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Re-Examination of Sibling Cross-Sterility in the Ascidian, *Ciona intestinalis*: Genetic Background of the Self-Sterility

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ABSTRACT—Self-sterility of solitary ascidians is a typical example of the allogeneic recognition, though its molecular mechanism remains an open question. In this paper we analyze the fertility between siblings from selfed and crossed eggs to understand the genetic basis of self-sterility in the ascidian, *Ciona intestinalis*. First, we show that the self-sterility is strict and stable, and the individuality expressed in gametes is highly diversified in the wild population that we used. Secondly, we show one-way cross-sterility and reciprocal cross-sterility within the siblings that are self-sterile but fertile with non-siblings. Thirdly, we show self-sterility and cross-sterility share some natures and both are closely related to the sperm capacity not to bind to the vitelline coat of the autologous eggs or the eggs sterile to the sperm concerned.

In all, this paper shows that the self-sterility is genetically governed by a multiple-locus system, and that most probably individual-specific determinants are haploid expression in sperm and diploid expression in eggs, given they recognize self but not non-self.

Key words: self-sterility, allogeneic recognition, sperm binding, *Ciona intestinalis*, ascidians

INTRODUCTION

Allogeneic recognition is the recognition of self and non-self between conspecific individuals. It is one of the most strict recognition processes and is widely spread throughout the animal kingdom from the sponges to the vertebrates (for a review see Cooper, 1992). Since the capacity of allogeneic recognition is shown mostly by experimental grafting of tissues or organs, the physiological significance of allogeneic recognition is not necessarily clear in many invertebrates. In the ascidians, however, two remarkable events of naturally occurring allogeneic recognition are known besides artificially induced allogeneic reactions. One is colony specificity in the compound ascidians, such as *Botryllus primigenus*, which form a clonal colony with genetically defined capacity to recognize self and non-self colonies (Oka and Watanabe, 1957; for a review see Saito *et al*., 1994). The other is self-sterility in solitary ascidians such as *Ciona intestinalis* (Morgan, 1923, 1938; Rosati and De Santis, 1978) and *Halocynthia roretzi* (Fuke, 1983). Self-sterility in the ascidians, which are simultaneous hermaphrodites, is the phenomenon that fertilization between eggs and sperm from the same individual is blocked though they are spawned concomitantly. Since the players of this interesting game are sperm and eggs, both of which are easily obtained as a uniform and single cellular population, the ascidian self-sterility seems to be a good model system to study the mechanism of allogeneic recognition in the invertebrates.

Morgan published a series of papers on the self-sterility in *C. intestinalis* (Morgan 1923, 1938, 1939, 1942, 1944). He found that the vitelline coat serves as the barrier against self-fertilization and that the self-sterility is abolished by treatment of eggs with acidic sea water. Besides these facts, it is now known that the eggs acquire self-fertility first and, during a very last phase of egg maturation, they establish self-sterility. The acquisition of self-sterility in eggs is reconstructed *in vitro* in *C. intestinalis* (De Santis and Pinto, 1991) and in *H. roretzi* (Fuke and Numakunai, 1996). It is also known that the acquisition is exactly coincident with the translocation of a low molecular weight substance, most likely a peptide, from the follicle cells to the vitelline coat with the aid of a heat-shock protein and proteasome. This peptide is claimed to be the one that is depleted from the vitelline coat by acidic sea water (Pinto *et al*., 1995; Marino *et al*., 1998, 1999). However, individual-specific factors

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including the peptide remain yet to be identified.

Morgan (1942, 1944) extensively investigated the genetic problem of self-sterility in *C. intestinalis* by using acid treatment to make otherwise self-sterile eggs self-fertile. His findings on the genetics of self-sterility are summarized as follows: First, cross-sterile combinations are hardly found in any wild populations, suggesting that the individuality recognized by the gametes is highly diverse. Secondly, cross-sterile combinations are found within the siblings from selfed eggs and those from crossed ones, suggesting that self-sterility is genetically governed. He recognized two types of cross-sterile combinations; reciprocally cross-sterile combinations in which both ways of the gamete combination were sterile, and one-way cross-sterile combinations in which one way was sterile whereas the other was fertile. Taking these findings into account, he proposed that the individuality was determined by haploid expression in sperm but by diploid expression in eggs (haploid-sperm hypothesis), and that self-sterility was governed by a multiple-locus system (multiple-locus hypothesis). Under haploid-sperm hypothesis, if sperm cannot fertilize eggs sharing at least one allele with them, the occurrence of one-way cross-sterility is easily accountable (Fig. 1). From the incidence of reciprocal cross-sterility, he estimated that a minimum of five independent loci should be present if haploid-sperm hypothesis was correct.

It is known that self-sterility in *C. intestinalis* is not very strict and rather unstable in some populations (Morgan, 1938, 1942, 1944; Rosati and De Santis, 1978; Kawamura *et al*., 1987). The strictness of self-sterility appears to differ from one population to another, and it changes seasonally within a population (De Santis, personal communication) or day-by-day for an individual (Kawamura *et al*., 1987). Such features were not carefully considered in Morgan's experiments. Furthermore, recent progress has revealed that, self-sterility in *C. intestinalis* coincides with some changes in sperm physiology. First, in the self-sterile animals, spermatozoa scarcely bind to the vitelline coat of glycerinated autologous eggs (Rosati and De Santis, 1978; Kawamura *et al*., 1987), to which we refer in this article as the failure of binding. Secondly, even in the rare case that they successfully bind to the vitelline coat, the sperm flagella cease beating within five minutes after insemination, to which we refer as the sperm inactivation (Kawamura *et al*., 1987). Blockade of sperm during the course of fertilization is known to occur not only at the level of sperm binding to the vitelline coat but at the level of sperm penetration through it (Kawamura *et al*., 1987). It is not known yet whether cross-sterility and self-sterility share the same mechanism or not. Furthermore, to our best knowledge, no one has ever confirmed Morgan's finding of cross-sterility within the siblings except that Kawamura (1989) reported preliminary results. In all, these situa-

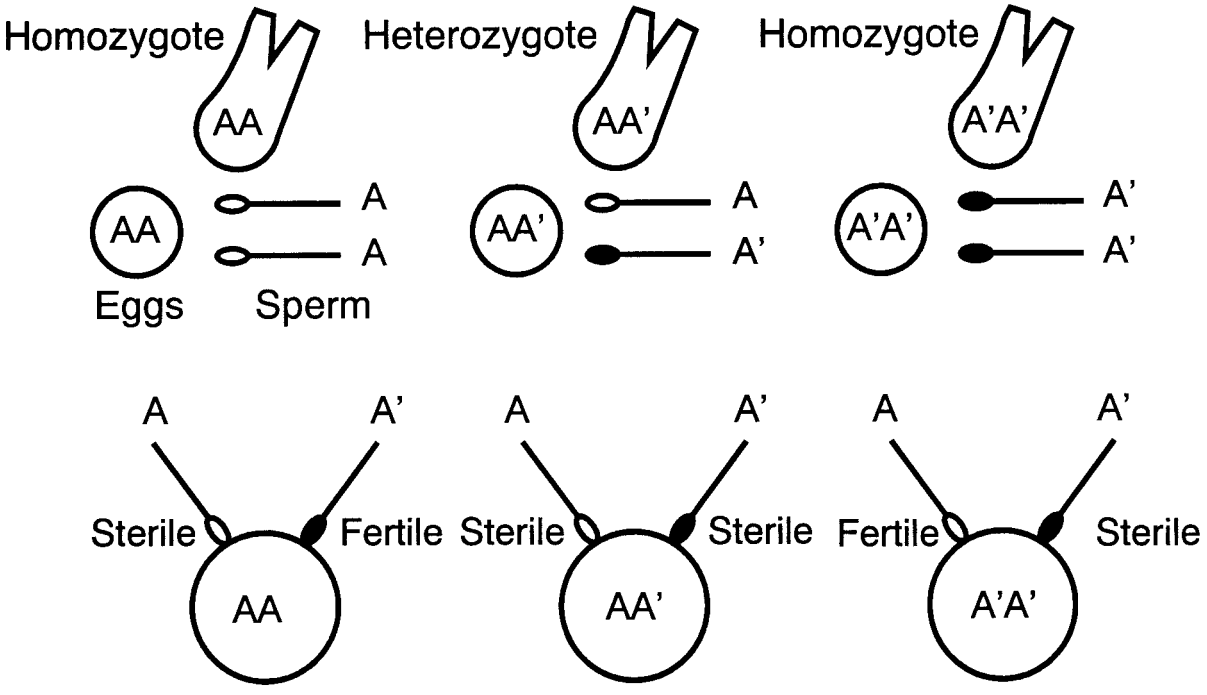


Fig. 1. A model for the occurrence of one-way cross-sterility in the single-locus system. According to haploid sperm hypothesis by Morgan (1942, 1944), it is assumed that functional expression of individual-specific factor(s) for self-sterility is haploid in sperm but diploid in eggs, and that the combination sharing one allele is sterile. Homozygotes of the gene concerned (AA or A'A') produce a homogeneous population of sperm with respect to the locus (A or A'), while heterozygotes (AA') produce a heterogeneous population of sperm (A and A'). No spermatozoa from the homozygote are fertile to the eggs from the heterozygote. However, since half of the spermatozoa are fertile to the eggs from the homozygote, the combinations of heterozygous sperm and homozygous eggs are fertile. Even in the case of multiple-locus system, one-way cross-sterility is accountable by an essentially similar model.

tions prompted us to re-examine the cross-sterility within the siblings from selfed eggs and those from crossed ones.

Here, we report the breeding of F_1 siblings from both selfed and crossed eggs, the occurrence of the cross-sterility within the siblings, and the relation between the cross-sterility, failure of binding and sperm inactivation. We discuss also a possible genetic background of self-sterility.

MATERIALS AND METHODS

Sea water

Filtrated sea water was used throughout the breeding of siblings in the laboratory. For bioassays, artificial sea water composed of 460 mM NaCl, 10 mM KCl, 25 mM $MgCl_2$, 25 mM $MgSO_4$, 10 mM $CaCl_2$, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; Dojindo Lab., Kumamoto, Japan), pH 8.0 was used. Acidic sea water was prepared by adjusting the pH of 50 mM glycine in filtrated sea water to 3.0 with HCl.

Animals and Gametes

Ciona intestinalis collected in Tokyo Bay was used throughout the present study. The gametes were collected from the gonoduct with a Pasteur pipette. The eggs washed with sea water and undiluted (dry) sperm were stored at 4°C until use. The concentration of sperm was determined according to Vacquier (1986) with some modification. Turbidity of sperm suspension was measured with a spectrophotometer and related to a standard curve constructed by hemocytometer counts.

For sperm binding assays, the eggs were glycerinated according to Rosati and De Santis (1978) with slight modification; briefly, the eggs were treated with increasing concentrations of glycerol up to 40% (v/v) in sea water and kept at 4°C overnight. Glycerinated eggs were washed five times with sea water and used for the binding assays.

For selfing, the self-sterility was abolished by acid treatment (Morgan, 1939; Kawamura *et al*. 1987; Byrd and Lambert, 2000); briefly, the eggs were kept in acidic sea water for 5 min, washed with normal sea water three times, and then mixed with autologous sperm. Since the acid treatment detached the follicle cells from the vitelline coat, follicle-free eggs were treated with acidic sea water for fertilization assays in order to exclude any possible indirect effects of these cells on fertilization from consideration. Follicle-free eggs were prepared by the method of Fuke (1983) with slight modification, briefly the intact eggs were treated with a buffered isotonic saline (460 mM NaCl, 1% EDTA and 10 mM Tris-HCl (Sigma Chemicals, St. Louis, MO), pH 8.0 for 1 hr and then washed with sea water three times.

Breeding of F_1 siblings

Self-sterile, healthy and mature animals were randomly selected from the wild populations to use as the starting parents. Gametes from two animals were reciprocally cross-inseminated to breed crossed siblings, and acid treated eggs from both animals were self-inseminated to obtain selfed siblings. About 1×10^4 eggs suspended in 10 ml sea water were inseminated with 10^7 sperm. Fertilized eggs were washed with sea water and reared at the density of 2×10^2 eggs/ml in 10-cm Petri dishes at 20°C, and the tadpole larvae at 16 hr after insemination were transferred into nail-scratched plastic dishes ($5–10\times10^2$ larvae/10 cm-dish). The dishes were placed in the dark for 2–3 days to allow the larvae to settle and metamorphose, and then the dishes were transferred into a container with 1 l of sea water per dish. The juveniles feeding on the diatom, *Chaetoceros gracilis*, were reared at 20°C in the container with daily renewal of sea water. After cultivation of juveniles for 1–2 months, the dishes were fixed in a plastic cage to be hung in the sea at a depth of 4–5 m. The animals matured within another period of 1–2 months in the sea. In order to get rid of the contamination from wild populations, dishes without juveniles were placed in the same cage as a control and all dishes in the cage were inspected from time to time.

Fertilization assay

All assays were done in 48-well multi-dishes at 20°C. One hundred eggs in 0.1 ml of sea water were mixed with 1×10^6 sperm in 0.1 ml of sea water, incubated for 1 hr, fixed by the addition of 0.2 ml of 2M sulfuric acid, and scored for fertilization by cleavage (Hoshi *et al*., 1981). Populations with a fertilization ratio greater than 80% were regarded as fertile, less than 20% as sterile, and between 20 and 80% as incomplete sterile.

Sperm binding assay

Sperm binding to the vitelline coat was observed in sea water containing 0.02% gelatin (Wako Pure Chemicals, Osaka, Japan) to block non-specific adsorption of sperm to the glass surface (unpublished observation). One hundred glycerinated eggs and 1×10^7 sperm suspended separately in 0.1 ml each of gelatin sea water were mixed, incubated for 20 min at 20°C, then fixed with the same volume of 10% formalin in sea water. Sperm nuclei were stained with 0.1 μg/ml of 4, 6-diamidino-2-phenylindol dihydrochloride (DAPI; Sigma-Aldrich Japan, Tokyo) in sea water for 30 min at room temperature. The nuclei of sperm bound to the vitelline coat of glycerinated eggs in an area of 2.5×10^3 μm^2 were counted under an epifluorescent microscope. Numbers of bound sperm were normalized by that to non-sibling eggs as 100%.

Observation of sperm inactivation after binding

After insemination as mentioned above, glycerinated eggs were mounted on the slide glass and observed under a phase-contrast microscope for the flagellar movement to assess sperm inactivation after binding to the vitelline coat.

RESULTS

Breeding of F_1 siblings

In May 2001 the breeding of siblings was started in our laboratory. No contamination of *C. intestinalis* from the wild populations was found throughout the cultivation in the sea, while other sessile organisms grew well on the dishes.

Four different combinations of gametes are possible from a pair of animals as shown in Fig. 2A, and thus the siblings from each combination were separately reared; namely, two populations of selfed siblings from the acid treated eggs (E1S1 and E2S2) and two populations of reciprocally crossed siblings (E1S2 and E2S1). The selfed siblings grew much slower and were less viable than the crossed siblings both in the aquarium and in the sea. The difference in the body size between the selfed and the crossed was already significant 10 days after fertilization (Fig. 2B). The lower viability of the selfed siblings was resulted not only from a slower growth rate of juveniles but also from the failure of metamorphosis of the larvae (unpublished observation). Some of the selfed siblings survived although growth of the population was not synchronous, and only a small number of juveniles grew large enough within one month after fertilization to be placed in the sea. In contrast to the selfed, crossed siblings grew much better and more synchronously in the aquarium as shown in Fig. 2B. In

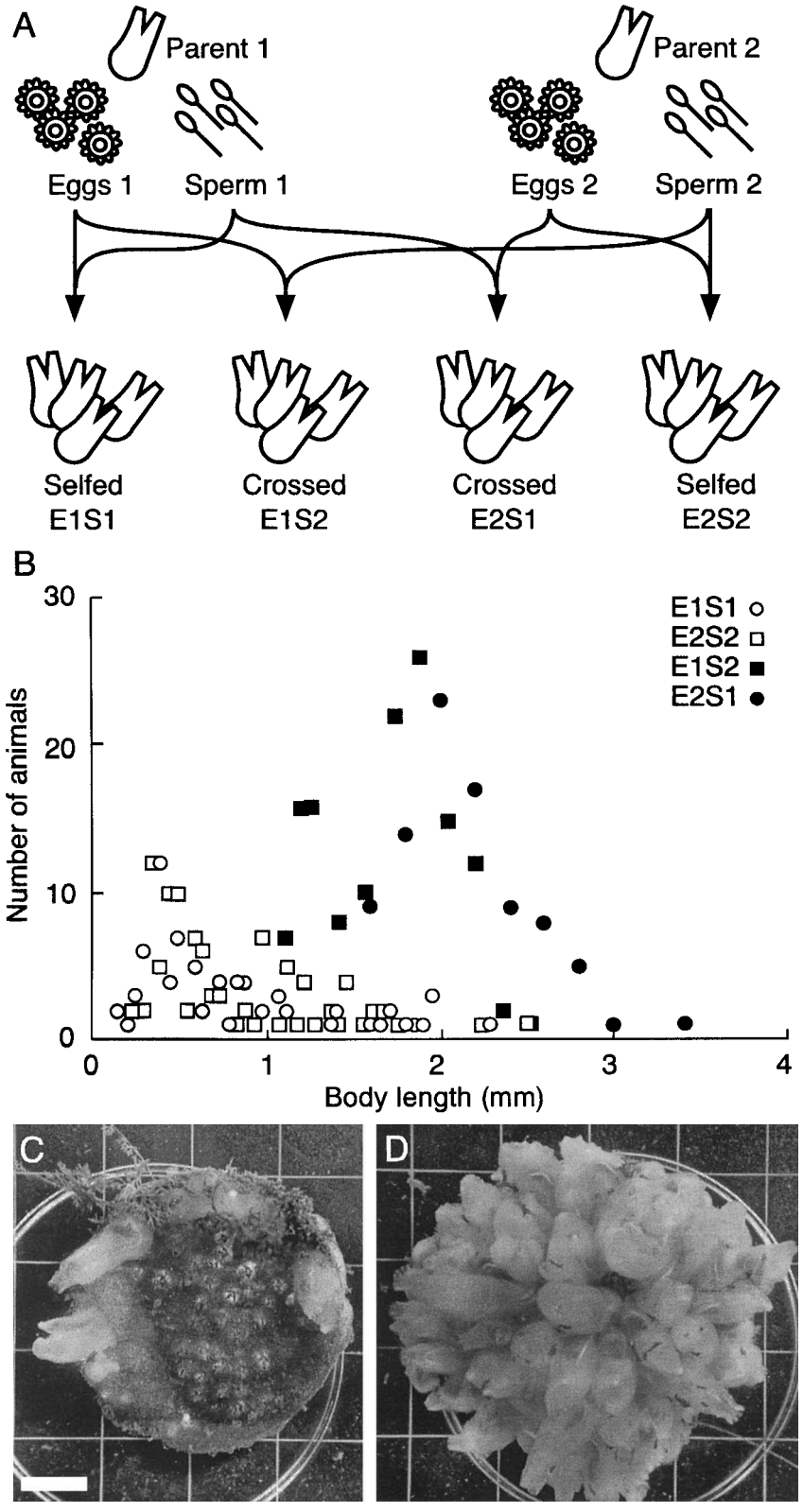


Fig. 2. Breeding of selfed and crossed siblings in *C. intestinalis.* A. Schematic diagram of the production of siblings. Gametes from a pair of parents (animals 1 and 2) were reciprocally crossed for the crossed siblings (E1S2, E2S1) and selfed after acid treatment of eggs for the selfed siblings (E1S1, E2S2). B. Body length of the siblings 10 days after fertilization. Selfed siblings (open circle, E1S1; open square, E2S2) were significantly smaller than the crossed (closed circle, E2S1; closed square, E1S2). $P<0.05$ by t-test and Cochran-Cox test. C. and D. Appearance of animals after the cultivation in the sea for one month. Many were lost in the selfed siblings (C) , but many sexually matured and healthy adults of a similar size remained in the crossed ones (D). Scale bar, 2 cm.

fact, within a month after fertilization, many of them grew up too large to keep growing under the limited supply of foods in the aquarium. Thus, they were placed in the sea at such stage of growth.

Differences between the selfed and the crossed became more evident after they are placed into the sea. As a result, only a small number of animals sexually matured in the selfed siblings (Fig. 2C, Table 1) while many matured quite synchronously in the crossed siblings (Fig. 2D, Table 1). All individuals sexually matured in the sea were used for fertilization assays.

Table 1. Number of the siblings bred.
Data for a typical breeding is summarized in this table.

	Selfed siblings		Crossed siblings	
	E1S1[a]	E2S2	E1S2	E2S1
One month after fertilization[b]	21	80	198	592
Two months after fertilization[c]				
Total	9	77	138	134
Sexually mature[d]	0	23	105	122

[a] Expression of siblings is given in Fig. 2A.
[b] Animals right before cultivation in the sea.
[c] Animals one month after cultivation in the sea.
[d] Animals with gametes.

Fertilization within a wild population

First we asked whether the wild population in Tokyo Bay had self-fertile animals. We have never found so far any self-fertile individuals, even a single animal, under the conditions used. Then we asked whether the incidence of cross-sterility was very low, if any, within the wild population in Tokyo Bay as reported by Morgan (1942). Three independent groups of 24 animals each were tested for selfing and crossing with the others in the group, resulting in 1,656 crossings and 72 selfings from 72 animals. Neither the reciprocally cross-sterile combination nor one-way cross-sterile combination was found. From these two sets of data, we conclude that the self-sterility is strict and stable, and the individuality expressed in gametes is highly diversified in the wild population from Tokyo Bay.

Cross-sterility between siblings

We examined gametes from 11 selfed and 10 crossed siblings that shared the mother with the selfed siblings, in a pairwise sterility panel of all 420 combinations with selfed combinations as negative control and the combinations with non-siblings as positive control (Fig. 3A). All animals used in this assay were self-sterile but fertile with non-siblings. Notable animals were found in the selfed siblings (animals 1–11); namely animals 1–3 produced cross-sterile eggs with sperm from any selfed siblings, while their sperm were fertile with eggs from any siblings except these three. They constitute most part of one-way cross-steriles (pale gray), and the animals of this type were found in all batches so far examined (data not shown). Moreover, sperm from some selfed siblings were one-way sterile to the eggs from crossed siblings sharing the mother with the selfed (combinations of animals 4 and 5 with animals 12–14 and 17–19, and animals 10 with animals 15–16), however all the complementary combinations resulted in fertile (eggs from SS versus sperm from CS). Within crossed siblings (eggs from CS versus sperm from CS), only reciprocal cross-steriles were observed. Even if one-way cross-steriles were found in some batches of crossed siblings, the incidence of reciprocal cross-steriles was always significantly higher than that of one-way cross-steriles (Table 2).

Combinations of hermaphroditic parents produce two populations of siblings (such as E1S2 and E2S1 in Fig. 2A) which share the same genetic potential. Indeed, similar patterns of reciprocal cross-sterility were found in both populations as shown in Fig. 3B (animals 1, 5 and 7, and animals 2–4, 6 and 8).

Relation between sperm binding, sperm inactivation and sterility in siblings

One of the most remarkable features in self-sterility in *Ciona* is the failure of sperm binding to the vitelline coat of autologous eggs (Rosati and De Santis, 1978; Kawamura *et al.*, 1987). We therefore examined whether this is the case also in cross-sterile combinations. Gametes from eight selfed siblings and two crossed siblings that shared the mother with the selfed siblings, were tested by pairwise assays with respect to both sterility and failure of binding. Fig. 4A shows a quite similar pattern of sterility observed in another batch of selfed siblings (Fig. 3A), indicating the reproducibility of our assays. Although the sperm binding to the vitelline coat was quantitatively assayed, each combination gave a result in an all-or-none manner (Fig. 4B). Fig. 4C shows that most of the sterile combinations including one-way cross-steriles results in the failure of binding except for three cases designated by asterisks. It shows also that the siblings apparently segregate into three discrete groups (animals 1–2, 3–6 and 7–8). On the other hand, substantial numbers of sperm bind to the vitelline coat in all fertile combinations, even in the cases of the complementary combination of one-way cross-sterility such as E3S1, E7S1, E7S2, E8S1, E8S2, E3S10, E7S9 and E8S9 (Fig. 4B).

Fig 5 summarizes the relation between the cross-sterility and the failure of sperm binding to the vitelline coat within crossed siblings. Two reciprocally cross-sterile combinations (animals 1 and 3, and animals 7 and 8) and three (or five if incomplete sterile cases are incorporated) one-way cross-sterile combinations (E1S4, E3S4, and E5S6; E2S4 and E9S10 for incomplete sterility) were observed (Fig. 5A). All of these combinations were categorized as the failure of binding (Fig. 5B). Failure of binding was observed in several fertile combinations as designated with asterisk in Fig. 5B. The failure of binding was always reciprocal and no one-way failure of binding occurred as far as we examined. Although the siblings apparently segregate into four discrete groups in terms of sperm binding (Fig. 5B), it is denied by the exami-

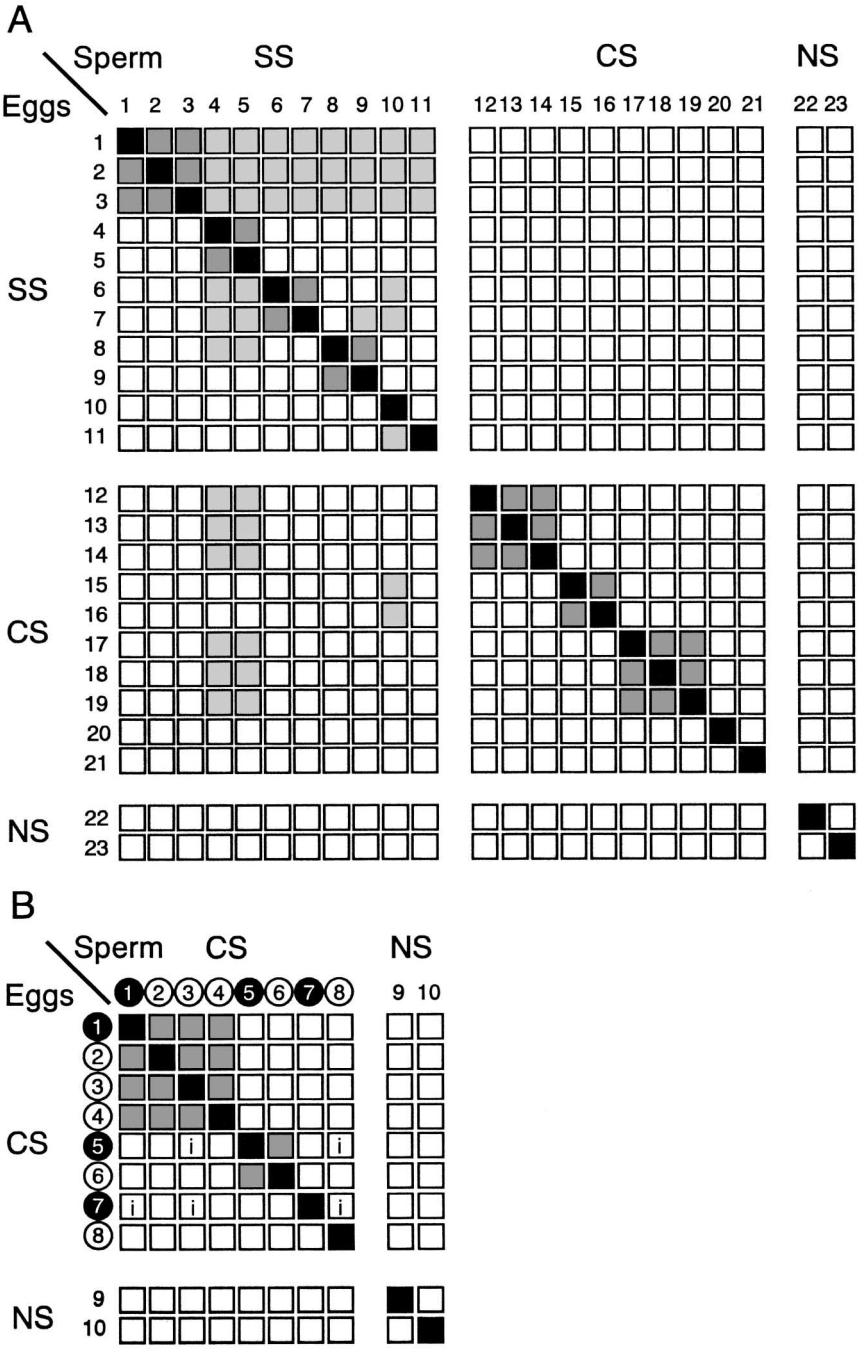


Fig. 3. Cross-sterility within the siblings. Individual animals were arbitrarily numbered and arranged for eggs vertically and for sperm horizontally. White, pale gray, dark gray and black cells express fertile, one-way sterile, reciprocally sterile and self-sterile combinations, respectively. A. Reciprocally pairwise sterility panel. All combinations of the gametes from 11 selfed siblings (SS; animals 1–11), 10 crossed siblings (CS; animals 12–21) and 2 non-siblings (NS; animals 22 and 23) were scored for fertilization. Note that all animals were self-sterile but cross-fertile with any non-siblings. Both one-way (pale gray) and reciprocally cross-sterile (dark gray) combinations appeared. For more detailed explanation, see the text. B. Reciprocally pairwise sterility panel between the siblings from one pair of animals crossed in the opposite directions. Siblings from E1S2 (CS with closed circle; animals 1, 5 and 7) and E2S1 (CS with open circle; animals 2–4, 6 and 8), and two non-siblings (NS; animals 9–10) as the control were scored. Reciprocally cross-steriles appear regardless of the direction of parental cross. Cells with the symbol i represent incomplete steriles.

nation in larger scale as summarized in Fig. 5C. Instead, they appear consisting of four groups or more but do not segregate into discrete groups, indicating the failure of binding is governed by a multiple-locus system.

Kawamura *et al*. (1987) reported that inactivation of sperm occurred if self-sterile sperm exceptionally bound to the vitelline coat of the autologous eggs. Therefore, we asked whether the cross-sterility was accompanied with

Table 2. Incidence of cross-sterile pairs within siblings.

	Total pairs	Reciprocally cross-sterile pairs		One-way cross-sterile pairs		Cross-fertile pairs	
	n	n	%	n	%	n	%
Selfed siblings							
1[a]	55	6 (6)	11 (11)	34 (34)	62 (62)	15 (15)	27 (27)
2[b]	15	5 (5)	33 (33)	4 (7)	27 (47)	6 (3)	40 (20)
3	28	9 (9)	32 (32)	11 (14)	39 (50)	8 (5)	29 (18)
4	28	11 (13)	39 (46)	11 (10)	39 (36)	6 (5)	21 (18)
Total	126	31 (33)	25 (26)	60 (65)	48 (52)	35 (28)	28 (22)
Crossed siblings							
5[a]	45	7 (7)	16 (16)	0 (0)	0 (0)	38 (38)	84 (84)
6[c]	28	7 (7)	25 (25)	0 (6)	0 (21)	21 (15)	75 (54)
7[d]	45	2 (2)	4 (4)	3 (5)	7 (11)	40 (38)	89 (84)
8	45	7 (7)	16 (16)	1 (7)	2 (16)	37 (31)	82 (67)
9	55	4 (4)	7 (7)	2 (2)	4 (4)	49 (49)	89 (89)
10	36	5 (7)	14 (19)	1 (4)	3 (11)	30 (25)	83 (69)
11	45	4 (5)	9 (11)	2 (5)	4 (11)	39 (35)	87 (78)
Total	299	36 (39)	12 (13)	9 (29)	3 (10)	254 (231)	85 (77)

Numbers in the parentheses represent the values if incomplete steriles are regarded as sterile.
Data shown in Fig. 3A[a], Fig. 3B[c], Fig. 4A[b] and Fig. 5A[d]

sperm inactivation. As far as we have observed, sperm inactivation is always associated with the failure of binding. In fact, sperm come around the eggs regardless of selfing or crossing, presumably in response to sperm activating and attracting factor (Yosida *et al*., 1993), and they drive eggs by beating the flagellum. In case of fertile combinations, after a while sperm bind to the vitelline coat firmly and keep beating the flagellum for almost 1 hr. However, in case of sterile combinations, even sperm appear to touch the vitelline coat, most of them fail to bind firmly to it and stop beating the flagellum shortly. The rate of sperm inactivation varied in different combinations, and fertile combinations without sperm binding (Figs. 3–5) and incomplete sterile combinations tend to be less inactivated than sterile combinations. Because it was impossible to quantify the sperm inactivation by microscopic observation, we did not go further on this issue.

Abolishment of cross-sterility by acid treatment

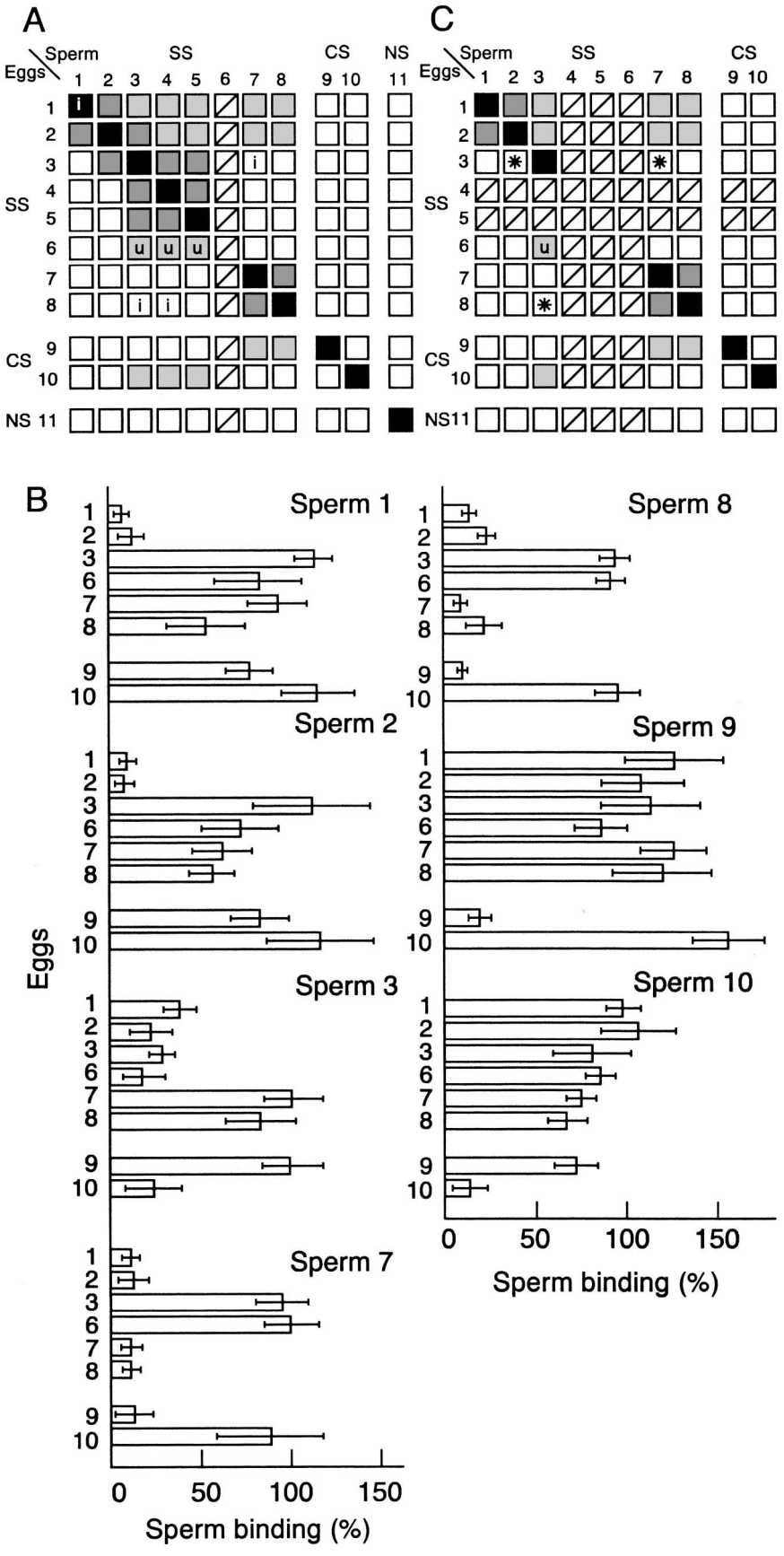
We then asked whether acid treatment of eggs abolished cross-sterility between the siblings as it did self-sterility. As shown in Fig. 6A, acid treatment of eggs abolished reciprocal cross-sterility (E3S1; columns 3) as well as self-sterility (E1S1, columns 1), but it did not affect the fertilization of fertile combinations of siblings (E2S1; columns 2) and of non-siblings (E4S1; columns 4). Acid treatment of eggs abolished reciprocal cross-sterility in the complementary combination (E1S3) as well (Data not shown). These results suggest that the sterility is caused by the same or closely related mechanisms in both self- and cross-sterility. Moreover, the sterility and fertility corresponded exactly to the failure and success of sperm binding to the vitelline coat (Fig. 6B), suggesting that recognition of self and non-self governs the sperm binding and fertilization by the same or closely related mechanisms.

DISCUSSION

This study provides experimental evidence for the following issues of high importance to understand the mechanism of self-sterility in *Ciona intestinalis*: First, the highly diversified individuality expressed in the gametes from the wild population. Secondly, the occurrence with rather high incidence of reciprocal cross-sterility and one-way cross-sterility in both selfed and crossed siblings in contrast to the absence of cross-sterile combinations in the wild population so far as we examined. Thirdly, the coincidence of the sterility, both self- and cross-sterility, with the failure of sperm binding to the vitelline coat, except for a few combinations. Also, our results with breeding of siblings support the inbreeding suppression that may interfere with the establishment of inbred strains (Kano *et al*., 2001).

Highly diversified individuality expressed in the gametes of wild population

We have never found a self-fertile individual in the wild population of *C. intestinalis* in Tokyo Bay so far as we tested by using our assay system. It is likely that the wild animals we collected are much stricter and much more stable in the self-sterility than those from Tosa Bay, Japan (Kawamura *et al*., 1987), the Gulf of Naples (Rosati and De Santis,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Sperm binding (%)

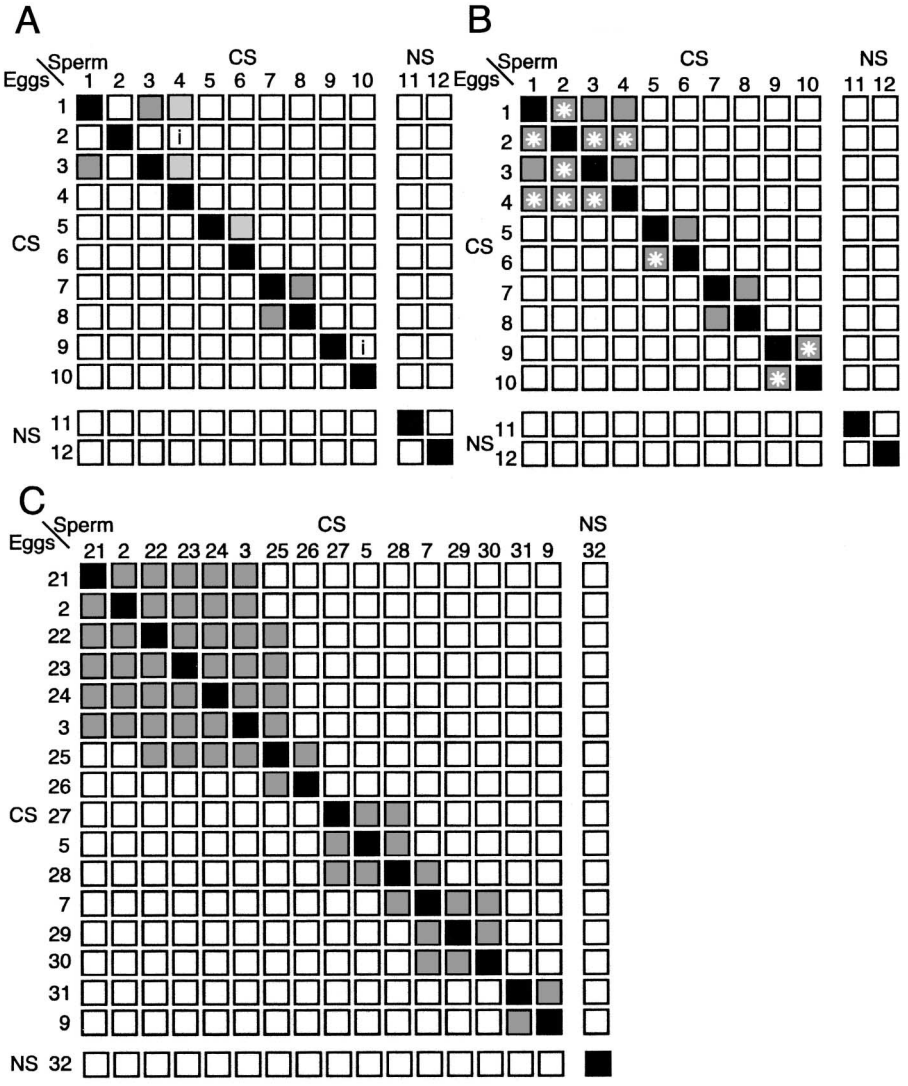

Fig. 5. Relation between the cross-sterility and the failure of binding in crossed siblings. A. Reciprocally pairwise sterility panel. All combinations of the gametes from ten crossed siblings (animals 1–10) and two non-siblings (animals 11 and 12) were scored for fertilization. All the symbols are the same as used in Fig. 4A. Note the occurrence of both reciprocal and one-way cross-steriles. B. Pairwise sperm-binding panel between the animals used in (A). All the symbols are the same as used in Fig. 4C except for the asterisks. The combinations scored as the failure of binding occurred reciprocally only. Such combinations were more than cross-sterile ones. The cross-fertile combinations scored as the failure of binding are represented by asterisks. C. Pairwise sperm-binding panel in a larger scale. All animals except for animal 32, a non-sibling control, belong to the same battery of siblings including those used in (B) (animals 3, 5, 7 and 9). All the symbols are the same as used in Fig. 4B. Note that the siblings do not segregate into four groups as expected for a single-locus system.

1978), and Corona del Mar, California (Morgan, 1938, 1942, 1944), though direct comparison is required to conclude the difference in strictness and stability of self-fertility among populations. It is also worthy to note that no cross-sterile combination was recorded out of 1,656 crossing from 72 animals. All these data confirm Morgan's finding that the

Fig. 4. Relation between the cross-sterility and the failure of binding in selfed siblings. A. Reciprocally pairwise sterility panel between selfed siblings (SS; animals 1–8), crossed siblings that shared mother with the selfed (CS; animals 9 and 10), and a non-sibling (NS; animal 11). Cells with the symbol u represent unidefined steriles, with which we could not determine either one-way or reciprocal because of the shortage of sperm. Cells with an oblique line represent combinations not examined. For other symbols, see Fig. 3. Although animal 1 was incompletely self-sterile, all others were self-sterile. Note the appearance of both reciprocal and one-way cross-steriles. B. Sperm binding to the vitelline coat of the siblings used in (A). Sperm bound to the vitelline coat of glycerinated eggs were counted and normalized by the number of sperm bound to non-sibling eggs as 100% (means±SD; n=10). C. Pairwise sperm-binding panel between the siblings. All the data in (B) are summarized to compare the failure of binding with sterility. White, pale gray, dark gray and black cells represent success, one-way failure, reciprocal failure and self-failure in sperm binding to the vitelline coat, respectively. Cells with an oblique line represent combinations not examined. Note that three cross-sterile combinations represented by asterisks are scored as the success in sperm binding.

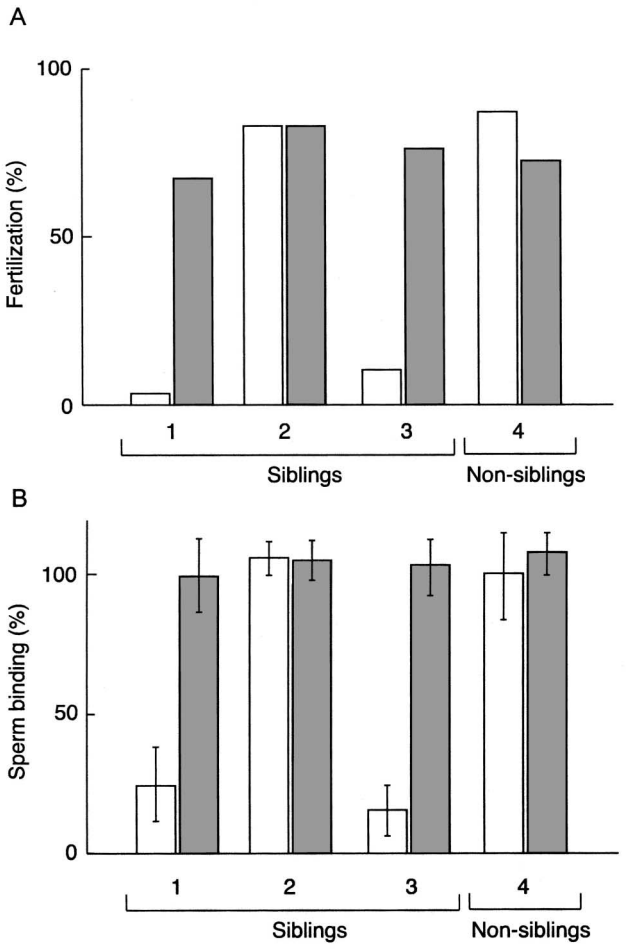

Fig. 6. Effects of acid treatment of eggs on the cross-sterility (A) and cross-binding (B) in siblings. A. Follicle-free eggs from two reciprocally cross-sterile siblings (animals 1 and 3), a fertile sibling to animals 1 and 3 (animal 2) and a non-sibling (animal 4) were treated with (solid columns) or without (open columns) acid sea water, and then inseminated with sperm from animal 1. Acid treatment abolished the cross-sterility (animal 3) as well as self-sterility (animal 1). Fertility for the combinations of eggs from animals 2 and 4, and sperm from animal 1 was not affected. B. Sperm binding assay was carried out with glycerinated eggs before (open columns) or after (solid columns) acid treatment. Sperm bound to the vitelline coat were counted and normalized by the number of sperm bound to non-sibling eggs as 100% (means±SD; n=10). Note the clear correlation between failure of binding and sterility.

highly diverse individuality is expressed in the gametes of wild population.

Cross-sterility between siblings

We found cross-sterile combinations within both selfed and crossed siblings as reported (Morgan, 1942, 1944), indicating that the self-sterility is genetically governed (Figs. 3–5). This conclusion is further supported by the fact that the cross-sterile combinations are found within a battery as well as between two batteries of crossed siblings by reciprocal insemination of a pair of animals, because these two batteries should be identical in genetic composition (Fig. 3B). The data summarized in Table 2 yet support the conclusion. Namely, a significantly higher incidence of cross-sterility within selfed siblings than that within crossed ones is consistent with the extent of genetic diversity expected for siblings.

The incidence of cross-steriles obtained in this study is apparently much higher than those reported by Morgan (1942, 1944). In fact, we have realized that the animals used by Morgan to score the cross-sterility included some self-fertile siblings. Considering this fact, we have found that the difference is not significant in the case of crossed siblings. However, the difference is still significant in the case of selfed siblings. Because we have found much lower viability of the selfed comparing to the crossed, which Morgan did not mention, we cannot deny the possibility that the selfed offspring reflect some selection with respect to the genotype concerned.

One-way cross-sterility

Morgan proposed the haploid-sperm hypothesis to explain the data he obtained without the evidence or theoretical basis. Here we try to examine the genetic background of self-sterility by interpreting the pattern of the occurrence of cross-steriles within the F_1 siblings. Even though we consider only single-locus model to make arguments simpler, the story can be applied to multiple-locus system at least qualitatively.

Selfed siblings should be divided into either homozygote or heterozygote in any Mendelian locus. In the selfed offspring, we found that the eggs from some animals (animals 1–3 in Fig. 3A, and animals 1 and 2 in Fig. 4) were sterile with sperm from any siblings, indicating that these animals are heterozygous in the gene concerned. Therefore, the other members in the battery are regarded homozygous. Combinations of sperm from the homozygotes and eggs from the heterozygotes should be sterile, while the reverse combination should be fertile. Since such combinations could occur between the selfed siblings and the crossed siblings that share the mother with the selfed, we predicted that one-way cross-steriles occur in the combinations between sperm from the selfed and eggs from the crossed. Indeed, it is the case as shown in Fig. 3A.

Sterility and Failure of Binding

It is clear that cross-sterility and self-sterility share some features, if not all, because in both cases sterility coincides with the failure of binding (Figs. 4 and 5). Treatment of the eggs with acidic sea water abolishes coincidentally the self-sterility, cross-sterility and failure of binding, that is the failure of sperm binding to the vitelline coat of autologous eggs (Fig. 6).

The coincidence of the failure of binding with cross-sterility (Fig. 4) may indicate that self/non-self recognition at the level of sperm binding is also genetically governed in a similar way. However, there are some fertile combinations of crossed siblings in which sperm binding is scored as the failure (Fig. 5). This apparent contradiction may be accountable

if the difference in nature between fertilization scores and binding scores is considered. Fertilization is a phenomenon reflecting the presence of one, or some very small number, of successful sperm, whereas sperm binding reflects the binding capacity of the most sperm in a population. If only a few sperm in a population can bind, this population is scored as the failure of binding, nevertheless they may succeed in fertilization. It may be worthy to mention that sperm binding was observed in an all-or-none manner (Figs. 4 and 6), suggesting that sperm binding has a threshold.

The failure of binding was always reciprocal and one-way failure of binding never appeared within crossed siblings in the present study (Fig. 5B). This result suggests a very low incidence of homozygous animals in terms of the genes concerned, because one-way type inhibition predicts that the haploid-sperm hypothesis summarized in INTRODUCTION is correct. However, since sperm binding is just one step for fertilization, we cannot deny the possibility, though it is unlikely, that one-way cross-sterility results from accidental errors in the regulation of a step in fertilization. To discuss this kind of problems, our knowledge on each step leading to fertilization is too much limited at present, though some information is available on the binding to and penetration through the vitelline coat (Hoshi *et al*., 1985, 1994).

Number of loci involved in self-sterility

Morgan (1944) concluded that more than five loci are involved under the haploid sperm hypothesis without saying the basis for calculation clearly. Since the individuality expressed in the gametes is highly diversified in the wild population, if self-sterility is governed by a single-locus system under haploid sperm hypothesis, the selfed siblings must segregate into three groups including a group of heterozygotes and two different types of homozygotes, and similarly the crossed siblings must segregate into four. The result summarized in Fig. 3A is not consistent with such prediction and thus we also conclude that self-sterility is governed by a multi-locus system. This conclusion is supported also in sperm binding to the vitelline coat, an important element for self-sterility (Fig. 5C). We cannot estimate the number of loci involved in self-sterility mainly because the practical panel size of pair-wise assay is much limited for the calculation; the size increases enormously as the number of involved loci increases.

Self or non-self

If sperm recognize self, the ordered pattern of cross-steriles in our study (Figs. 3 and 4) is easily accountable by haploid sperm hypothesis in which at least phenotypically haploid sperm react with diploid eggs. This hypothesis is based upon the heterogeneous sperm population produced by a single heterozygotic animal. Such inequality in a sperm population is known in mice. Male carrying one complete *t*-haplotype transmit it to virtually all offspring (Olds-Clarke and Peitz, 1985). However, genetically haploid spermatids are thought to be phenotypically diploid in general (Braun *et al*., 1989), though haploid expression is known in mice (Matsumoto *et al*., 1993). Phenotypically haploid sperm would be produced by allelic exclusion of germ cells before the formation of syncytium by incomplete cytokinesis, though such examples are not yet found.

Alternatively, if fertilization occurs when gametes recognize non-self, it is not necessary to assume haploid sperm. Instead, a mechanism must exist in which an egg transmits positive signal for fertilization to the sperm when the egg recognize non-self signal on the sperm. Although it is widely believed that the gametes recognize self in *C. intestinalis* (Morgan, 1942), there is an important piece of experimental evidence for the recognition of non-self (Kawamura *et al*., 1991).

In all, this paper clearly show that the self-sterility is genetically governed by a multiple-locus system, and that most probably individual-specific determinants are haploid expression in sperm and diploid expression in eggs, given they recognize self but not non-self. Yet, it is an open question whether ascidian gametes recognize self like plants (Watanabe *et al*., 2001) or non-self like fungi (Kothe, 1999). At present, we do not have enough evidence for either hypothesis. It is urged to isolate and identify the individual-specific peptide by Marino *et al*. (1998) and non-self factor by Kawamura *et al*. (1991) to solve this key problem to understand the mechanism of self-sterility.

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