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Authors: Ishii, Teruhisa, Sawada, Tomoo, Sasaki, Katsuaki, and Ohtake, Shin-Ichi

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Study of Color Variation in the Solitary Ascidian *Halocynthia roretzi*, Collected in the Inland Sea of Japan

Teruhisa Ishii[1]*, Tomoo Sawada[2], Katsuaki Sasaki[3], and Shin-Ichi Ohtake[4]

[1]*Division of Biology, Department of Natural and Environmental Sciences, Akita University, Akita 010-8502, Japan*
[2]*Department of Human Science, Yamaguchi University School of Medicine, Ube 755-8505, Japan*
[3]*Towa-cho Nagisa Aquarium, Aquatic Wildlife Breeding Center, Towa-cho, Yamaguchi 742-2601, Japan*
[4]*Department of Biology, Nihon University, School of Medicine, Itabashi 173-8610, Japan*

ABSTRACT—In the vicinity of Yashiro Island in the Inland Sea of Japan, the solitary ascidian (tunicate) *Halocynthia roretzi* with tunics of various colors were collected. Samples of these animals were sorted into three groups on the basis of visual observation of tunic color. The red group includes animals with dark-red, light-red, or orange tunics. The pink group includes animals with tunic colors ranging between red and white. The white group includes only animals with completely white tunics. Animals in the white group lacked color internally, with the exception of the hepatopancreas and the gonads in breeding season; the epidermis and gill basket were white. In contrast, animals of both the red group and the pink group were colored internally, with red-orange epidermis and yellow gill basket. Alloreactivity was tested by mixed-hemocyte incubation between different animals belonging to the same color group and between animals belonging to different color groups. Alloreactivity between animals of the white group was 56.3%, between animals of the pink group was 60.0%, and between animals of the red group was 69.3%. The relatively high frequency of compatible combinations among the white animals is discussed.

Key words: solitary ascidian, white *Halocynthia roretzi*, alloreactivity, MHI assay, color variation

INTRODUCTION

Halocynthia roretzi, the one of common solitary ascidian in Japan, is found most of Japanese coast line, and is known to be an ascidian species of fisherman's cultivation for food in northern district. Despite that the reports considering genetic variation of any ascidian species have been very few, so far, there were some reports about the variations, which would reflect genetic variations, in *Halocynthia roretzi.* Numakunai and his colleague had reported about three different types (A, B, and C) of *Halocynthia roretzi* which are distinguishable by their different spawning seasons; Type A spawns in the morning in November, Type B spawns in the evening or at night from late October to November, and Type C spawns in the afternoon in April (Numakunai and Hoshino, 1973; 1974). As the differences in the feature among three types, Type A has long papillae (tunic protrusions) and shows a light pink external tunic color, Type B has short thin papillae, brownish red or reddish orange, and Type C has short thick papillae, bright red (Numakunai *et al*., 1981). These three types have no difference in the color components qualitatively, but pigment-deposited sites in tunic differ among three types (Numakunai *et al*., 1981). As a conspicuous variant of body color, here we studied a local group with white and whitish tunic in the Inland Sea of Japan.

There have been several records that "so-called white *Halocynthia roretzi*" having pale-colored tunics were collected from Japan (Tokioka, 1953; Nishikawa, 1995). But no biological study has been attempted on those "so-called white *H. roretzi*", and the color inside of the body has not been noted. The animals with white or pale pink tunics have been thought to have yellowish-colored body inside, same to the animal having red or orange tunic. The genetics of tunic color differences has not been studied either.

Not many phenotypic markers are available to analyze genetic correlation with the tunic color variation in *H. roretzi*.

* Corresponding author: Tel. +81-18-889-2681;
FAX. +81-18-889-2681.
E-mail: tishii@ipc.akita-u.ac.jp

Alloreactivity of hemocytes is the distinct one which is thought to reflect genetic diversity in this species. Fuke (1980) found an allogeneic cytotoxicity “contact reaction” in the hemocytes of *H. roretzi*. This cytotoxic reaction is indicative of somatic cellular self-nonself recognition occurring in this species, which also shows this recognition in its germ cells via self sterility. Although it has not been proven that these phenomena are based on the recognition of genetically

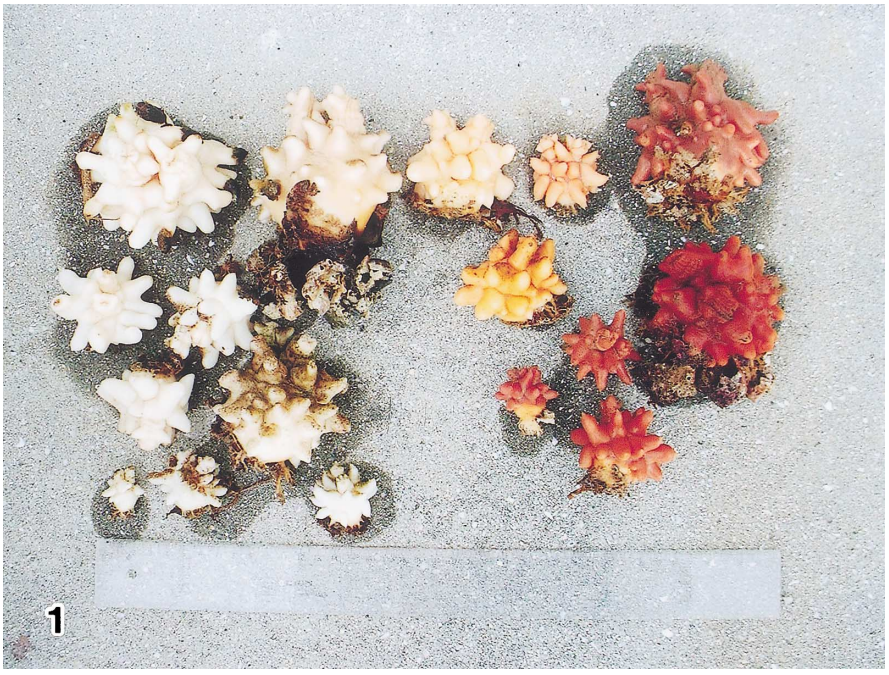

Fig. 1. *Halocynthia roretzi* of various tunic colors, obtained from the vicinity of Yashiro Island in the Inland Sea of Japan in August 2002. The animals in this figure are arranged by tunic color, with those at the far left having absolutely white tunics, those at the far right having red or orange tunics, and those in the center having colors that range in between the two extremes. The ruler at the bottom of the figure is about 40 cm in length.

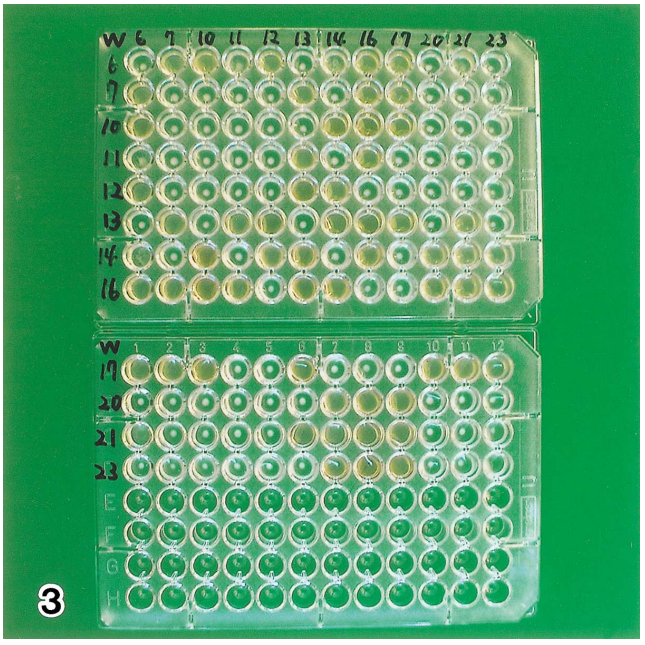

Fig. 3. Mixed-hemocyte incubation assay for pairs of animals in the white (W) group, after 18-hr incubation at 18°C. The alloreactivity results for these microplates are recorded in Table 3.

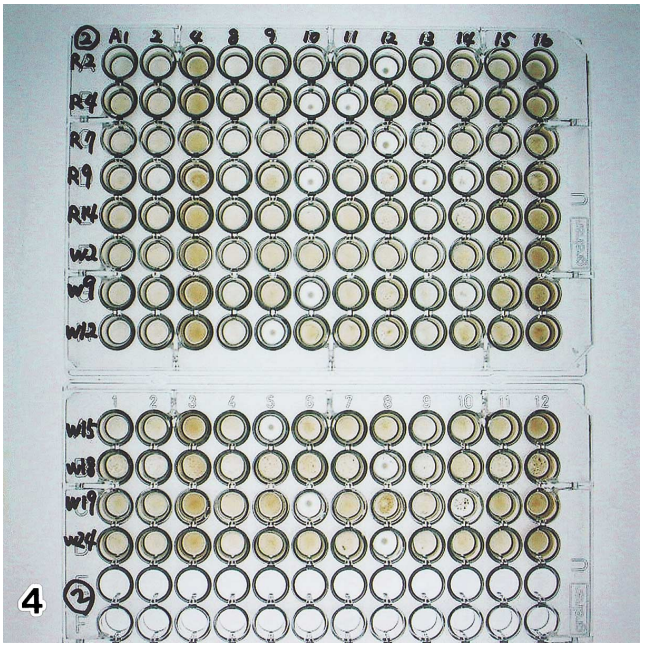

Fig. 4. Mixed-hemocyte incubation assay for white (W) versus Mutsu Bay (A) and red (R) versus Mutsu Bay combinations, after 22-hr incubation at 22°C. The alloreactivity results for these microplates are recorded in Table 4.

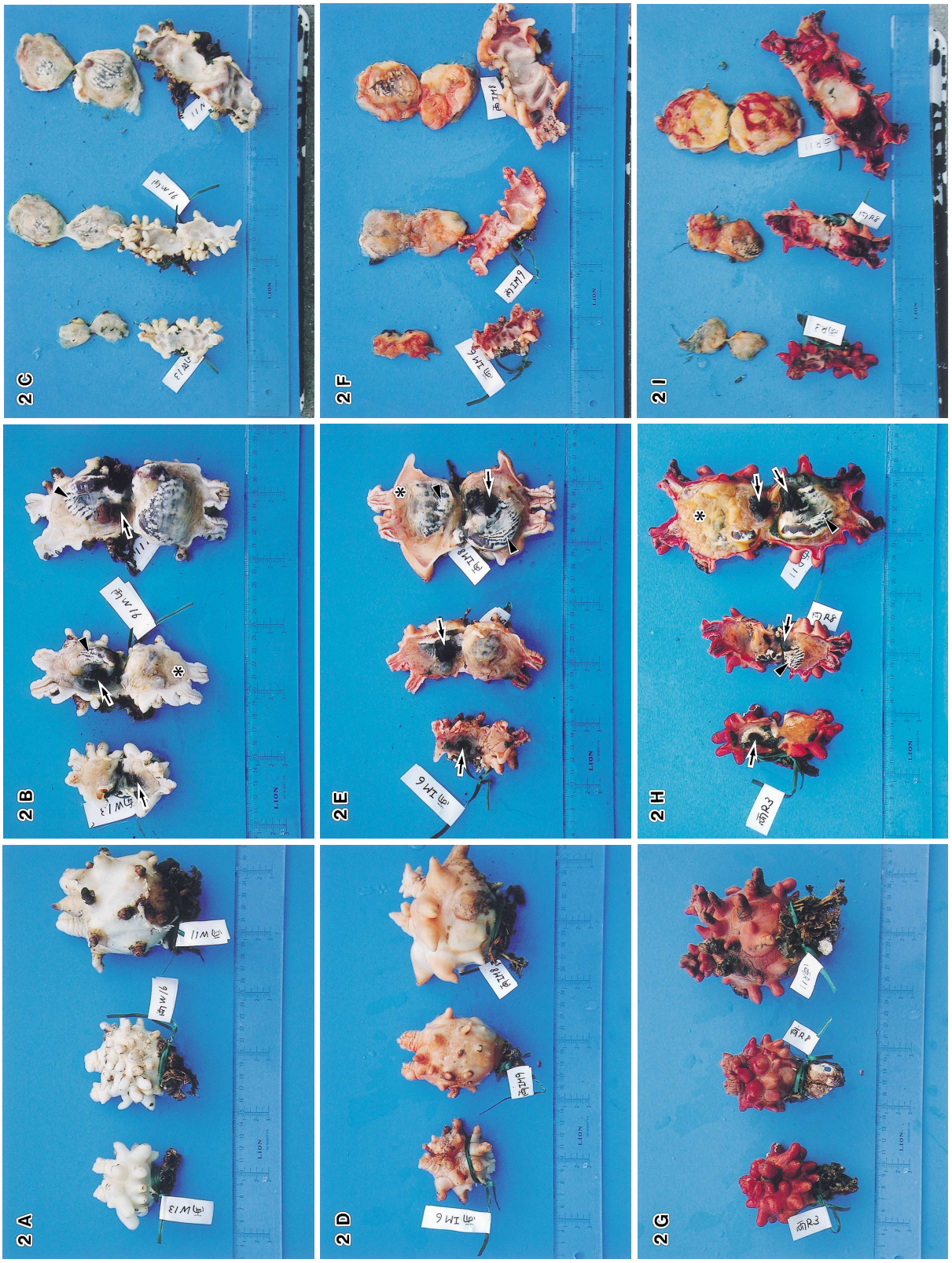

Fig. 2. Animals of the three color groups. These animals were collected in November or December 2002, were kept in an aquarium supplied with running seawater and were dissected in January 2003. A–C: White group; D–F: Pink group; G–I: Red group. A, D, and G: External views. B, E, and H: Internal views by dissecting along the plane that links the two siphons. Arrowheads indicate gonads. Arrows indicate hepatopancreas. Note that the gill baskets in B (for example; asterisk in center animal) are white, whereas they are yellow in E (for example; asterisk in right animal) and H (for example; asterisk in right animal). C, F, and I: Separation of the tunic (bottom row of each photograph) from each body (upper row). The epidermis (upper row) in C is white, whereas it is red-orange in F and I. The ruler in all pictures is same. The numbers on this ruler graduate in centimeters.

Table 1. Alloreactivity of White (W), Pink (P), and Red (R) Animals With Other Animals of These Groups and With Animals From Mutsu Bay

Animal Identification	W+	W–	P+	P–	R+	R–	M+	M–
W1	6	5	3	0	1	1		
W2	13	4	3	0	5	2	12	0
W3	5	2	3	0	1	1		
W4	5	2						
W5	5	2						
W6	7	4						
W7	4	7						
W8	2	1	0	3	2	2		
W9	2	4			1	4	11	1
W10	4	7						
W11	2	9						
W12	6	11			2	3	11	1
W13	7	4						
W14	8	3						
W15	3	3			2	3	11	1
W16	9	2						
W17	9	4	2	1	4	0		
W18	6	0			4	1	11	1
W19	5	1			3	2	11	1
W20	3	8						
W21	5	6						
W22	2	1	2	1	2	2		
W23	3	10	0	3	3	1		
W24	3	3			1	4	11	1
W25	8	3	1	2	0	2		
W26	8	3	2	1	1	1		
W27	5	2	1	2	1	1		
W28	5	2	2	1	1	1		
W29	3	4						
W30	2	5	2	3	1	1		
W31	5	2						
Total of Ws	160	124	21	17	35	32	78	6
P1	5	3	2	2	2	2		
P2	6	2	3	1	4	0		
P3	5	3	2	2	2	2		
P4	1	3	2	3	3	1		
P5			5	0				
P6	2	2	2	3	2	2		
P7			2	3				
P8			5	0				
P9	1	3	2	3	3	1		
P10	1	0	4	0	2	0		
P11	0	1	1	3	2	0		
Total of Ps	21	17	30	20	20	8		
R1	3	4	1	2	1	0		
R2	3	4			1	3	11	1
R3					5	6		
R4	5	2			10	3	10	2
R5					6	5		
R6	0	1	5	0	1	0		
R7	5	2			11	2	11	1
R8					9	2		
R9	2	5			6	7	10	2
R10	1	3	0	3	6	5		
R11					7	4		
R12	4	0	2	1	9	2		
R13	3	4	3	0	1	0		
R14	2	5			2	2	12	0
R15	3	1	3	0	10	1		
R16					7	4		
R17	1	0	3	2	1	0		
R18	3	1	3	0	11	0		
Total of Rs	35	32	20	8	104	46	54	6

The numbers in the W+ column indicate the number of animals tested belonging to the white (W) group that showed alloreactivity with the white animal in that row. The numbers in the W– column indicate the number of animals tested belonging to the white group that did not show alloreactivity with the white animal in that row.

controlled differences between self and nonself molecules in *H. roretzi*, research by Fuke and Nakamura (1985) suggests that several different alleles might control allogeneic compatibility in this species. Sawada and Ohtake (1994) developed the mixed-hemocyte incubation (MHI) assay, which is a convenient method for testing allogeneic cytotoxicity between a large number of *H. roretzi* pairs at the same time, using mixtures of allogeneic hemocytes on U-bottomed microplates. It is thought that the frequency of alloreactivity reflects the size of genetic repertoire in the local group.

One purpose of the present study was to provide a detailed description of the color variation of this solitary ascidian living in the Inland Sea of Japan, as the first biological record of "so-called white *Halocynthia roretzi*." We also examined the correlation between the allogeneic cytotoxicity reaction and tunic color variation to see how it might relate to genetic background.

MATERIALS AND METHODS

Animals

Ascidians, *Halocynthia roretzi*, were collected from the vicinity of Yashiro Island, Yamaguchi Prefecture, in the Inland Sea of Japan. Type C animals (judged by external observation; with short thick papillae and bright red tunic color) were collected from Mutsu Bay, Aomori Prefecture, in the northern part of Japan. Animals from the Inland Sea of Japan were kept in an aquarium supplied with running seawater. Animals from Mutsu Bay were kept in a tank in the laboratory supplied with seawater.

Grouping

The animals that were collected from Yashiro Island were sorted by visual observation into three groups according to tunic color. The three groups were the red group, the pink group, and the white group. The red (R) group includes animals with dark-red, light-red, or orange tunics. The pink (P) group includes animals with tunic colors in the range between red and white. The white (W) group includes only animals with absolutely white tunics. After grouping them by tunic color, some animals (more than 14) from

Table 2. Alloreactivity of Animals Within and Between Groups

Combination	+	–	Alloreactivity (%)
W vs W	80	62	56.3
W vs P	21	17	55.3
W vs R	35	32	52.2
P vs P	15	10	60.0
P vs R	20	8	71.4
R vs R	52	23	69.3
W vs M	78	6	92.9
R vs M	54	6	90.0
W vs W+P+R	136	111	55.1
P vs W+P+R	56	35	61.5
R vs W+P+R	107	63	62.9
W+P+R vs W+P+R	223	152	59.5
M vs W+R	132	12	91.7

The "+" column indicates the presence of an alloreaction. The "–" column indicates the absence of an alloreaction.

each group were dissected to observe the color inside the body. Individual animals in the groups were identified as follows: R1, R2, and so forth for the red group; P1, P2, and so forth for the pink group; and W1, W2, and so forth for the white group. The animals from Mutsu bay were all same as the group red (R) in colors of tunic and the inside body.

Mixed-Hemocyte Incubation (MHI) assay

To test for alloreactivity (also known as *contact reaction* or *allogeneic cytotoxicity*) between two animals, we carried out the mixed-hemocyte incubation (MHI) assay as described by Sawada and Ohtake (1994). Briefly, hemolymph, including hemocytes, in the space beneath the epidermis was harvested from tunic papillae using plastic syringes (without anticoagulant). Hemolymph (100 µl from each individual) was mixed from pairs of animals and incubated in U-bottomed 96-well microplates at 18–23°C. After 11 to 29 hours of incubation, microplates with brown pigmentation and cell aggregation were judged to be *allopositive*, indicating the presence of alloreactivity between the two animals of that combination. When alloreactivity was absent between the two animals, the hemocytes in the hemolymph mixture settled into the form of a white button on the bottom of the microplates (see Sawada and Ohtake, 1994). We tested more than twice for the all combinations examined, using the same pairs of animals.

Combinations

We collected 31 animals for the white (W) group, 11 animals for the pink (P) group, and 18 animals for the red (R) group. We also used 12 animals of Type C from Mutsu Bay (M). All the animals from Mutsu Bay were bright red tunics. To study alloreactivity, we tested 142 combinations of W versus (vs.) W, 38 combinations of W vs. P, 67 combinations of W vs. R, 25 combinations of P vs. P, 28 combinations of P vs. R, 75 combinations of R vs. R, 84 combinations of W vs. M, and 60 combinations of R vs. M. All combinations were tested more than twice, using the same pairs of animals.

RESULTS

The animals from Yashiro Island showed a variety of tunic colors (Fig. 1). During observation while snorkeling, we noticed that the animals of different colors were sympatric, that is, they inhabited a small area of several square meters on the same side of the seawall. For the purposes of this study, we divided the tunic color variations into three separate groups as described in the Materials and Methods section.

We dissected some individuals from each color group and found that animals of the white group had no pigmentation in their epidermis or gill baskets (Fig. 2, A–C). In these white animals, the hepatopancreas was brown and the gonads in the breeding season were the same color as the gonads of animals of the other groups (Fig. 2B). In contrast, animals of the pink group had red-orange epidermis and yellow gill baskets (Fig. 2, D–F). Animals of the red group also had red-orange epidermis and yellow gill baskets (Fig. 2, G–I).

The results of alloreactivity tests as determined by the MHI assay are shown in Tables 1 and 2. Table 1 shows the results of all combinations in MHI assay. For example, animal W1 showed alloreactivity with 6 of 11 other white animals, with all 3 of the pink animals tested, and with 1 of the 2 red animals tested.

Alloreactivity between animals of the same group was 56.3% in the white group, 60.0% in the pink group, and 69.3% in the red group (Table 2). However, alloreactivity of animals from the Inland Sea with animals from Mutsu Bay was 92.9% for white animals (W vs. M) and 90.0% for red animals (R vs. M; Table 2). In the bottom part of Table 2, it is seen that, in total, the white group showed 55.1% alloreactivity. Data for other total combinations are also presented.

Fig. 3 shows the representative microplates of the MHI assay for combinations of animals within the white group, and the results (the presence or absence of alloreactivity) of these microplates are presented in Table 3. Hemocytes in autologous control combinations and in 35 of the nonself

Table 3. Alloreactivity of Animals Within the White Group as Determined by the Mixed-Hemocyte Incubation (MHI) Assay Shown in Fig. 3

Animal Identification	W6	W7	W10	W11	W12	W13	W14	W16	W17	W20	W21	W23
W6	–	+	+	–	+	–	+	+	+	–	+	–
W7	+	–	–	–	–	+	–	+	+	–	–	–
W10	+	–	–	–	–	–	+	+	+	–	–	–
W11	–	–	–	–	–	+	–	+	–	–	–	–
W12	+	–	–	–	–	+	+	–	–	–	–	–
W13	–	+	–	+	+	–	+	+	+	–	+	–
W14	+	–	+	–	+	+	–	+	–	+	+	+
W16	+	+	+	+	–	+	+	–	–	+	+	+
W17	+	+	+	–	–	+	–	–	–	+	+	+
W20	–	–	–	–	–	–	+	+	+	–	–	–
W21	+	–	–	–	–	+	+	+	+	–	–	–
W23	–	–	–	–	–	–	+	+	+	–	–	×

See the Materials and Methods section for a description of the MHI assay. The presence and absence of alloreactivity are indicated by + and –, respectively. In the autologous control of W23 vs. W23, we could not detect a white button of hemocytes because of an experimental mistake.

Table 4. Alloreactivity of Animals From Mutsu Bay (Labeled A1 Through A16) With Animals of the Red Group or of the White Group as Determined by the Mixed-Hemocyte Incubation (MHI) Assay Shown in Fig. 4

Animal Identification	A1	A2	A4	A8	A9	A10	A11	A12	A13	A14	A15	A16
R2	+	+	+	+	+	+	+	−	+	+	+	+
R4	+	+	+	+	+	−	−	+	+	+	+	+
R7	+	+	+	+	+	+	+	−	+	+	+	+
R9	+	+	+	+	+	−	+	+	−	+	+	+
R14	+	+	+	+	+	+	+	+	+	+	+	+
W2	+	+	+	+	+	+	+	+	+	+	+	+
W9	+	+	+	+	+	−	+	+	+	+	+	+
W12	+	+	+	+	−	+	+	+	+	+	+	+
W15	+	+	+	+	−	+	+	+	+	+	+	+
W18	+	+	+	+	+	+	+	−	+	+	+	+
W19	+	+	+	+	+	−	+	+	+	+	+	+
W24	+	+	+	+	+	+	+	−	+	+	+	+

See the Materials and Methods section for a description of the MHI assay. The presence and absence of alloreactivity are indicated by + and −, respectively.

combinations accumulated in the center of the bottom of the wells to form white buttons. In contrast, hemocytes in reactiveonself combinations were spread out on the bottom of the wells in the form of brown precipitation.

Fig. 4 shows another MHI assay microplates for combinations of animals from Mutsu Bay (labeled A1 through A16) with animals of the red group or of the white group. The results are shown in Table 4. Hemocytes in 12 of the nonself combinations accumulated in the center of the bottom of the wells to form white buttons. In contrast, hemocytes in most of the nonself combinations were spread out on the bottom of the wells in the form of brown precipitation.

DISCUSSION

White Subgroup of *Halocynthia roretzi*

Halocynthia roretzi has been found along almost the entire coastline of Japan. Whitish or white animals of *H. roretzi* have been reported to live in the Inland Sea of Japan (Nishikawa, 1995). There have been a few reports about variations within this species other than the white tunic. Observations by Numakunai and Hoshino (1973; 1974) and Numakunai *et al*. (1981) about the three sympatric types (Types A, B, and C), which vary in spawning behavior and have minor differences in external features, were made on *H. roretzi* in Mutsu Bay, in the northern part of Japan. No difference was found in internal anatomy among three types (Numakunai *et al*., 1981) and three types are included morphologically in the same species. Animals of the three different types in Mutsu bay have red-orange epidermis. Coexistence of the three types has been observed only in Mutsu Bay. The animals from the other coast of Japan, except in the vicinity of Hokkaido, were reported as monotypical, being classified as Type C.

Here we report another phenotypic variants in *H. roretzi* from the Inland Sea of Japan that are distinguished by variation in tunic colors, the groups having red, pink, and white tunic. The red group includes dark-red, light-red, and orange animals. The pink group includes pink animals and whitish animals. The white group includes only the animals whose tunic color is absolutely white. On the basis of their external features, but ignoring tunic color, *H. roretzi* of the Inland Sea that belong to the white and pink groups were tentatively classified as Type C.

We compared the internal (epidermis and gill basket) pigmentation of animals from the three groups. We dissected more than 26 animals from the white group and confirmed that they had neither red, orange, nor yellow color internally, other than the hepatopancreas (brown) and the gonads (yellow to light-brown because of oocytes). We dissected more than 14 animals from the pink group and found color internally, red-orange epidermis and yellow gill basket. Also we dissected more than 17 animals from the red group and found color internally as well as pink group, red-orange epidermis and yellow gill basket. Animals of the three color groups are sympatric, living in a small area of several square meters on the same face of the seawall. This fact suggests that the white tunic color has a genetic basis, rather than being caused by differences in nutrition or other environmental factors and these observations led us to hypothesize that the animals of the pink group may have originated by genetic crossing between the red group and the white group.

White ascidians of the Inland Sea have been thought to be colored internally despite having a white appearance externally. This article is the first report to confirm the existence of the white group —having a white tunic and lacking color internally— of *H. roretzi.* Therefore, we believe that the earlier observations of white *H. roretzi* probably included animals of the pink group.

Correlation Between Alloreactivity and Tunic Color

We examined the correlation between alloreactivity and tunic color in the three color groups of *H. roretzi* for the purpose of investigating the possible contribution of genetics to color variation. According to previous studies (Fuke, 1980; Fuke and Nakamura, 1985; Sawada and Ohtake, 1994; Akita and Hoshi, 1995), the alloreactivity of *H. roretzi* hemocytes may be determined by genetics. In the solitary ascidians, these alloreactivity among allogeneic hemocytes are observed only in *Halocynthia roretzi*.

The presence of nonalloreactive combinations, in W vs. M and R vs. M, demonstrate that the animals from Yashiro Island and the animals from Mutsu Bay belong to the same species. Hemocytes of *Halocynthia roretzi* always react against xenogeneic hemocytes. It might be also meaningful that , in Fig. 4, both A4 and A16 lines of wells showed strong colors. As Arai *et al.* (2002) mentioned the correlation between the degree of brown pigmentaion and the intensity of cytotoxicity, there may exist some different cytotoxicity levels among alloreactivity-positive combinations and there may be animals possessing high-cytotoxicity and the ones having lower cytotoxicity. However, we checked only the existence or non-existence of the alloreactivity in this study.

On average, animals of Types A, B, and C from Mutsu Bay showed approximately 85% alloreactivity (Fuke and Nakamura, 1985). No differences in the alloreactivity ratio were detected between the Types A to B and A to C or B to C. In contrast, alloreactivity of the animals from the sea around Yashiro Island was 59.5%, on average. The animals around Yashiro Island seemed to be genetically related. Higher alloreactivity of the animals from Yashiro Island against the animals from Mutsu Bay —92.9% in W vs. M and 90.0% in R vs. M (see Table 2)— showed that the groups of animals of different colors from Yashiro Island are somewhat genetically isolated from the Mutsu Bay animals.

As seen in Table 2, the alloreactivity ranged from 56.3% (within the white group) to 69.3% (within the red group), exhibiting a little difference. This small difference suggested that the genetic factors for “the white tunic” is not linked to any certain “alloreactivity type”. However, it may be important to note that the alloreactivity within the white group was the lowest among the three groups and that the pink group exhibited an intermediate alloreactivity value (60.0%) between those of the white and red groups.

Future Investigations

We reported here about the tunic color variants in *H. roretzi*. By the loss of color in inside body, white animals seemed to lose a certain enzymatic process of orange pigmentation in inside organs as well as tunic. However, there is no proof that this color loss is linked to some particular physiological defects. In assessing the distribution and the behavior of viriform cells in the tunic of the red-colored *H. roretzi*, we are interested in these cells' activity in white animals. The viriform cell is one of hemocyte types and is especially abundant in tunic in *H. roretzi*. When *H. roretzi* send out new and white attachment villi in laboratory aquaria, many viriform cells were observed just under the cuticle of the new and white attachment villi, and broken viriform cells were observed in the region of coloration into brown (Ohtake *et al.*, 2001). The viriform cells (we examined Type C so far) have phenoloxidase (PO) activity as detected by histochemical method (unpublished data), and we focuses on PO activity in white animals.

PO is released in the contact reaction of *H. roretzi* hemocytes (Akita and Hoshi, 1995). *H. roretzi* hemocytes released PO in response to sheep red blood cells or yeast cells, and PO showed antibacterial activity in the presence of plasma of *H. roretzi* (Hata *et al.*, 1998). The possibility that the lowest alloreactivity percentage of the white group could be resulted from somewhat low activity of PO in them is not eliminated.

As a base for further studies, it must be first confirmed that white tunic phenotype is genetically determined.

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