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Colony Specificity in the Xenogeneic Combinations among Four *Botrylloides* Species (Urochordata, Ascidiacea)

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ABSTRACT—Xenogeneic rejection reactions were histologically examined among four compound ascidians of the genus *Botrylloides; B. simodensis, B. lentus, B. fuscus* and *B. violaceus,* to compare with the allogeneic rejections of these species. When the incompatible conspecifics were brought into contact, hemolytic rejections occurred at the point where the tunic of the two colonies was partially fused. Xenogeneic contact at their growing edges induced hemolytic rejection in some combinations (*B. simodensis-B. lentus, B. lentus-B. fuscus,* and *B. fuscus-B. violaceus*), while conspicuous reaction was not found in the other combinations. Since the hemolytic rejection requires the partial fusion of tunic, the occurrence of hemolytic rejection suggests that the tunic cuticle of the colonies does not discriminate the facing colony from conspecifics. On the other hand, whereas cut surface contact between incompatible conspecifics induced intense rejection in *B. simodensis,* it resulted in fusion (formation of vascular connection) even in the combination in which the growing edge contact resulted in rejection. In xenogeneic combination, the cut surface contact of colonies always resulted in an intense rejection reaction except for *B. fuscus-B. violaceus* in which hemolytic reactions did not occur. The absence of hemolytic rejection suggests that the effector system for rejection reaction is not activated in this combination. Activity of phenoloxidase, a key enzyme of the rejection reaction, indicates lower reactivity in *B. lentus, B. fuscus* and *B. violaceus* than that in *B. simodensis.*

Key words: colonial ascidian, xeno-recognition, hemolytic rejection, viviparity, phenoloxidase

INTRODUCTION

Allogeneic recognition is one of the most fundamental interactions between individuals. Although its occurrence is often represented by allograft rejection, tissue graft is an artificial treatment that never occurs in nature. On the contrary, colony specificity is a natural occurrence of allogeneic recognition, which reported in many phyla of colonial animals. In compound ascidians, colony specificity has been mainly studied in the species of the family Botryllidae, botryllid ascidians (reviewed in Saito *et al*., 1994). Colony specificity in these species is manifested by the fusibility between two colonies juxtaposed at their natural growing edges or at the artificial cut surfaces. If the colonies are compatible, they fused into single mass sharing the common vascular systems. If they are incompatible, the colonies do not fuse and a rejection reaction is usually induced. As for the Japanese *Botrylloides*, the occurrence of colony specificity has been studied in one ovoviviparous species, *Botrylloides simodensis* (Mukai and Watanabe, 1974; Hirose *et al*., 1990; 1997; cf. Saito *et al*., 1981), and three viviparous species, *B. violaceus* (Hirose *et al*., 1988), *Botrylloides fuscus* (Hirose *et al*., 1994; 1997), and *B. lentus* (Okuyama *et al*., 2002). Whereas the eggs of ovoviviparous species are heavily yolked and the embryo size hardly increases during the development in the atrial brooding pouch, the eggs of viviparous species are devoid of yolk granules and the embryos grow larger (e.g., about 3 times in *B. lentus* and more than 10 times in *B. violaceus*) (Saito and Watanabe, 1985; Mukai *et al*., 1987; Zaniolo *et al*., 1998). All of the four *Botrylloides* species show colony specificity when two allogeneic colonies are brought into contact at their growing edges. The rejection lesion is formed in the restricted area where the tunic is partially fused with the tunic of the facing colony (subcuticular rejection, SCR), and it is hardly observable under the binocular stereomicroscope. Blood cells (mainly

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morula cells) are infiltrated into the tunic partially fused with incompatible colony, and they are broken down discharging their vacuolar contents (Hirose *et al*., 1997).

When colonies are brought into contact at their cut surfaces instead of their growing edges, the colonies always fuse with compatible colonies establishing a common vascular system, but the allogeneic response of the ovoviviparous species *B. simodensis* is much different from that of viviparous species. In *B. simodensis*, the cut surface contact of the incompatible colonies causes intense rejection reaction; many blood cells are broken down in the tunic around the contact area and new walls are formed in the tunic, separating the rejection lesion from the colonies. The rejection lesion can be seen as a black line along the contact border between the incompatible colonies. These rejection reactions occur within a few days after the contact. In contrast to the cut surface rejection of *B. simodensis*, the other three species always fuse and form a common vascular system even in the incompatible combinations at the growing edges. This cut surface fusion among incompatible colonies is referred as "surgical fusion" and is thought to be caused by the absence of allo-reactivity in the vascular system in these three species that are all viviparous. In viviparous species, the embryo encased in the brood pouch is situated in the hemocoel or the lumen of blood vessels, and thus, the absence of allo-reactivity may be necessary to allow the brooding of their embryos that are semi-allogeneic to the parent colony (Okuyama *et al*., 2002). Whereas the major effector cells for the rejection reaction are morula cells that are distributed throughout the vascular system, they do not respond to allogeneic tissue in the case of surgical fusion. Therefore, allo-recognition should be carried out by another type of cells that trigger the rejection reaction setting the effector cells to work. Phenoloxidase (PO) is one of the key enzymes in the allo-rejection reaction of botryllids, and activated PO is released from morula cells and is directly involved in the inflammatory rejection reaction (Ballarin *et al*., 1998; Shirae and Saito, 2000; Shirae *et al*., in press). PO activity of hemolysate of *B. fuscus* is much lower than that of *B. simodensis*, suggesting lower allo-reactivity in viviparous species compared to ovoviviparous species (Shirae and Saito, 2000).

Colonial contact of xenogeneic combination occurs in nature, because several *Botrylloides* are sympatric species (Cf. Saito and Watanabe, 1985). Therefore, colonies need to avoid fusing with xenogeneic colonies as well as allogeneic colonies. The recognition system of the *Botrylloides* may simply recognize non-self colonies, not discriminating allogeneic colonies from xenogeneic ones. Alternatively, the colonies may possess xeno-recognition system other than allo-recognition system. However, xenogeneic reactions are not well described in colonial ascidians to date. Although the occurrence of xenogeneic rejection was observed between *B. simodensis* and *B. violaceus* under the stereomicroscope (Hirose *et al*., 1988), the other xenogeneic combinations remained to be investigated. It is unknown whether the absence of allo-reactivity also effects on the reaction against xenogeneic tissues. Here, we reported the histological features of xenogeneic reaction among the four *Botrylloides; B. simodensis, B. lentus, B. fuscus,* and *B. violaceus*. The xeno-reaction at the growing edges and the cut surfaces varied depending on the combinations. Moreover, PO activity of hemolysate was compared among the four species to compare the reactivity of the effector system for rejection reaction in *Botrylloides.*

MATERIALS AND METHOD

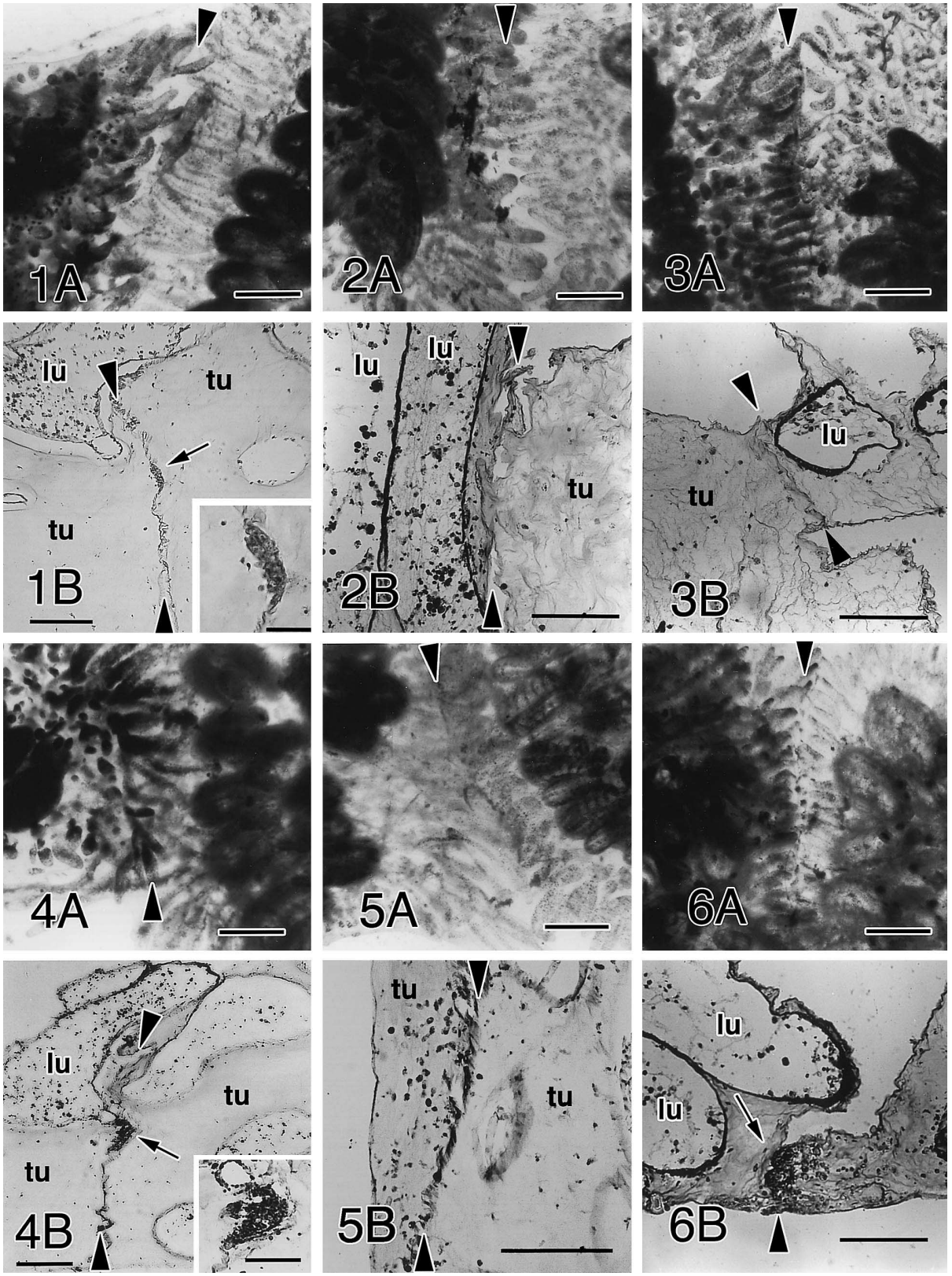
Colonies of *B. simodensis, B. lentus, B. fuscus,* and *B. violaceus* were collected in the vicinity of Shimoda. The colonies were attached on glass plates and reared in culture boxes immersed in Nabeta Bay near the Shimoda Marine Research Center (SMRC), University of Tsukuba. Well growing colonies were cut into pieces of about 15×15 mm for the fusion experiment, as follows. On the glass slide, the two colony pieces were brought into contact either at the cut surface or at the growing edges. After 1–2 hr incubation in a moisture chamber, the glass slides were placed in running seawater in the laboratory. One or two days after the contact, the experimental animals were observed and photographed under the binocular stereomicroscope and then fixed in 10% formalin-seawater. The paraffin sections were stained with Delafield's hematoxylin and eosin-orange G.

PO activity of hemolysate in *B. lentus* and *B. violaceus* was detected as previously described (Shirae and Saito, 2000). Colonies were preincubated with filtrated seawater (FSW) containing 10 mM L-cystein (pH 7.5) for 5 min to prevent hemocyte clotting and their blood was collected from the blood vessels cut with razor blades. The collected blood was centrifuged at 780 *g* for 15 min and resuspended in FSW to a cell concentration of 10^9 cells /ml. Twenty μl of hemocyte suspension was lysed with 0.1% tween 20 in phosphate buffered saline (PBS: 0.8% NaCl, 0.02% KCl, 0.02% KH_2PO_4, 0.115% Na_2HPO_4, pH 7.2) and 160 μl of PBS on ice. Twenty μl of the hemolysate was mixed with a reaction mixture consisting of 490 μl of PBS, 200 μl of L-DOPA-saturated PBS, and 290 μl of 20.7 mM MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride) in PBS containing 4% N, N'-dimethylfolmamide at 25°C. One minute after mixing, the absorbance at 505 nm was read using a Shimadzu UV-1200 spectrophotometer. PO activity in the hemolysate sample from each colony was measured in triplicate. For *B. violaceus*, six colonies were examined and for *B. lentus*, four colonies were examined.

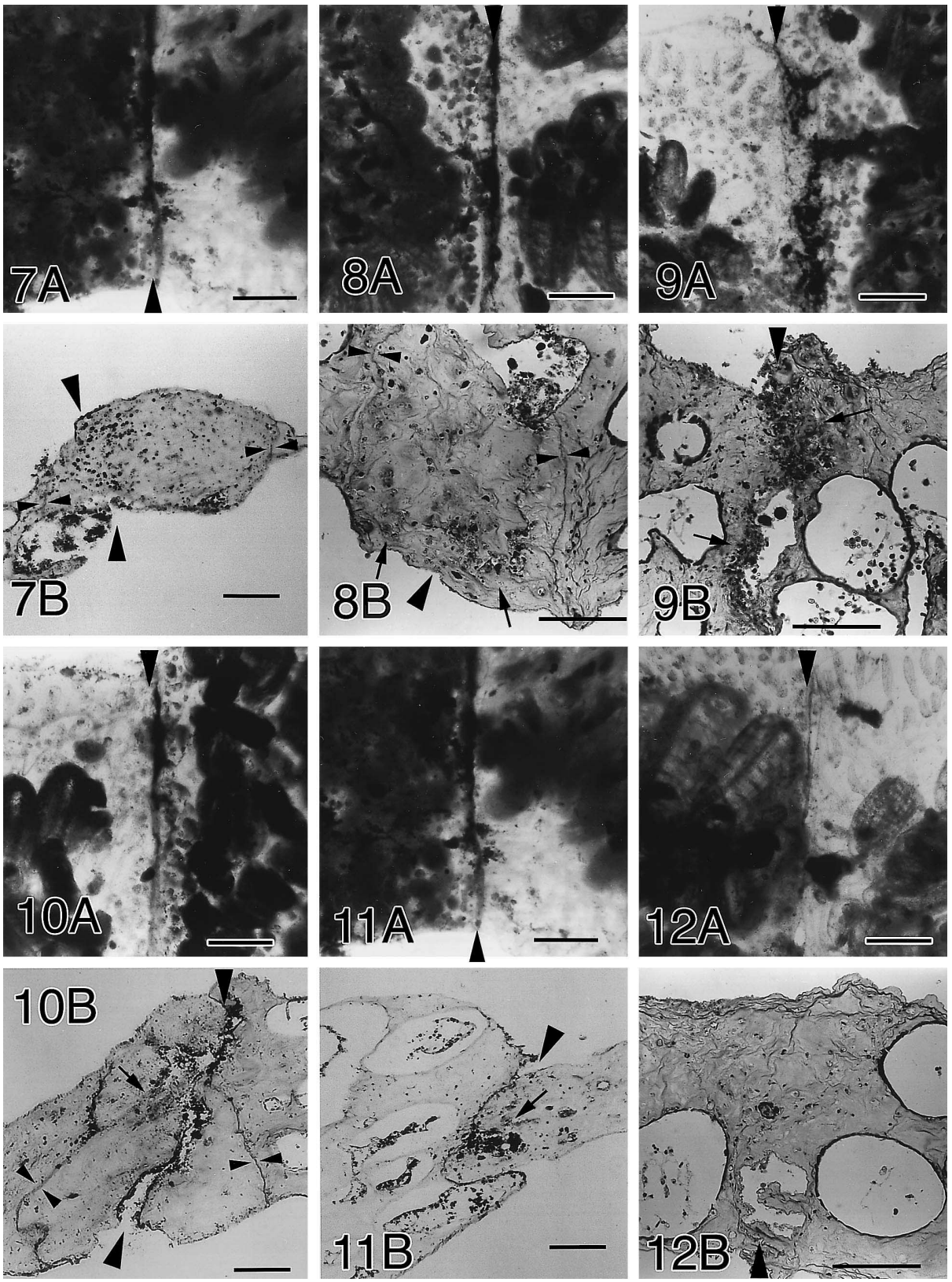
RESULTS

Xenogeneic reactions induced by growing edge contact

Colonial fusion never occurred in any xenogeneic combinations. At the contact area, the colony peripheries of both colonies were seen to push against each other, but their vascular ampullae never penetrated into the tunic of the facing colony. Signs of rejection were not clearly visible in the contact area under the binocular stereomicroscope (Fig. 1A–6A). In histological sections, subcuticular rejection (SCR) occurred in some combinations, i.e., *B. simodensis-B. lentus, B. lentus-B. fuscus,* and *B. fuscus-B. violaceus* (Fig. 1B, 4B, 6B). The tunic of the contacting colonies was partly fused, and the infiltrated hemocytes were aggregated and disintegrated there (insets in Fig. 1B, 4B). Many of these



Figs. 1–6. Xenogeneic reactions induced by the growing edge contact (A, stereomicroscope; B, histological section). Two to five days after the contact. Arrowheads indicate the contact border. Arrows point the lesion of subcuticular rejection (SCR). lu, lumen of the vascular ampullae, tu, tunic matrix. Scale bar: 1 mm for A, 100 μm for B, 50 μm for insets in 1B and 4B. Fig. 1. *B. simodensis* (left) and *B. lentus* (right). Inset, enlargement of SCR. Fig. 2. *B. simodensis* (left) and *B. fuscus* (right). Fig. 3. *B. simodensis* (left) and *B. violaceus* (right). Fig. 4. *B. lentus* (left) and *B. fuscus* (right). Inset, enlargement of SCR. Fig. 5. *B. lentus* (left) and *B. violaceus* (right). Fig. 6. *B. fuscus* (left) and *B. violaceus* (right).



Figs. 7–12. Xenogeneic reactions induced by cut surface contact (A, stereomicroscope; B, histological section). Two to three days after the contact. Arrowheads indicate the contact border. Arrows point hemocytes broken down in the tunic. Scale bar; 1 mm for A, 100 μm for B. Fig. 7. *B. simodensis* (left) and *B. lentus* (right). Fig. 8. *B. simodensis* (left) and *B. fuscus* (right). Fig. 9. *B. simodensis* (left) and *B. violaceus* (right). Fig. 10. *B. lentus* (left) and *B. fuscus* (right). Fig. 11. *B. lentus* (left) and *B. violaceus* (right). Hemocyte infiltration was observed, particularly in the tunic of the left colony, but hemolysis did not occur. Fig. 12. *B. fuscus* (left) and *B. violaceus* (right).

hemocytes were morula cells that stained well with eosin. This rejection reaction is essentially the same as SCR in allogeneic rejection in these *Botrylloides* species.

In contrast, neither partial fusion of the tunic nor hemolytic rejection occurred in the other combinations (*B. simodensis-B. fuscus, B. simodensis-B. violaceus,* and *B. lentus-B. violaceus*) (Fig. 2B, 3B, 5B). In these cases, the tunics were simply in contact without any connections with the counterpart, and the facing tunic cuticles were often interdigitated. In some combinations, particularly *B. lentus-B. violaceus*, although hemocyte infiltration was occasionally found in the tunic, the hemocytes neither aggregated nor broke down (Fig. 5B). Since this reaction does not involve hemolytic interaction between the colonies, it is referred as "nonfusion without cuticular fusion" here.

Xenogeneic reactions induced by cut surface contact

A hemolytic rejection reaction occurs in the all combinations except for *B. fuscus-B. violaceus*, and the rejection lesion was observed as a conspicuous black line along the boundary of the colonies (Fig. 7A–11A). This rejection reaction was essentially the same as the allogeneic rejection induced by cut surface contact between incompatible colonies of *B. simodensis*. Many hemocytes, mainly morula cells, were infiltrated and disintegrated in the tunic around the contact area forming the rejection lesion (arrows in Fig. 7B–11B), and boundary structures (new walls) appeared to separate the colonies from the rejection lesion (small arrowheads in Fig. 7B, 8B, 10B). Among these combinations in which hemolytic rejection occurred, the extent of rejection lesion was minimum in the xeno-rejection between *B. lentus* and *B. violaceus*; the black line was not so dark and the amount of hemocyte infiltration and disintegration was much smaller than the other combinations (Fig. 11).

In contrast, the rejection reaction was not conspicuous in the combinations between *B. fuscus* and *B. violaceus* (Fig. 12B). Two days after the contact, neither black lines nor interconnection of blood vessels were observed under a binocular stereomicroscope. In histological sections, the tunics of both colonies were fused for a few days after the contact, and many tunic cells were seen in the contact area. There were, however, no signs of hemocyte infiltration and disintegration in the tunic. This inconspicuous non-fusion reaction is referred as "nonfusion without hemolytic reaction". The contacting colonies usually separated within one week.

PO activity of hemocytes

Fig. 13 shows the PO activity (Δ A_{505}/min $\times$100) of the hemolysates in the first minute in *B. lentus* and *B. violaceus* compared with those of *B. simodensis* and *B. fuscus* that were previously reported (Shirae and Saito, 2000). The PO activities of viviparous species (*B. lentus, B. fuscus, B. violaceus*) were much lower than the activity of the ovoviviparous species (*B. simodensis*).

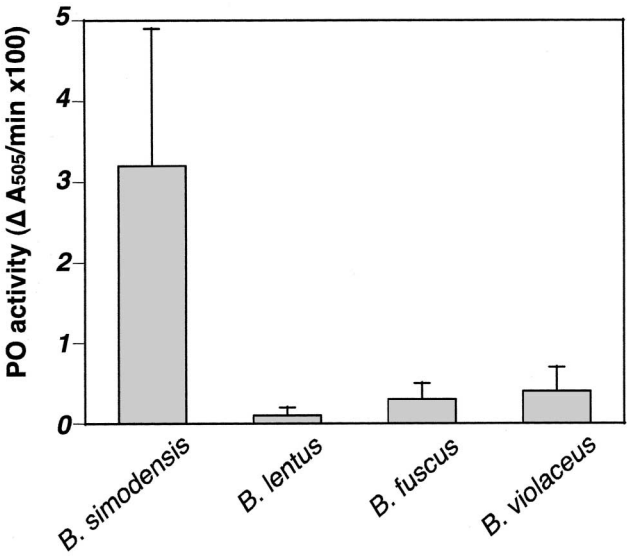


Fig. 13. Phenoloxidase (PO) activity in the hemolysates of the four *Botrylloides*. Error bars, standard deviation. The data of *B. simodensis* and *B. fuscus* is referred from Shirae and Saito (2000).

DISCUSSION

Xenogeneic fusion were never established in the all combinations among the four *Botrylloides* species, while the xenogeneic reactions varied depending on the type of contact between the colonies (growing edge or cut surface contact) and the species combination. Table 1 summarizes the reactions induced by the two types of contact in allogeneic and xenogeneic combinations. The phylogenetic relationship among the four species may reflect on the mode of xenogeneic reaction. Phylogeny of *Botrylloides* species was mainly discussed based on the mode of sexual reproduction, and *Botrylloides* species are thought to have progressed from ovoviviparity to viviparity and from a short brooding period to a long period (Saito and Watanabe 1985; Saito *et al*., 2001). This phylogenetic view arranges the present four species as following order; *B. simodensis* (the most primitive), *B. lentus* , *B. fuscus* , and *B. violaceus* (the most advanced).

In the case of growing edge contact, SCR was induced in some combinations (*B. simodensis-B. lentus, B. lentus-B.*

Table 1. Allogeneic and xenogeneic reaction induced by the growing edge or cut surface contact of the colonies in four *Botrylloides*

	Reactions* (growing edge / cut surface)			
Species	*B. simodensis*	*B. lentus*	*B. fuscus*	*B. violaceus*
B. simodensis	SCR/R	SCR/R	NFc/R	NFc/R
B. lentus		SCR/SF	SCR/R	NFc/r
B. fuscus			SCR/SF	SCR/NFh
B. violaceus				SCR/SF

* NFc, nonfusion without cuticular fusion; NFh, nonfusion without hemolytic reaction; R, intense rejection; r, rejection less intense than "R"; SCR, subcuticular rejection; SF, surgical fusion.

fuscus, and *B. fuscus-B. violaceus*); hemocytes (mainly morula cells) aggregated at the tunic partially fused with the counterpart and disintegrated there. In SCR between allogeneic combinations, the initial interaction between the colonies is dissolution of the tunic cuticles resulting partial fusion of the tunics (Hirose *et al*., 1997), and the morphological process of xenogeneic SCR is very similar to that of allogeneic SCR. In other words, the colonies could not discriminate the xenogeneic colony at the tunic cuticle, and thus the dissolution of the tunic cuticle might proceed in the same way as allogeneic SCR. The occurrence of xenogeneic SCR may suggest that the phylogenetic relationship is so close that they overlook the tunic fusion with xenogeneic colonies. This is consistent with the phylogeny deduced from the mode of sexual reproduction (described above). However, it is uncertain whether the colonies discriminate allo-grafts from xeno-grafts or they simply recognize the grafts as "nonself". On the other hand, neither partial fusion of the tunic nor hemolytic rejection occurred (i.e., nonfusion without cuticular fusion) in the combinations *B. simodensis-B. fuscus, B. simodensis-B. violaceus,* and *B. lentus-B. violaceus,* suggesting that the colonies did not recognize the contacting colony as conspecifics and/or closely related species. It is possible that the differences of chemical constitution of the tunic cuticles may not allow the dissolution of the cuticles in these xenogeneic combinations. Partial fusion of the tunic, i.e., dissolution of the tunic cuticle, appears to be essential for the occurrence of SCR. We supposed that the cuticle dissolution occurs when a colony recognizes the facing colony as a candidate of "self" in order to promote more precise self-nonself recognition in the tunic. The mechanism of cuticle dissolution should be an important key to disclose the process of the allo-/xeno-recognition in colonial ascidians.

The cut surface contact of incompatible conspecifics always results in "surgical fusion" in the viviparous *Botrylloides, B. lentus, B. fuscus,* and *B. violaceus* (Hirose *et al*., 1988; 1994; Okuyama *et al*., 2002), and thus allo-recognition sites are supposed to be absent in their vascular systems. Furthermore, Shirae and Saito (2000) and the present study have shown that the PO activity of hemolysate in these viviparous species is much lower than the ovoviviparous *Botrylloides,* i.e., *B. simodensis,* suggesting the lower reactivity of the effector system in the viviparous species than the ovoviviparous species (Fig. 13). Our preliminary results suggest that the low PO activity in the viviparous species reflects not only lower amount of morula cells per unit volume of blood but also the lower PO activity of each morula cell as compared to *B. simodensis*. In the present study, the cut surface contact of xenogeneic colonies never resulted in interspecific fusion in the all combinations among the three species as well as the other combinations, indicating the xeno-grafts were recognized as nonself even in the species that can not discriminate incompatible conspecifics from compatible conspecifics at their cut surface. In the all xenogeneic combinations except for *B. fuscus-B. violaceus,* cut surface contact of colonies induced intense rejection involving black line formation; a number of hemocytes (mainly morula cells) infiltrated and broke down around the contact border, discharging the vacuolar contents of the hemocytes. This process is the same as that of allo-rejection induced by the cut surface contact in *B. simodensis* (Hirose *et al*., 1990). Xenogeneic recognition may activate the effector system in the similar way of allo-rejection. Although the PO activities in the viviparous species are much lower than that of the ovoviviparous species, hemolytic rejection occurred in some combinations of viviparous species (*B. lentus-B. fuscus* and *B. lentus-B. violaceus*). This indicates that the effector system in these viviparous species is capable of promoting the hemolytic rejection reaction at the cut surface. This may also suggest that the low PO activity is not the primary reason for the occurrence of surgical fusion in the viviparous species. By contrast to the hemolytic rejection described above, cut surface contact between *B. fuscus-B. violaceus* resulted in inconspicuous nonfusion reaction. Although there is no vascular fusion, neither black line formation nor hemocyte infiltration occurs (i.e., nonfusion without hemolytic reaction). It is probable that the xenogeneic contact between *B. fuscus-B. violaceus* does not activate the effector system in the both species.

Since SCR is induced by growing edge contact of allogeneic colonies, the occurrence of allogeneic surgical fusion does not mean the absence of effector system for rejection reaction in the vascular system but does mean the absence of allo-recognition sites there. The viviparous botryllids are thought to have evolved from ovoviviparous species, and thus, the viviparous *Botrylloides* are assumed to have "lost" the allo-recognition site in the vascular system. In botryllid ascidians, the absence of allo-reactivity in the vascular system is exclusively found in viviparous species in which embryos develop in the vascular system of their mother colony (Okuyama *et al*., 2002). The embryos expressing the paternal genome are semi-allogeneic for their mother colony. Since semi-allogeneic colonies are fusible in short term but result in separation or resorption in log term incubation (Rinkevich and Weissman, 1987; 1992), the embryos of viviparous species are possible targets of the allo-recognition system of the mother colony. Therefore, the loss of allo-reactivity is supposed to be essential to acquire the viviparity in order to avoid rejecting their embryos. Among the three viviparous species, the brooding period of *B. lentus* is the shortest (about 10 days), that of *B. fuscus* is about 2 weeks, and that of *B. violaceus* is about 1 month (Saito and Watanabe, 1985). The non-hemolytic xeno-reaction between *B. fuscus* and *B. violaceus* may suggest that the longer brooding period requires a less reactive effector system in the vascular system. On the other hand, the failure of surgical fusion between these viviparous colonies indicate that their vascular system probably possesses another recognition system that recognize xenogeneic materials as non-self.

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