai, 1977). In addition, we showed that the brooding organs were formed from the branchial sacs in *Botryllus sexiensi* and *Botryllus delicatus* in this study. The structure of the brooding organ of *B. delicatus* is more complicated than that of *B. sexiensi*. Therefore, with respect to the formation of brooding organs, the evolutionary “stream” of the botryllids might be divided into two “currents”. One is the current of making the brooding organ from a part of the branchial sac, and the other is the current of making the brooding organ from the peribranchial epithelium. *B. sexiensi*

and *B. delicatus* are in the former current. These species use a part of the branchial sac in front of the ovary as the brooding organ. In contrast, *B. schlosseri*, *B. primigenus*, and all species of *Botrylloides* use a part of the peribranchial epithelium near the ovary to make the brooding organ.

From the developmental point of view, it is thought that the branchial sac and the peribranchial epithelium have the same origin. During asexual reproduction, a thickening part of the peribranchial epithelium of the mother zooid appears as the beginning of a bud in botryllids (Berrill, 1941; Watanabe, 1953). Then, it curves into a closed sphere with a cavity (Stage 3), and this cavity is divided into three parts. All of the organs, including the branchial epithelium and the peribranchial epithelium, are made from this cavity at the same time (Stage 4). Moreover, it was reported that the branchial epithelium and the peribranchial epithelium express the same differentiation marker on the cell surface in the colonial ascidian *Polyandrocarpa misakiensis* (Fujiwara and Kawamura, 1992). In addition, both the branchial epithelium and the peribranchial epithelium were found to be derived from the endodermal tissue of the sexually reproduced larva in *Halocynthia roretzi* (Hirano and Nishida, 2000). Judging by these observations, the branchial epithelium and the peribranchial epithelium are considered to have the same origin. Therefore, perhaps there is no great difference between the two evolutionary currents with regard to the origin of the brooding organ.

	<i>Botryllus</i>					<i>Botrylloides</i>
position of ovary	anterior to testis					posterior to testis
structure of brooding organ	<i>B. delicatus</i>	<i>B. sexiensi</i>	<i>B. scalaris</i>	<i>B. schlosseri</i>	<i>B. primigenus</i>	<i>B. simodensis</i>
before ovulation						
after ovulation						
existence of sac-like brooding organ	yes		no		yes	
origin of brooding organ	branchial sac		no	peribranchial epithelium		
evolutionary current	←			→		

Fig. 9. Diagram summarizing the characteristics of brooding organs in botryllid ascidians.

In botryllid ascidians, however, the existence of a mature oocyte is necessary for the formation of a brooding organ. A bud without mature oocytes does not make any brooding organs. It is supposed that only the existence of a mature oocyte induces the formation of a brooding organ. According to Mukai (1977), the formation of a brood pouch in *Botrylloides* and in *Botryllus primigenus* was induced by the presence of an egg follicle. In the botryllids that make the brooding organ from the branchial sac, including *Botryllus delicatus* and *Botryllus sexiens*, it is of interest to learn how the branchial sac gets the information for making the brooding organ and how the part that becomes the brooding organ is determined in the branchial sac. The mature oocyte must give a "signal" to the branchial sac across the peribranchial cavity filled with seawater, because there is no direct connection between the oocyte and the branchial sac. On the other hand, in the botryllids that make the brooding organs from the peribranchial epithelium, the brooding organ is made of tissue that is adjacent to the ovaries; thus, information transfer between the mature oocyte and the peribranchial epithelium may be easier in this type of botryllid. In this respect, the difference between these two types of botryllids might be important even though the brooding organs of these two groups have the same developmental origin. Studies of the structure of the brooding organ in many more species may clarify the phylogenetic relationships of botryllid ascidians.

ACKNOWLEDGEMENTS

This work is dedicated to the late Professor H. Mukai (1938-1998), Gunma University, with deep respect. We are grateful to him for his kind advice and helpful suggestions. We thank the staff of the Shimoda Marine Research Center, University of Tsukuba, for their kind assistance and hospitality during this work. This study is contribution No. 656 from the Shimoda Marine Research Center, University of Tsukuba.

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(Received September 18, 2000 / Accepted November 7, 2000)