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Source: Zoological Science, 20(6) : 765-771

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.20.765

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ZOOLOGICAL SCIENCE **20**: 765–771 (2003) © 2003 Zoological Society of Japan

Studies on Japanese Botryllid Ascidians. III. A New Species of the Genus *Botryllus* with a Vivid Colony Color from the Vicinity of Shimoda

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ABSTRACT—The morphology and life history of a new species of the genus *Botryllus* belonging to the family Botryllidae are described in detail. This ascidian was collected from the stony shore in the cove near Shimoda Marine Research Center, University of Tsukuba (Shimoda, Shizuoka Prefecture, Japan). The ascidian colony was easily distinguished from colonies of other botryllids because it was very thin and bright pink in color. The arrangement of ovary and testis in this ascidian was the same as that in other species of the genus *Botryllus*. This ascidian was prolific, with 1-5 embryos on each side of a zooid, and the embryos of this ascidian developed in the peribranchial cavity without any brooding organs as in *Botryllus scalaris*. We observed the processes and features of the allorecognition reaction in colony specificity and found that allorejection occurred after fusion of the vascular system between two incompatible colonies. This manner of allorejection is also shown in *B. scalaris* and *Botryllus delicatus*; however, the reaction speed of allorejection is faster than that of *B. delicatus* and similar to that of *B. scalaris*. These results indicate that this ascidian might be closely related to *B. scalaris*.

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

INTRODUCTION

At Shimoda Marine Research Center (SMRC), University of Tsukuba, Shizuoka Prefecture in central Japan, several species of botryllid ascidians have been used for developmental and immunological studies for the last 4 decades, and many colonies of botryllids have been collected from the shore in the vicinity of Shimoda. These colonies have been cultured in the cove in front of SMRC (Nabeta Bay) for those studies. In 1981, from the observation on the life history of a botryllid known as *Botrylloides violaceus*, it became clear that there was some confusion in the taxonomy of this botryllid, that is, that *B. violaceus* was a polyphyletic taxon (Saito *et al*., 1981b). Actually, there are few differences in the morphology of zooids and colonies among botryllids, so such confusion in the taxonomy of botryllids is understandable. Careful observation of the life history and morphology of colonies and zooids was needed to classify botryllids precisely, and as a result many colonies of botryllid ascidians were collected from the shore around Shimoda and cultured throughout the year, so their morphology and life history could be observed in detail. These studies revealed that many species of botryllids were living in the shore around Shimoda, and most of them were unknown species. To date, four species of the genus *Botryllus* and four species of the genus *Botrylloides* have been reported as new species from the vicinity of Shimoda (Saito *et al*., 1981a, b; Saito and Watanabe, 1985; Okuyama and Saito, 2001a, 2002). Some botryllids have still not been identified, and their features have not yet been studied in detail.

On the other hand, there is an interesting phenomenon known as “colony specificity” in botryllid ascidians, which is a kind of allogeneic recognition like transplantation immunity in vertebrates. Colony specificity has been examined among many botryllids, most of which are living around Shimoda, including *Botryllus scalaris* (Saito and Watanabe, 1982; Shirae *et al*., 1999), *Botryllus primigenus* (Oka and Watanabe, 1957a; Tanaka and Watanabe, 1973), *Botryllus schlosseri* (Boyd *et al*., 1990), *Botryllus delicatus* (Okuyama and Saito, 2001a), *Botryllus promiscuus* (Okuyama and Saito, 2002), *Botrylloides simodensis* (Mukai and Watanabe, 1974; Hirose *et al*., 1997), *Botrylloides lentus* (Okuyama *et al*., 2002), *Botrylloides fuscus* (Hirose *et al*., 1994, 1997), and *Botrylloides violaceus* sensu stricto (Hirose *et al*., 1988). These immunological studies using botryllid ascidians showed that the manner of allogeneic rejection is different

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among botryllid species, and they suggested that the rejection manner may become one of the characteristics used for the classification of botryllids (Saito *et al*., 1994, 2001). Furthermore, the developmental and morphological studies of brooding organs in botryllids showed that it is very important for the classification of botryllids to determine whether the brooding organs are formed, or how the brooding organs are formed (Mukai, 1977; Okuyama and Saito, 2001b; Saito *et al*., 2001).

In the present study, we observed the morphology and life history of an unknown botryllid with a vivid body color by culturing several colonies of this species all through the year. We also examined the process of allogeneic rejection in colony specificity of this species as well as the formation of brooding organs. Then, we compared the results of this study with the features of other known botryllid ascidians.

MATERIALS AND METHODS

Animals

To observe the morphology, life history, and colony specificity of this species, several colonies were collected from the stony shore in the lower intertidal zone and subtidal zone (0–5 m in depth) in the cove known as Nabeta Bay in front of SMRC. Collected colonies were fastened to glass slides with cotton thread and cultured in boxes immersed in seawater (13–25°C) in the cove, where the environment was the most natural and undisturbed. Colonies were cleaned every two weeks by removing mud and other sessile organisms from the colony surface, and their morphology was observed under a stereomicroscope (Nikon SMZ-10).

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 specimens 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), then sectioned at 7 μm and stained with Delafield's hematoxylin and eosin gelblich (Merck). The sections were observed under a light microscope (Nikon Optiphoto).

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 20°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 rejection.

The holotype and paratypes are deposited in University of Tsukuba (TKB), Ibaraki Prefecture, Japan.

RESULTS

Botryllus puniceus Saito and Nagasawa n. sp.

Type series: HOLOTYPE: a colony (TKB-anim.2359); 2.5×5.5 cm; Y. Saito; 27 Dec. 1983. PARATYPES: a colony (TKB-anim.2360); 2.5×4.5 cm, with gonads; Y. Saito; 21 May 1984, two colonies (TKB-anim.2361); Y. Saito; 19 Nov. 1983, larvae (TKB-anim.2362); Y. Saito; 5 Jun. 1983, and oozooids (TKB-anim.2363); Y. Saito; 4 Jun. 1984.

Type locality: Shimoda, Japan.

Description

Colonies of this ascidian are found only at the stony shore in Nabeta Bay near SMRC. They encrust the surfaces of stones at a depth of 0–5 m. In the shallow habitat they often compete with colonies of *Botryllus scalaris* and *Botrylloides fuscus*. At the depth of 3–5 m, this ascidian is dominant, as colonies of other botryllids are very scarce, although the population density of this ascidian is smaller than that of the other two species in this cove. Colony size varies from a disc of a few millimeters in diameter to more than 10 cm across. Colony thickness is usually 0.8–2.2 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 usually bright pink (Fig. 1a). In addition, a few white pigment cells (a type of blood cell) are sometimes deposited 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. Blastozooids are always connected with one another by a common vascular system. The periphery of the colony is fringed with sausage-shaped vascular ampullae 400–1200 μm in length and 100–200 μm in width.

Zooids (Fig. 2a) are 2.8–3.8 mm in length and situated almost horizontally. 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 8–11 rows of stigmata on each side, and 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 lamina 5–6. 3. 3. 5–6 endostyle (periods represent longitudinal vessels; Fig. 2a). The anterior edge of the intestinal loop attains anteriorly the level of the seventh or eighth transverse vessel, and the anus opens at the level of the seventh transverse vessel. Many blood cells colored dark pink or red are deposited along each side of the endostyle in the range from the first to the eighth 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 9–10 longitudinal plications and a very elongate prominent pyloric caecum.

Asexual reproduction occurs throughout the year. Usually, each zooid produces a single bud on the left side and 1–2 buds on the right side of the body. The cycle of the alternation of generations (known as takeover; Watanabe, 1953) is about one week. Sexual reproduction can be

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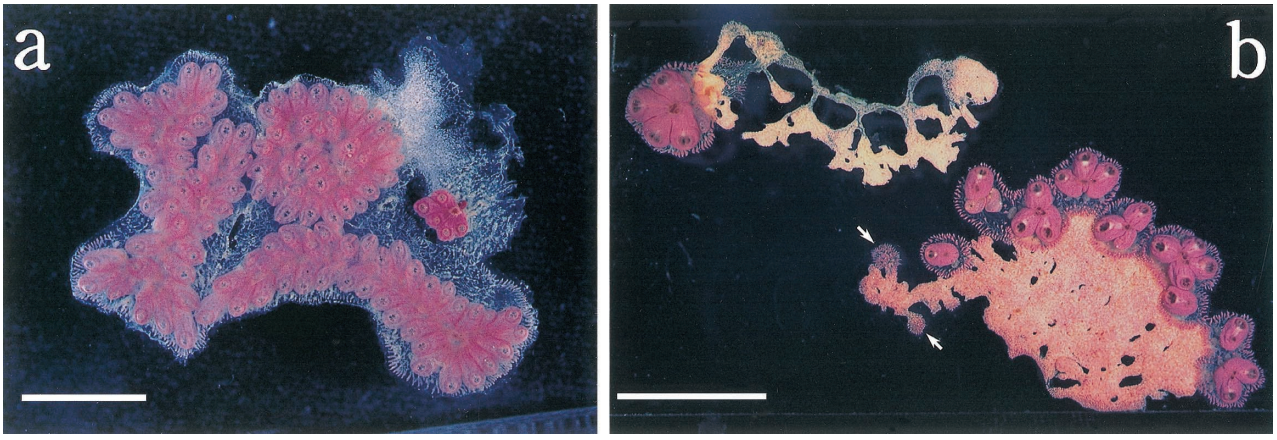

Fig. 1. Photographs of living colonies of *Botryllus puniceus* n. sp. (a) A young colony. (b) Colonies recovering from the degenerating form of a densely packed vascular system. New blastozooids are produced by vascular budding (arrows). Scale bar is 1 cm.

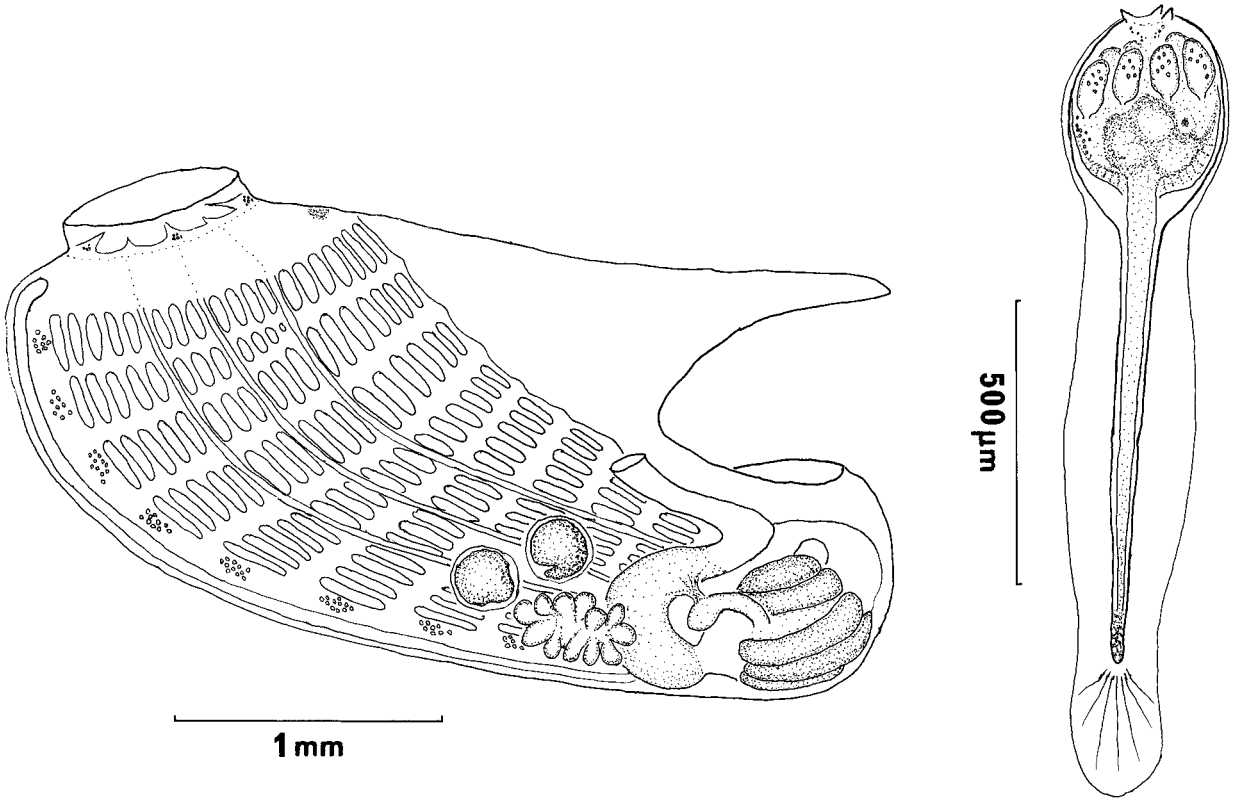

Fig. 2. *Botryllus puniceus* n. sp. (a) A zooid from the left side. (b) A larva from the left side.

observed from April to June (14–22°C), with a peak in late May. The testis is situated along the anterior edge of the circum-intestinal gland area on the left side and at the level of the ninth or tenth row of stigmata on the right side, ventrolateral to the ovary. It consists of several (10–12) lobes forming a rosette that is 450–550 µm long, 300–400 µm wide, and milky-white. Eggs mature in the ovary of a bud and reach a maximum size of about 240 µm just before ovulation, and they are orange. Usually one to three and sometimes five eggs mature on each side of the body of the new blastozooids. When the blastozooid opens its branchial and atrial siphons, eggs are ovulated into the peribranchial cavity and fertilized there soon. No brooding organs for embryos form (Fig. 3). Ovulation takes place synchronously in all zooids of a colony. The release of sperm occurs about two days after ovulation in the same zooids, so self-fertilization rarely occurs under natural circumstances. The fertilized egg develops into a tadpole larva in the peribranchial cavity of the parent zooid, and about one week after fertilization, the larva swims out of the parent colony before degeneration of the parent zooid.

Larvae usually swim out from their parent colony

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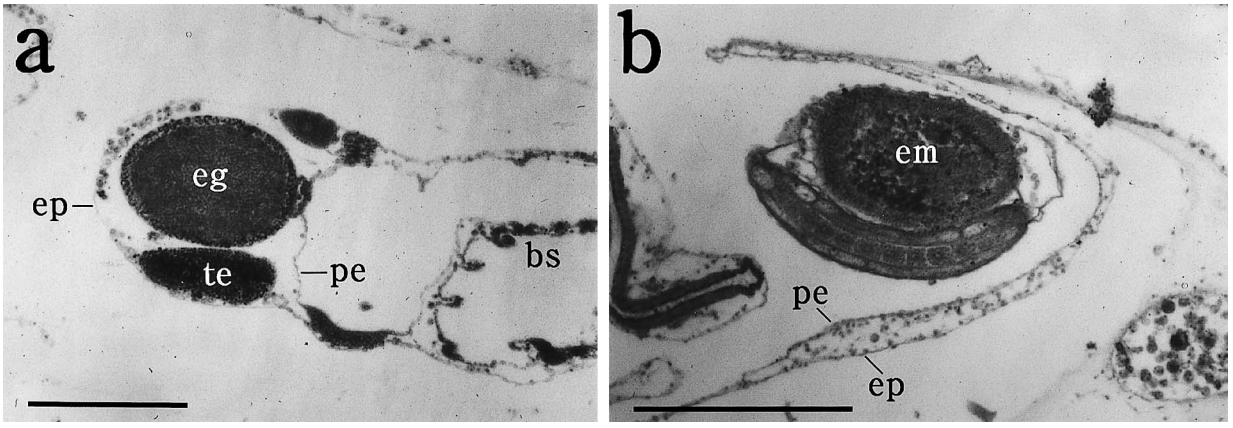

Fig. 3. Cross sections of a bud and a functional zooid of *Botryllus puniceus* n. sp. (a) A mature egg before ovulation in an ovary of a bud. Neither the peribranchial epitherium nor the branchial sac forms any brooding organs. (b) An embryo of the late developmental stage in the peribranchial cavity of a zooid. There is no brooding organ around a developing embryo. bs, branchial sac; eg, egg; em, embryo; ep , epidermis; pe, peribranchial epitherium; te, testis. Scale bars are 200 μm.

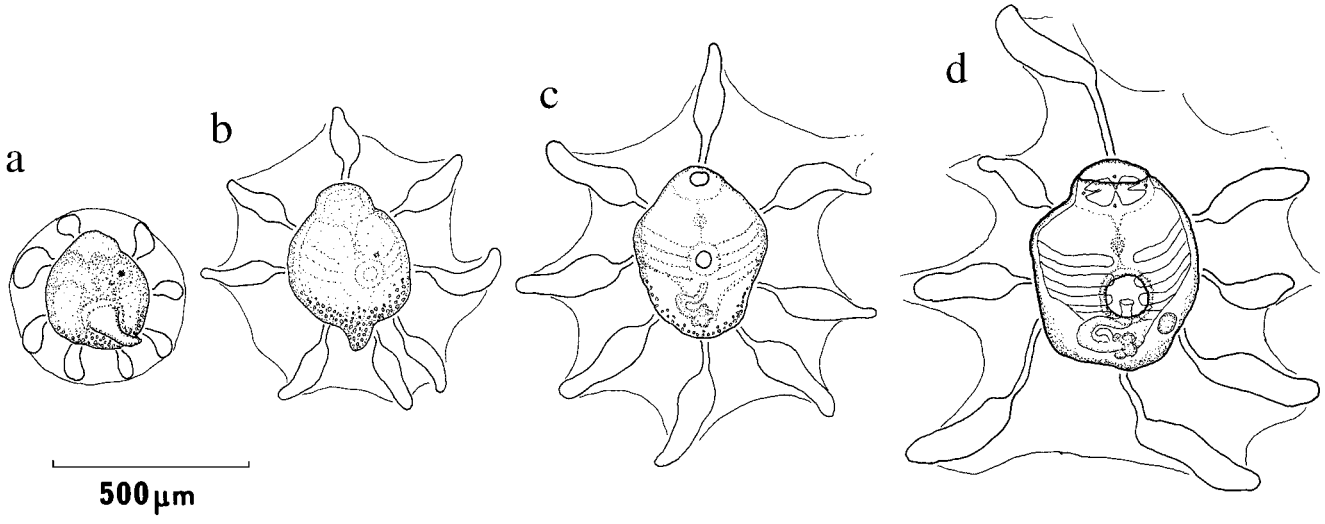

Fig. 4. *Botryllus puniceus* n. sp. Stages in metamorphosis from larval attachment to a functional oozooid. (a) An oozooid, one hour after larval attachment. Tail absorption is in progress. (b) The same, 12 hr after attachment. Tail absorption is almost completed. (c) The same, 24 hr after attachment. Metamorphosis is almost completed. (d) The same, 48 hr after attachment. Both branchial and atrial siphons open, followed by completion of a functional oozooid. The first bud appeared on the right side of the body.

between 10 a.m. and 2 p.m., most often around noon. The live larva (Fig. 2b) is about 1.5 mm long and orange. The trunk is about 350 μm and oval in outline, and it 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. Four to ten hr 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. 4a–d). The larva becomes a functional oozooid by opening its siphons and beginning to feed about 30 hr after the attachment. An oozooid (Fig. 4d) is about 500 μm long and 400 μm wide and has 4 long transverse stigmata (protostigmata) on each side of the branchial sac. There is one inner longitudinal blood vessel on each side of the branchial sac. The oozooid has four large branchial tentacles. The stomach has four or five longitudinal plications and a long pyloric caecum. The anus opens at the level of the third protostigma. About two days after attachment, the first pallial bud is formed on the right side of the body of the oozooid.

When colonies of this species are exposed to unfavorable conditions, such as high (>25°C) or low (<13°C) seawater temperature, their blastozooids degenerate and only the vascular system remains. The remaining vascular vessels gradually form clusters. These colonies consisting of only vascular systems can live more than one month, and they recover by vascular budding (noted by Oka and Watanabe, 1957b, 1959) if the surrounding environmental

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conditions improve sufficiently (Fig. 1b).

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 to form a single colony, or they rejected each other. The processes of the allorecognition reaction of this new botryllid are illustrated schematically in Fig. 5.

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 hr 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 common vascular network of each colony, then continued to extend into the tunic matrix of the facing colony (Stage 3). Twelve to fourteen hr after contact, a majority of the tips of these ampullae came into contact with the proximal part of the ampullae of the facing colony, while a few of them contacted the internal vessels directly (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 internal vessels in the center of the colony. Thus, the two colonies became a single unit (Stage 6).

In the case of rejection, the manner of allorejection in this ascidian was similar to that in *Botryllus scalaris* and *Botryllus delicatus* (Saito and Watanabe, 1982; Shirae *et al.*, 1999; Okuyama and Saito, 2001a). Fusion of both the tunic matrices and ampullae occurred in the same manner as that observed in the case of fusion (Stage 1–5). That is, even between incompatible colonies, the vascular systems of these two colonies became connected with each other. However, immediately after the exchange of blood began through the fused vessels between the two colonies, signs of rejection appeared at the vascular interconnection. The first detectable change was that blood cells began to cluster there (Stage 6'). Then, blood vessels became opaque and contracted around the interconnection, and blood flow was occluded. Finally, a few hours later, contracted vessels become amputated near the connected parts, and many blood cells dispersed from the amputated vessels into the tunic (Stage 7').

Remarks

The new species described here shows the remarkable features in its appearance, that is, the colony is very thin and its color is striking bright pink. The present species is closely related to *Botryllus scalaris* in the morphology of colonies, the manner of sexual reproduction, and the manner of allogeneic rejection (Saito *et al.*, 1981a; Saito and Watanabe, 1982). The colony thickness of this botryllid is thin, usually 0.8–2.2 mm, and blastozooids are arranged in ladder systems. The colony of *B. scalaris* is also thin, 1.0–1.5 mm in thickness, and zooids are also arranged in ladder systems. Furthermore, neither species forms any brooding organs for developmental embryos, and in both species allogeneic rejection occurs only after the fusion of blood vessels between incompatible colonies. However, colonies of *B.*

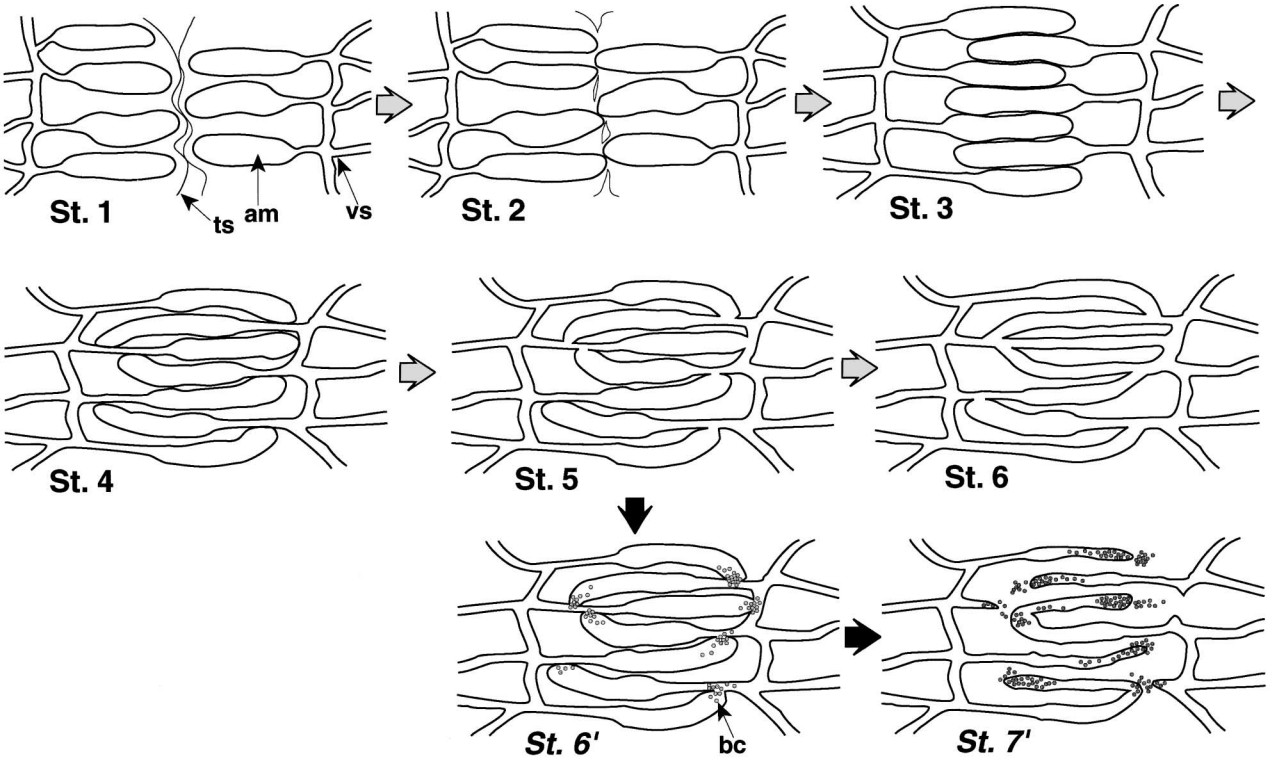

Fig. 5. Scheme showing the processes of fusion (Stages 1–6) and rejection (Stages 1–7') in *Botryllus puniceus* n. sp. am, ampulla; bc, blood cell; ts, tunic surface; vs, vascular system.

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scalaris are yellowish-brown, and the blastozooid is much smaller than that of the present species, that is, the former is about 1.5 mm in length and the latter is 2.8–3.8 mm. The number of stigmatal rows of *B. scalaris* (eight rows) is fewer than that of the present species, and the number of mature eggs in a zooid of the former (one or two eggs on each side of the body) is also fewer than that of the latter. The season of sexual reproduction of *B. scalaris* is from June to November, with a peak in August (Saito *et al*., 1981a), but in the present species it is from April to June, with a peak in late May. In the present species a colony is often transformed into the type of colony consisting only of vascular systems when it is exposed to unfavorable conditions, but that type of colony transformation is not shown in *B. scalaris*. In addition to those differences, the fusion of tunic matrices, not to mention the fusion of vascular vessels, never occurs between two colonies of the present species and *B. scalaris*. If they belong to the same species, the fusion of tunic matrices must occur between those two colonies at least, considering the manner of allogeneic recognition known to take place in these species (cf. "*Colony specificity in this new botryllid*" in RESULTS; Saito and Watanabe, 1982).

The present species is also similar to *Botryllus delicatus* in the manner of allogeneic rejection in colony specificity. A colony of *B. delicatus* forms a mass of vascular vessels without any zooids when it is exposed to unfavorable conditions, which is the same what the present species does. However, the colony color of *B. delicatus* is very different from that of the present species. That is, the colony of *B. delicatus* is basically yellow in color and orange, white, or purple pigment cells are deposited around the branchial siphon and on the atrial languet of respective zooid, while the colony of the present species is pink and sometimes with the deposition of a few white pigment cells on the atrial languet. Furthermore, the colony of *B. delicatus* is very soft and much thicker than is the present species.

The present species also resembles *Botryllus planus* Van Name of the West Indies in the arrangement of zooid in a colony and in zooid structure (Van Name, 1945). However, *B. planus* colonies are dark colored, such as purple, purplish brown, and blackish, while the body color of the present species is bright pink. There are fewer stigmatal rows in the present species than in *B. planus* (11–13), and only a single mature egg is found on each side in *B. planus*. Further, the size of a mature egg of this species (about 240 μm in diameter) is much smaller than that of *B. planus* (360–380 μm in diameter).

It is likely that the present species may safely be defined as a new species related most closely to *B. scalaris*. 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 (cf. Okuyama and Saito, 2001a, 2002). Thus, we classified this new ascidian as a member of *Botryllus* because of its arrangement of gonads and named the species after its colony color, bright pink---*Botryllus puniceus* Saito and Nagasawa n. sp.

Etymology

The specific name, *puniceus*, is derived from the Latin for bright-red, referring to the colony color.

DISCUSSION

When we collect colonies of a botryllid species on the coast, the colony color becomes a most important marker for us to distinguish one species from another. Nevertheless, in the taxonomy of botryllid ascidians, the colony color has not been regarded as important. The difference of color does not always reflect the difference of species. In the past, colonies with several variations of colony color in *Botryllus schlosseri* were described as other species of *Botryllus* (Alder and Hancock, 1912), and an adjunct problem is that it is very difficult to describe the colony color precisely and objectively, because people's perception of color varies. Even so, the person who has collected many colonies on the coast and observed them sufficiently can distinguish subtle differences among similar colors, so information about the appearance of a colony, especially its color, might be very useful and necessary for field researchers, such as those in ecology and environmental biology who deal with live colonies of botryllids, to clarify species names. In our recent studies on the taxonomy of botryllids, we have reported as much information on the color of colonies, zooids, larvae, and so on as possible. The colony of the present species is bright pink, and species of botryllid ascidians of this color have never before been seen around Shimoda. Furthermore, there are not any other reports on botryllids with such color from Japanese coast. Thus, this might be a case where the body color can be used as an important characteristic for the species identification.

In our recent studies, we have reported on the colony specificity, morphology, and life history of various botryllid ascidians. Colony specificity is commonly recognized in botryllid ascidians, and it has become clear that the manner of allogeneic rejection between incompatible colonies might be diversified among botryllid species, though the process of fusion between compatible colonies is the same in all botryllid ascidians. Therefore, the manner of allogeneic rejection should be a useful characteristic for classifying botryllids (Saito *et al*., 2001). The manner of allogeneic rejection of the present species is almost the same as that of *B. scalaris* and *Botryllus delicatus*. In those species, allogeneic rejection occurs after the fusion of vascular vessels between two incompatible colonies (Saito and Watanabe, 1982; Shirae *et al*., 1999; Okuyama and Saito, 2001a). In *B. scalaris* the rejection reaction proceeds rapidly soon after the fusion of vascular vessels. A few min after the fusion of vascular vessels, the blood flow between two incompatible colonies is interrupted by the aggregates of blood cells in the vascular lumen of the fused vessels (Shirae *et al*., 1999). On the other hand, in *B. delicatus* the rejection reaction proceeds

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gradually, and three to four hr after the fusion of vascular vessels the blood exchange is completely stopped by the cluster of blood cells (Okuyama and Saito, 2001). In the present species, the rejection reaction proceeds relatively quickly after the fusion of vascular vessels, and so the present species might be more closely related to *B. scalaris* than *B. delicatus*. In most of the botryllid ascidians examined thus far, morula cells play the most important role in allogeneic rejection, but in *B. scalaris* phagocytes play the leading role (Shirae *et al*., 1999). Therefore, it may be necessary to examine what type of blood cells play the leading part in allogeneic rejection in the present species.

As colony specificity is a kind of allorecognition, fusion of tunic matrices or vascular vessels never occurs between two xenogeneic colonies. That is, if two colonies fuse with each other, they belong to the same species. Furthermore, in species that show allogeneic rejection after tunic fusion, two colonies are not the same species if tunic fusion does not occur between them. Between a colony of the present species and a colony of *B. scalaris*, tunic fusion never occurs, which indicates that the present species is different from *B. scalaris*. Thus, colony specificity can be used as an expedient to examine whether two colonies are the same species or not, though the manner of allogeneic rejection in colony specificity is one of the useful characteristics for the classification of botryllid ascidians. We believe knowing the manner of allogeneic recognition of species should be a necessary part of determining the taxonomy of botryllids.

ACKNOWLEDGEMENTS

We express our gratitude to Dr. Hiroshi Watanabe, Professor Emeritus of University of Tsukuba, for his kind advice and helpful suggestions. We thank the staff of Shimoda Marine Research Center, University of Tsukuba, for their kind assistance and hospitality during this work. This study is contribution No. 690 from Shimoda Marine Research Center.

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(Received February 3, 2003 / Accepted March 19, 2003)

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