ZOOLOGICAL SCIENCE 9: 983–988 (1992)

Self-Nonself Recognition in the Colonial Protochordate *Botryllus schlosseri* from Mutsu Bay, Japan[1]

BARUCH RINKEVICH[2] and YASUNORI SAITO[3]

[2]*National Institute of Oceanography, Israel Oceanographic & Limnological Research, Tel-Shikmona, P.O.B. 8030, Haifa 31080, Israel and*
[3]*Shimoda Marine Research Center, University of Tsukuba 5-10-1 Shimoda-shi, Shizuoka 415, Japan*

ABSTRACT—Wild *Botryllus schlosseri* collected from a 5×5 m area in Mutsu Bay (Aomori Prefecture, Japan) were tested for alloresponses in intrapopulation colony allorecognition assays (CAAs). Results indicate that rejection patterns are similar to those recorded previously in the populations from Monterey and Santa Barbara, California, from the Mediterranean coast of Israel, and from the Venetian lagoon, Italy. The only difference was the marked accumulation of bright-yellow blood cells in the tips of interacting ampullae. Pairwise CAAs which were performed on all combinations (n=91) from 14 colonies resulted in 12.1% fusions, which gives a populational estimation of 32 alleles on the fusibility locus. A fusibility chart for these 14 genotypes revealed 18–19 different, not equally frequent, allorecognition alleles, of which 12 were assigned to only one genotype, each; 5 occurred twice, and each one of 2 alleles was present in three different genotypes. It is concluded that the sampled area was too small to represent the probable higher number of fusibility alleles residing in this population.

INTRODUCTION

It is now almost 90 years that studies on the colonial tunicate *Botryllus schlosseri* (Pallas) have shown its capacity for colony specificity [1], a histocompatibility system which resembles the major histocompatibility complex (MHC) of the vertebrates in many aspects [2]. The genetic basis for the colony specificity resides in a single, highly polymorphic haplotype (called the fusibility/histocompatibility locus, Fu/HC [3]), which possesses multiple codominantly expressed alleles. Interacting colonies which do not share any allele on this locus reject each other, while colonies which share in common at least one allele on the Fu/HC locus may undergo a natural transplantation (fusion) by forming vascular anastemoses between their peripheral ampullae [2, 4–6].

B. schlosseri is a cosmopolitan inhabitant of shallow water, hard bottom communities [7, 8]. This species has been studied for intrapopulation allorecognition responses in 4 remote localities: the population from the Venetian lagoon, Italy [6, 9], from the Mediterranean coast of Israel [10], from Woods Hole, Massachusetts, Atlantic Ocean [2, 11, 12], and from the Monterey and Santa Barbara areas, California, Pacific Ocean [11–15]. Results indicated that allogenic interactions between ampullae of noncompatible *Botryllus* conspecifics exhibited not only the species-specific characteritics of rejection processes, but also population-specific rejection types which were constantly expressed even in interpopulation encounters [11, 12]. When two incompatible colonies of the Woods Hole population came into tunic-tunic contact, a limited fusion of the cortical layers of both colonies prevailed, which resulted in a continuous tunic matrix between both partners. This permitted a reciprocal ampullae penetration which was followed by ampullae amputation and/or haemorrhages formation, the development of dark-brown necrotic areas, points of rejection (PORs). In the other 3 studied *B. schlosseri* populations, on the other hand, the outer layers of the tunics did not fuse during allogeneic encoun-

Accepted July 1, 1992
Received April 16, 1992
[1] Contribution number 541 from the Shimoda Marine Research Center.
[3] To whom all correspondence should be addressed.

ters. As a result, the cortical layers were clearly observed as demarcating lines between colonies. PORs, therefore, were developed without reciprocal ampullae penetration.

The above studies [12, 13] have suggested that an extended comparison of intrapopulation allogeneic interactions would be of great benefit for a better understanding of self/nonself histocompatibility alloresponses of this cosmopolitan species. Here we study allorecognition of a *B. schlosseri* population from Mutsu Bay, Japan, and try to evaluate the polymorphism pattern of the Fu/HC locus by calculating frequencies of fusion within a population sampled from a small area.

MATERIALS AND METHODS

Wild *B. schlosseri* colonies were collected (November 1991) in Mutsu Bay, Aomori (Aomori Prefecture), where they were grown in shallow water on floating Pecten culturing cages. All colonies were collected from a 5×5 m area. Large healthy colonies were removed from substrates by razor blades, tied with thin cotton fibers onto 2.5× 7.5 cm glass slides, and shipped to Shimoda Marine Research Center, where they were kept vertically in slots within a wooden culture box, submerged in Nabeta Bay, Shimoda. For the colony allorecognition assays (CAAs), we used small groups of zooids at the growing edges of colonies, carefully isolated from colonies. These subclones were attached in pairs on 5×7.5 cm glass slides, as described previously [11–15]. During the experiments, the colonies were kept in 17-liter standing seawater tanks, aerated by airstones and maintained at 17–18°C by aquarium heaters. CAAs were observed daily and cleaned during the observations by soft small brush. Food was supplied daily (artificial diet; Liquifry Marine, England).

RESULTS

Intrapopulation alloresponses

Contacts between extended ampullae of encounter colonies were established within 24 hr after forming the CAA. Tips of marginal ampullae were reciprocally positioned in tip-tip orientation where the cortical layers of the allogeneic partners were clearly observed as demarcating lines along contact areas. When compatible pairs were assayed, the tunic matrices eventually fused in limited or in broader areas. Ampullae of both partners, or of only one partner in a pair, penetrated into the tunic matrix of the other colony through the fusion areas and were positioned in tip-ampulla base (proximal part) orientations. This resulted, within less than 24 hr, in allogeneic anastomosis of blood vessels, the formation of chimeras. Chimeras were followed up for a period of one month. During this period, chimerism in several assays already ended in the resorption of all zooids of one partner each.

When nonfusible partners come into direct contact, tunics did not fuse together, so ampullae engaged each other reciprocally but indirectly, through both cortical layers (Fig. 1a-i). During this process, the tips of interacting ampullae, and sometimes also the tips of all peripheral ampullae (even those of the other sides of the colony), of one or both partners in a pair, became very distinctive in bright fluorescent yellow color. This resulted from aggregations of bright yellow blood cells which accumulated in the ampullar tips (Fig. 1a, b). This phenomenon was also observed in interacting colonies of the Monterey population [11, 12] and the Mediterranean colonies [10]. By employing histological examinations, we (Tertakover and Rinkevich, unpublished) characterized the accumulated cells as morula cells. However, the Mediterranean and Monterey populations did not exhibit the deeper intensity in color and the high frequency of cases as it is recorded in the Japanese population. In most of the yellow colored tips, the intense yellow color gradually abated and disappeared within the next 24–72 hr, while in some of the tips the color became dark-brown, blood cells infiltrated out of the ampullar tips, and the formation of PORs through haemorrhages was documented (Fig. 1a-i). In a few cases, ampullae were amputated from the peripheral blood vessels and gradually disintegrated. Only a few (1–8) out of many (up to tens) of interacting ampullae produced cytotoxic lesions (Fig. 1a, e). Following the acute phase of allogeneic response, where all PORs were developed within a short period of a

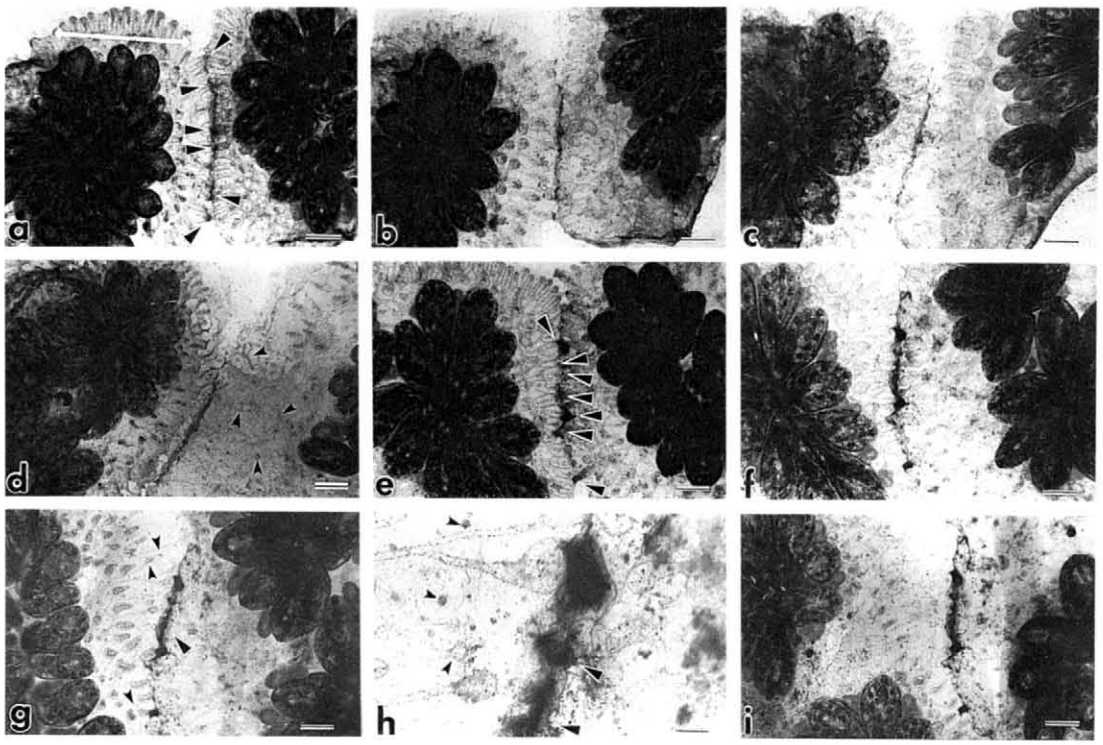


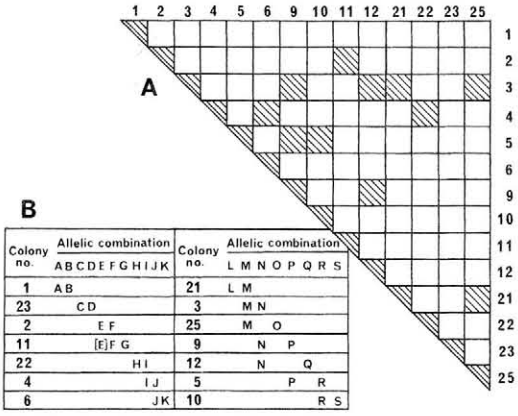
FIG. 1. Allogeneic interactions between *B. schlosseri* colonies: genotype combination 3 (left) vs 2 (a-d), and genotype combination 10 (left) vs 3 (e-i). a-48 hr after CAA. Six small reciprocal PORs along the contact area (arrowheads) out of 12 vs 17 interacting ampullae. Tips of ampullae (genotype 3) still possessed aggregations of bright yellow cells in the contact area and in other peripheral ampullae (confined between two white arrowheads). b-Two days thereafter. No more PORs were added. Ampullae were cleared of yellow cell aggregations, and only 9 vs 7 were still interacting. Genotype 2 started retreat growing [14]. c-Two days later. Only a few colony 2 ampullae were still in contact with those of colony 3. Colony 2 zooids retreated 1–2 mm. d-7 days later. Zooids of colony 2 retreated more than 3 mm from the initial location, leaving an unvascularized tunic matrix with rudiments of blood vessels/ampullae and masses of degenerated infiltrating blood cells (small black arrowheads). e-48 hr after doing CAA. 17 vs 14 interacting ampullae, respectively. 7 PORs (arrowheads) were all produced by colony 10, but some were not yeat well developed. In genotype 10, the upper peripheral ampullae still had the yellow color. f-Two days thereafter. All former PORs were fully developed and no more were added. Ampullae were cleared of yellow color. g-h-4 days later. Some of the large necrotic lesions were diffused and dispersed (arrowheads), forming a black line along the contact area between the two genotypes. Interacting ampullae of both colonies (predominantly of genotype 10) retreated, leaving behind degenerated ampullae and masses of dying cells (small black arrowheads). i-5 days thereafter. Ampullae of both genotypes reciprocally retreated, leaving degenerating areas. Scale of bars: a-g, i = 1 mm. h = 0.25 mm.

few days (usually within 48 hr), other ampullae continued to interact for longer periods, but without the formation of any more PORs (Fig. 1d, g-i). It was also evident in many cases that during POR development and thereafter, ampullae of one or both colonies in a pair withdrew from the contact areas, leaving a bare tunic which gradually deteriorated (Fig. 1d, h, i). This process was sometimes enhanced by the "retreat growth phenomenon" [14], where fewer buds than zooids per generation were developed in the contact area, resulting in a directional colony growth form, away from interacting zones (Fig. 1b-d).

Pairwise allorecognition assays

We examined 14 Japanese *B. schlosseri* colonies

in a pairwise allorecognition panel of all 91 combinations. Fusions were recorded in 11 (12.1%) allogeneic assays (Fig. 2a, striped squares) as well as in all the controls, the isogeneic combinations (Fig. 2a, striped triangles). An Fu/HC chart for the studied 14 genotypes (Fig. 2b) revealed 18 to 19 different fusibility alleles. Genotypes 11 and 2 are clearly distinguished from each other since they possess different color morphs. However, according to the predictions of the fusibility model (legend to Fig. 2b), they share in common either one or both Fu/HC alleles. Twelve out of the maximum 19 Fu/HC alleles (63.2%) are assigned to only one colony each, while 5 alleles (F, I, J, P, R) (26.3%) occurred twice, and 2 alleles (M, N) (10.5%) occurred three times.



Colony no.	Allelic combination A B C D E F G H I J K
1	A B
23	C D
2	E F
11	[E] F G
22	H I
4	I J
6	J K

Colony no.	Allelic combination L M N O P Q R S
21	L M
3	M N
25	M O
9	N P
12	N Q
5	P R
10	R S

FIG. 2. A pairwise allograft panel between 14 Japanese *B. schlosseri* colonies (A) and their Fu/HC allelic combination chart (B). A-Each rejecting CAA set is marked by a blank square, fusible pair by a striped square. All isografts resulted in fusions (striped triangles). B-The chart depicting predictions for a single Mendelian locus model of partial genetic matching for histocompatibility [2, 4–6], an exclusive heterozygosity of the Fu/HC locus in wild genotypes [2, 12], and a codominant expression by Fu/HC alleles [2, 4–6]. Colony 11 is marked also by allele E in brackets to note that it can share with colony 2 one or both alleles on the Fu/HC haplotype.

DISCUSSION

Intrapopulation allorejection processes in *B. schlosseri* from Mutsu Bay, Japan, are of like pattern as in the populations from the Venetian lagoon, from the Mediterranean coast of Israel and from the Monterey-Santa Barbara areas [6, 9, 10–15]. The only difference which we could detect which characterized the Japanese population from all the above is the marked accumulation of yellow colored cell aggregations in the tips of interacting ampullae. Therefore, out of the 5 studied *B. schlosseri* populations up to date, only the Woods Hole population differs significantly from all the others when comparing between the effector mechanisms which are expressed during intra- or interpopulation interactions [11, 12, 16]. Since *B. schlosseri* is found in many additional localities around the world [7, 8, 11], further study is required to find whether the Woods Hole population possesses a unique variation of alloresponses within this taxon.

Different populations of *B. schlosseri* are probably characterized by a highly polymorphic pattern of alloreactivity. In the Mutsu Bay population, sampling from a very small area of 5 by 5 m revealed only 12.1% of fusions. In the Woods Hole population, two different studies have found 6.2% of fusions out of 1,262 contiguous borders of colonies [17] and 4.2% fusions out of 500 pairs of colonies collected randomly along a 20 m transect [18]. Similar low percentage numbers of fusions were obtained from the Israeli *B. schlosseri* population (Porat and Rinkevich, unpublished). These studies and others [19] reflect the existence of approximately 100 histocompatibility alleles in each studied *B. schlosseri* population. In *Botryllus primigenus*, pairwise fusibility tests revealed a similar low percentage of fusions [5, 20, 21]. In *Botrylloides fuscus*, on the other hand, panels of pairwise combinations between colonies which were collected from 3 localities, 1–3 km apart, resulted in 64.0–74.0% of fusions. This indicates a much lower polymorphism on the Fu/HC locus of this species [22].

The 14 genotypes from Mutsu Bay which were collected randomly from a very small site possess 18 to 19 alleles on their fusibility locus, of which at least 2 are commonly shared with the Monterey, CA, population (colonies 5, 9, 11 fused with Monterey colonies [16]). Based on the results of 12.1% fusions, we may estimate [23] the numbers

of alleles on the Fu/HC haplotype in the Mutsu Bay population as approximately 32. However, it is clear that this estimation is minimal and that our sample does not represent the probable higher number of alleles residing in the Fu/HC locus of this population, since colonies were collected from a very limited (5×5 m) area. Moreover, calculations are based on the prediction that all the alleles on the fusibility locus are equally frequent [23], which is not the case of the present study (Fig. 2b). This skews the figure for the number of histocompatibility alleles and also marks the difficulty of using CAAs as the only implementation for assessing polymorphism on the Fu/HC haplotype. Some of these colonies responded to intrapopulation allogeneic encounters by the "retreat growth phenomenon" [14]. In assays resulting in fusion, one of the partners in the chimera was usually resorbed [3, 9, 13], a result which was recorded before in other *B. schlosseri* populations as well. These outcomes suggest that botryllid ascidians have highly complex systems of effector mechanisms, all controlled by a variety of histocompatibility genes.

ACKNOWLEDGEMENTS

Thanks are due to Dr. Numakunai for helping in colony collection and to T. Ishii and A. Dasai for general assistance. The staff of the Shimoda Marine Research Center is acknowledged for their hospitality and assistance. This study was supported by a grant from S. Price, North American Friends of IOLR, by a JSPS Fellowship for Research in Japan (B.R.), and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, nos. 03455008 and 01304007 (Y.S.).

REFERENCES

1 Bancroft, F. W. (1903) Variation and fusion of colonies in compound ascidians. Proc. Calif. Acad. Sci., **3**: 137–186.

2 Scofield, V. L., Schlumpberger, J. M., West, L. A. and Weissman, I. L. (1982) Protochordate allorecognition is controlled by an MHC-like gene system. Nature, **295**: 499–502.

3 Weissman, I. L., Saito, Y. and Rinkevich, B. (1990) Allorecognition histocompatibility in a protochordate species: Is the relationship to MHC semantic or structural? Immun. Rev., **113**: 227–241.

4 Oka, H. and Watanabe, H. (1957) Colony specificity in compound ascidians as tested by fusion experiments. Proc. Japan Acad., **33**: 657–659.

5 Oka, H. and Watanabe, H. (1960) Problems of colony specificity in compound ascidians. Bull. Mar. Biol. Stn. Asamushi, **10**: 153–155.

6 Sabbadin, A. (1962) La basi genetiche della capacita di fusione fra colonie in *Botryllus schlosseri* (Ascidiacea). Rend. Accad. Lincei, **32**: 1031–1035.

7 Berrill, N. J. (1950) The Tunicata. Ray Society, London.

8 Tokioka, T. (1953) Ascidians of Sagami Bay. Iwanami-Shoten, Tokyo.

9 Sabbadin, A. and Astorri, C. (1988) Chimeras and histocompatibility in the colonial ascidian *Botryllus schlosseri*. Dev. Comp. Immun., **12**: 737–747.

10 Lilker-Levav, T. (1992) Allogenic responses in *Botryllus schlosseri* and *Botrylloides leachi* from the Mediterranean coast of Israel. M. Sc. Dissertation, Tel-Aviv Univ. (with English summary)

11 Boyd, H. C., Weissman, I. L. and Saito, Y. (1990) Morphologic and genetic verification that Monterey *Botryllus* and Woods Hole *Botryllus* are the same species. Biol. Bull., **178**: 239–250.

12 Rinkevich, B. and Weissman, I. L. (1991) Interpopulational allogeneic reactions in the colonial protochordate *Botryllus schlosseri*. Int. Immun., **3**: 1265–1272.

13 Rinkevich, B. and Weissman, I. L. (1992) Chimeras vs genetically homogeneous individuals: potential fitness costs and benefits. Oikos, **63**: 119–124.

14 Rinkevich, B. and Weissman, I. L. (1988) Retreat growth in the ascidian *Botryllus schlosseri*: a consequence of nonself recognition. In "Invertebrate Historecognition". Ed. by R. K. Grosberg, D. Hedgecock and K. Nelson. Plenum, New York, pp. 93–109.

15 Rinkevich, B. and Weissman, I. L. (1992) Incidents of rejection and indifference in Fu/HC incompatible protochordate colonies. J. Exp. Zool., **263**: 105–111.

16 Rinkevich, B., Shapira, M., Weissman, I. L. and Saito, Y. (1992) Allogenic responses between three remote populations of the cosmopolitan ascidian *Botryllus schlosseri*. Zool. Sci., **9**: 989–994.

17 Karakashian, S. and Milkman, R. (1967) Colony fusion compatibility types in *Botryllus schlosseri*. Biol. Bull., **133**: 473.

18 Grosberg, R. K. and Quinn, J. F. (1986) The genetic control and consequences of kin recognition by the larvae of a colonial marine invertebrate. Nature, **322**: 456–459.

19 Scofield, V. L. and Nagashima, L. S. (1983) Morphology and genetics of rejection reactions between oozooids from the tunicate *Botryllus schlosseri*. Biol. Bull., **165**: 733–744.

20 Tanaka, K. and Watanabe, H. (1973) Allogeneic inhibition in a compound ascidian, *Botryllus primigenus* Oka. I. Processes and features of "nonfusion" reaction. Cell. Immun., **7**: 410–426.

21 Mukai, H. and Watanabe, H. (1975) Distribution of fusion incompatibility types in natural populations of the compound ascidian, *Botryllus primigenus*. Proc. Japan Acad., **51**: 44–47.

22 Hirose, E. (1990) Colony specificity and possible allorecognition sites in *Botrylloides* (Ascidiae, Compositae). Ph. D. Dissertation, Univ. Tsukuba, Japan.

23 Grosberg, R. K. (1988) The evolution of allorecognition specificity in colonial invertebrates. Quart. Rev. Biol., **63**: 377–412.