Mar. Biotechnol. 3, 58–67, 2001
DOI: 10.1007/s101260000048

MARINE BIOTECHNOLOGY

Genetic Relatedness and Variability in Inbred and Wild Populations of the Solitary Ascidian *Ciona intestinalis* Revealed by Arbitrarily Primed Polymerase Chain Reaction

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Abstract: As the first step in the establishment of inbred *Ciona intestinalis* strains, the genetic backgrounds of several local populations were evaluated on the basis of comparing 313 random amplified polymorphic DNA (RAPD) loci. Cluster analyses of individuals indicated that this species has local genetic characteristics, although various genetic distances among the populations were relatively low. The bulked RAPD analyses revealed that populations from Torihama and from Onagawa were the most distantly related among the 7 populations examined, suggesting that these two populations are the best candidates for outbreeding. Successive inbreeding in the laboratory was achieved using the Onagawa population. Although the genetic similarity of the F_4 progeny became distinctly higher than that of natural populations, F_5 progeny did not survive, presumably owing to homozygous lethal alleles.

Key words: ascidians, marine invertebrates, genetic markers, inbreeding in laboratory, AP-PCR, RAPD.

INTRODUCTION

Ascidians constitute a major class of benthic marine organisms within the subphylum Urochordata of the phylum Chordata, and they are important experimental animals for studying the genetic circuitry responsible for the specification of embryonic cells and for understanding basic chordate morphogenesis (reviewed by Satoh, 1994). Many studies indicate that the ascidian larva has the prototypical body plan basic to all chordates (reviewed by Satoh and Jeffery, 1995). Furthermore, ascidians have long been established as a powerful research model in many fields of bioscience, including developmental biology, evolutionary biology, physiology, cell biology, and immunology.

The genetics of ascidians is still poorly understood, however, because of difficulties in breeding them in the laboratory and because of their relatively long generation time. These technical limitations have often prevented progress in the study of ascidian biology, especially their developmental genetics. However, a solitary ascidian, *Ciona intestinalis,* is easily bred throughout the year and matures within 2 to 3 months in the laboratory. As self-fertilization can be induced artificially (Morgan, 1904; Kawamura et al., 1987), it should be possible to establish an inbred strain of this species in a relatively short time. Together with transgenic methods that have been developed for *C. intestinalis*

Received October 15, 1999; accepted July 18, 2000.

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using microinjection or electroporation methods (reviewed by Di Gregorio and Levine, 1998) and chemical mutagenesis methods (Sordino et al., 2000), a very detailed developmental genetic analysis of ascidian development is possible.

The size of haploid genome of *C. intestinalis* is approximately 162 Mbp, corresponding to an estimated gene number of 15,500 ± 3,700, which is 5.4% of the human genome (Simmen et al., 1998). Thus the relatively small genome of *C. intestinalis* will greatly facilitate genetic studies. In addition, gene disruption experiments seem to be more efficient in ascidians than in the other vertebrates, most likely because the extensive gene duplications that have occurred in vertebrates have not occurred in the ascidian genome (Holland et al., 1994). Again, this suggests that *C. intestinalis* has great potential for helping us to understand fundamental mechanisms responsible for the development of the chordate body plan.

For detailed developmental genetics of ascidians, the establishment of an inbred strain with a stable and known genetic background is desirable. To establish an inbred strain, we first surveyed the genetic background of this species. This species is cosmopolitan and widely distributed in subtidal waters. The swimming larvae of *C. intestinalis* adhere to firm objects at the seashore and metamorphose within a few days, and their migration must be limited by the short pelagic time span (Schmidtke and Engel, 1980). Thus we expected genetic polymorphisms among local populations, which could be used as genetic markers. However, genetic polymorphisms of *C. intestinalis* have been seldom studied, except for some morphological variations (Hoshino and Nishikawa, 1985) and the analysis of the ribosomal DNA spacer region (Matsumoto et al., 1992).

To study genomic polymorphisms within a species, randomly amplified polymorphic DNA (RAPD) markers have been useful to survey differences within the entire genome of an organism (Welsh and McClelland, 1990; Williams et al., 1990). Among the various methods used to collect RAPD markers, arbitrarily primed polymerase chain reaction (AP-PCR) using a radioisotope label is the most sensitive method for recognizing individuals. Small amounts of DNA that are sufficient for this sensitive analysis can be extracted from juveniles or swimming larvae. In addition, the bulked RAPD analysis can survey heterogeneous populations (Yu and Pauls, 1993).

In the present study, we examined genetic polymorphisms of *C. intestinalis* from 7 local populations in Japan using RAPD markers. Genetic similarities within each population and the genetic relatedness among different populations were determined. Furthermore, we established a pilot inbreeding program in the laboratory and monitored the genetic characteristics of this population. The present results provide useful genetic information for the future derivation of a stable inbred line of *C. intestinalis* for developmental genetic studies.

Materials and Methods

Collection of Adults

Ciona intestinalis adults were collected from 7 sites in Japan: 20 individuals from Onagawa Bay (OG) in Miyagi Prefecture; 10 from Kisaradu (KS) in Chiba Prefecture; 10 from Shinkiba (SK) in Tokyo; 29 from Shinyamashita (SY) in Kanagawa Prefecture; 26 from Torihama (TH) in Kanagawa Prefecture; 20 from Mukaishima (MS) in Hiroshima Prefecture, facing the Seto Inland Sea; and 19 from Maiduru (MD) in Kyoto Prefecture, facing the Sea of Japan (Figure 1). The KS and SK populations were excluded from the analysis of genetic variability because of the scarcity of specimens

Inbreeding in the Laboratory

Breeding in the laboratory was carried out in circular plastic containers (ϕ = 25 cm), filled with 2.5-L of seawater at 20°C, without circulation of the water. The seawater was filtrated using a Millipore filter (0.22 μm) in order to exclude contamination by foreign zygotes or juveniles. Filtered seawater (FSW) in containers was changed every 3 to 4 days. About 50 to 150 ml of cultured diatom, *Chaetoceros gracilis* per 2.5-L culture vessel was fed to the animals every day. Under these conditions, juveniles usually developed to sexual maturity within 2 to 3 months (Figure 2).

Successive inbreeding by self-mating has been carried out since 1998. The founder individuals were obtained from Onagawa Bay. Each adult was grown in a circular plastic container (ϕ = 25 cm) filled with 2.5-L of FSW. Spawning was induced by controlling the light and dark conditions in the following manner: a mature adult was isolated in a container (ϕ = 15 cm) filled with 500 ml of FSW under continuous light for 3 days, then maintained in the dark for a minimum of 3 hours, and then exposed to the light again. Spawned gametes were usually self-fertilized because of high

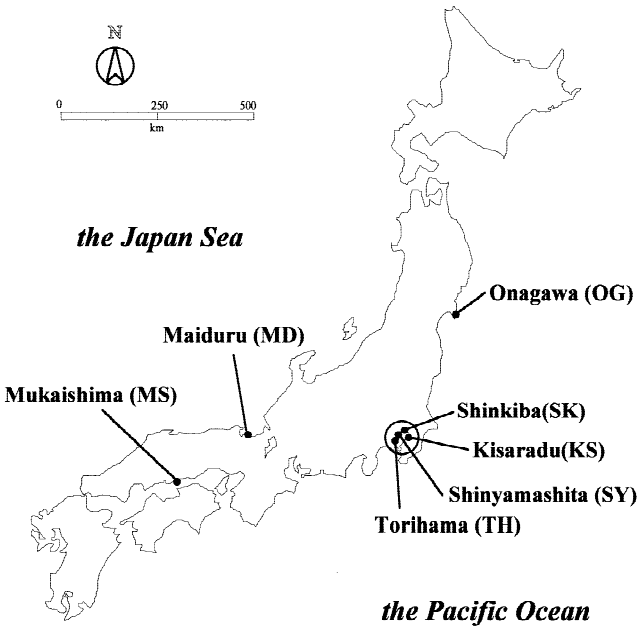

Figure 1. Locations of *C. intestinalis* collection sites in Japan. Specimens were obtained from Onagawa (OG), Kisaradu (KS), Shinkiba (SK), Shinyamashita (SY), Torihama (TH), Mukaishima (MS), and Maiduru (MD). The open circle indicates Tokyo Bay.

concentration of sperm. When self-fertilization did not occur naturally, acid treatment was used to remove the self-fertilization block according to Kawamura et al. (1987). The larvae were collected using a plankton mesh (100 μm) and transferred to plastic Petri dishes (ϕ = 9 cm). They settled and metamorphosed on the plastic dishes in the dark. Juveniles grown on dishes were transferred to another container filled with 2.5-L of FSW. Mature individuals were isolated after the testis matured to avoid contamination by zygotes from other individuals.

Genomic DNA Preparation and Bulking

Genomic DNA was extracted from the body wall muscle of an adult specimen according to Aljanabi and Martinez (1997), with minor modifications. To remove parasites from the surface of tissues, tissue sections were washed with 70% ethanol, and the lack of contamination was confirmed by examination with a binocular microscope. The extracted DNA was dissolved in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA. The approximate concentration of DNA was determined by comparing fluorescence intensities with those of molecular weight markers. When PCR products were not obtained, cetyltrimethylammonium bromide (CTAB) treatment was done as follows: the volume of DNA solution was adjusted to 350 μl, and 86 μl of 4 M NaCl and 108 μl of CTAB solution (5% CTAB, 0.35 M NaCl) were added and incubated at 65°C for 30 minutes. The DNA was extracted once with chloroform and precipitated with isopropanol.

For the bulked RAPD analyses, an equal amount of genomic DNA from 10 individuals per populations was used as templates in AP-PCR (Yu and Pauls, 1993).

AP-PCR Fingerprinting

AP-PCR was carried out essentially as described by Wada et al. (1995). Detailed conditions for this method were optimized according to Scott et al. (1992) and Williams et al. (1993). The annealing temperatures for low-stringency PCR and high-stringency PCR were modified to 34°C and 45°C, respectively. Thermal cycling was performed using a DNA Thermal Cycler PJ1000 (Perkin-Elmer Cetus, U.S.A.), and AP-PCR fingerprints were visualized by autoradiography using x-ray film (Fuji Photo Film Co., Ltd., Tokyo).

A total of 38 primers (Operon, U.S.A.) were tested on 4 specimens obtained from a wild population. Six of these primers were chosen on the basis of whether discrete PCR bands could be obtained. In the present study, cluster analysis and analysis of genetic similarity were carried out using three primers, OPA9, OPB7, and OPB8. The bulked RAPD analyses to examine genetic relatedness of individuals were carried out using 4 primers, OPA9, OPB7, OPB8, and OPB18. These primers were obtained from a commercial oligonucleotide supplier (Japan Bio Service, Saitama, Japan).

Analysis of RAPD Markers

PCR was carried out 2 or 3 times using the same template DNA, and only the reproducible markers were used for further analyses. They were scored manually according to the presence or absence of bands having the same relative mobility on the gels. The genetic similarity between 2 individuals (*GSI*) was estimated from Nei and Li's coefficient (1979) as follows:

$$GSI = 2N_{XY}/(N_X + N_Y),$$

in which N_{XY} is the number of RAPD markers shared by

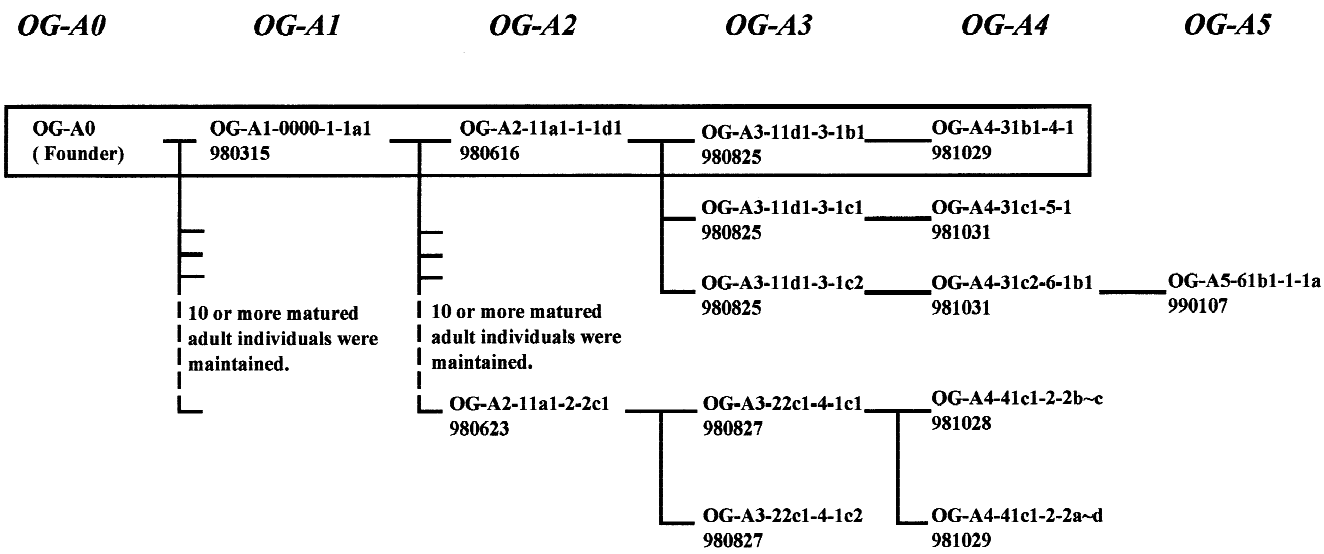

Figure 2. Record of successive inbreeding of the "OG-A" line of the solitary ascidian *C intestinalis.* The pedigree line surrounded by the rectangle was analyzed in the present study. Each number following "OG-A" indicates the generation. The date of fertilization is indicated below each pedigree name.

individuals X and Y, and N_X and N_Y are the total number of bands scored in individuals X and Y, respectively. The mean *GSI* value (*MGS*) was calculated using the formula $MGS = \Sigma GSI/S$, where S is the number of pairwise comparisons of n individuals, $S = n(n - 1)/2$. *MGS* indicates the genetic variability within each population or the genetic difference between populations. Genetic similarity between 2 bulked populations was estimated by the same formula used to estimate *GSI*. Significant differences between populations were estimated at $P < .01$ (**) and $P < .001$ (***) by the Mann-Whitney test.

Cluster analysis among individuals of local populations was examined by parsimony analysis and distance analysis. Phenograms were constructed by the parsimony method using the MIX program of PHYLIP (Felsenstein, 1993). The CONSENSE program was used to reduce these sets to a consensus tree. Bootstrap analysis was performed by sampling data using the SEQBOOT program of PHYLIP. Distance matrices among individuals were produced from *D* values according to the following formula:

$D = -\ln (GS)$ (Swofford et al., 1996).

Phenograms were constructed by the neighbor-joining method and the UPGMA method using the NEIGHBOR program of PHYLIP.

Results and Discussion

RAPD Markers for Genetic Analysis of Ascidian Populations

Using RAPD markers, we detected genetic polymorphisms within a population of *Ciona intestinalis* (Figure 3). By contrast, comparisons of 18S ribosomal DNA sequences did not reveal variability among 3 individuals of the OG population or among 3 local populations in Japan (Dr. Hiroshi Wada, personal communication)

When 77 independent markers are surveyed, it is theoretically expected that at least 1 marker would be specific for each of the 14 chromosomes of *C. intestinalis* (Taylor, 1967). RAPD markers usually reveal several independent genetic loci (Williams et al., 1993). Actually, we tested the possibility that RAPD markers were clustered in specific regions of the genome owing in similarities among sequences of primers. The 4 primers used in the present study shared no specific motifs. The average number of annealed nucleotides was about 3.5, which was estimated from the formula $b = l \cdot 4^{-2n} \cdot c$ (where b is the average number of amplified bands, l is the length of the amplified sequences in base pairs, n is the number of annealed bases, and c is the complexity of the genome; see Williams et al., 1993). These estimates indicate that the annealing during the low-stringency cycles of AP-PCR was random, and the 4 primers

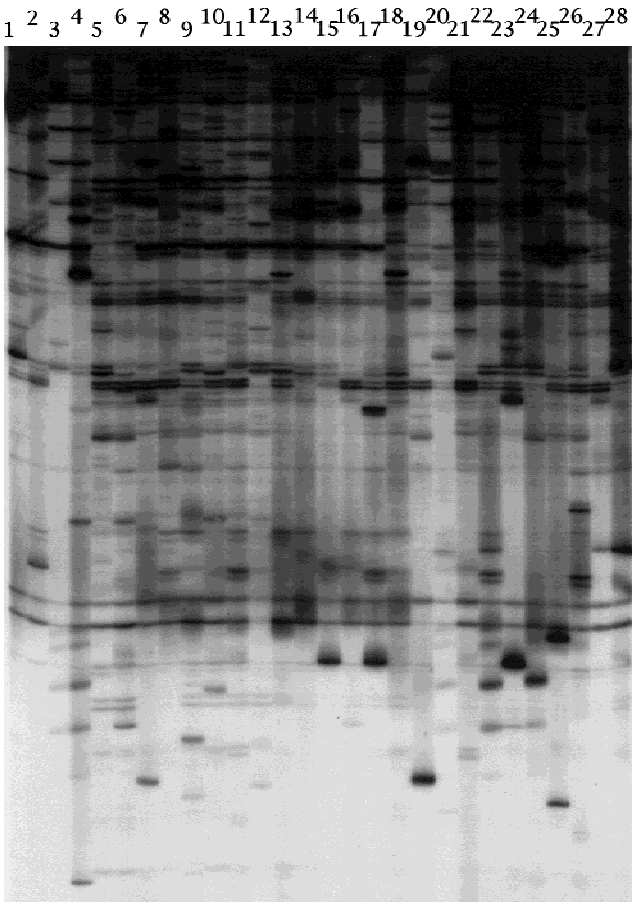

Figure 3. Examples of AP-PCR fingerprints using the primer OPB7. Four individuals were sampled from each local population. Each individual could be distinguished from the others. Lanes 1–4, OG population; lanes 5–8, KS population; lanes 9–12, SK population; lanes 13–16, SY population; lanes 17–20, TH population; lanes 21–24, MS population; and lanes 25–28, MD population.

should have generated independent RAPD markers. Thus the RAPD markers used in the present study were sufficient to cover the whole genome.

Local Specificities in Populations of *Ciona intestinalis*

To examine whether local specificities exist in populations of *C. intestinalis,* cluster analysis was carried out at the level of the individual using a parsimony method and 2 distance matrix methods. Using these 3 analyses with 313 RAPD loci, we found that most individuals were clustered according to the sites where they were collected (Figure 4). Local specificities were also supported by comparisons between *MGS*s within and among populations (Table 1). When the *MGS*

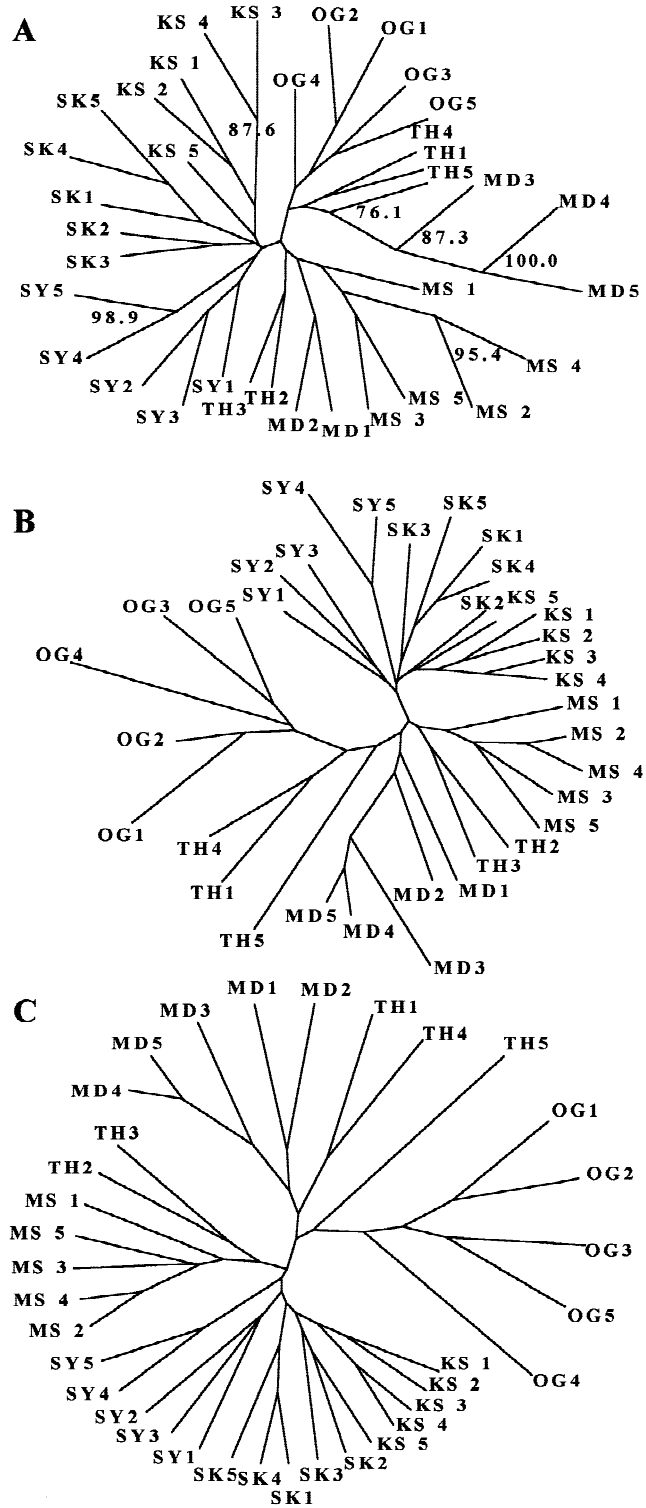

Figure 4. Phenograms at the level of local individuals. Five individuals were used from each local population. The numbers following each abbreviation indicate the number of independent individuals from each local population. **A:** Unrooted consensus tree from the parsimony analysis. Only bootstrap values above 70.0 are indicated. **B:** Unrooted neighbor-joining tree. **C:** Unrooted UPGMA tree.

Table 1. Comparisons Between Intrapopulation and Interpopulation Genetic Similarities by RAPD Analyses Among Five Individuals[1]

	OG	KS	SK	SY	TH	MS	MD
OG	*0.687 ± 0.054*	0.618 ± 0.058**	0.560 ± 0.054***	0.557 ± 0.050***	0.595 ± 0.043***	0.557 ± 0.048***	0.569 ± 0.027***
KS	***	*0.782 ± 0.036*	0.714 ± 0.035***	0.684 ± 0.030***	0.625 ± 0.059***	0.664 ± 0.035***	0.626 ± 0.044***
SK	***	*	*0.751 ± 0.050*	0.684 ± 0.031***	0.617 ± 0.068***	0.652 ± 0.037***	0.615 ± 0.052***
SY	***			*0.702 ± 0.057*	0.606 ± 0.048***	0.643 ± 0.035*	0.609 ± 0.055**
TH	***				*0.650 ± 0.054*	0.647 ± 0.049	0.621 ± 0.039
MS	***	***	***	***	***	*0.776 ± 0.044*	0.657 ± 0.041***
MD	***	**	**	***	***	*	*0.724 ± 0.086*

[1]The values are *MGS* values ± SD. The lower triangle values are equivalent to the values in the upper triangle.
Asterisks indicate that local specificities for each population in a vertical column are supported; each interpopulation *MGS* of the horizontal rows is significantly lower than the intrapopulation *MGS* indicated in italics.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Mann-Whitney test).

within a population is significantly larger than the *MGS* between that population and another, local specificity of the population is supported.

Each clustering of the OG and MS populations was supported by all 3 analyses. In particular, the OG population was found to be distantly related to the other populations (Figure 4, B and C). Each *MGS* within the OG and MS populations was significantly larger than every *MGS* between each of these two populations and the other populations (Table 1). This indicates that each of the two populations forms a local group.

Individuals within the KS, SK, and SY populations also tended to be clustered, although a few individuals did not belong to the same cluster (Figure 4, B and C). In addition, it was found by all 3 analyses that these 3 populations formed a single group. The *MGS* within the SY population was not significantly different from that between the KS and SK populations (Table 1). Conversely, the *MGS* within each of the KS and SK populations was significantly larger than that between each of these two and the SY population ($P < .001$). This may be related to the geographical distributions of the populations. The 3 sites where the specimens were collected are near each other in Tokyo Bay. Counterclockwise tides in Tokyo Bay might cause genetic flows from the KS and SK populations into the SY population, and these populations might thus form a group in Tokyo Bay.

Conversely, the TH population was not clustered in the same group as these 3 populations although the TH site is also located in Tokyo Bay. In the present study, TH individuals were not clustered into a single population. Two individuals, TH2 and TH3, seemed to be related to the MS population (Figure 4, B and C). Three individuals, TH1, TH4, and TH5, appeared to be related to the MD or OG population. Moreover, the *MGS* within the TH population was not significantly higher than the *MGS* between the TH population and the others, except the OG population. These results suggest that the local specificity of the TH population is low. This fact may cause the scattered positions of TH individuals in the 3 analyses.

The MD individuals were clustered in the 2 distance analyses, although they were divided into 2 groups in the parsimony method (Figure 4). The MD population is likely to be related to the OG population, although the MD site is on the opposite side of the Japanese Islands from the OG site (see Figure 1). The relatedness of the MD and OG populations is difficult to explain only by the geographical sites. The same is true for the relatedness between the TH population and the other 3 populations in Tokyo Bay.

The present study suggests that local specificities do exist in this species, although the genetic distances among the local populations are small. These might be related to the life history of the ascidian, which is a sessile organism. The migration of this species is usually limited; however, the animals may spread by adhering to something that drifts. If they adhere to the bottom of ships or marine products, artificial genetic flow could occur, and the relatedness among local populations would not be associated with geographical distributions. This would cause different topologies among the 3 phenograms. If this were an introduced species from foreign water, the small genetic distance among Japanese natural populations might be reasonable. It will be necessary in further investigations to use other ge-

Table 2. Genetic Similarities Among Seven Local Populations by Bulked RAPD Analyses*

	OG	KS	SK	SY	TH	MS	MD
OG	–	0.850	0.824	0.814	0.778	0.822	0.794
KS		–	0.802	0.868	0.800	0.861	0.812
SK			–	0.855	0.827	0.828	0.816
SY				–	0.856	0.900	0.861
TH					–	0.829	0.810
MS						–	0.876
MD							–

*Abbreviations for local populations are described in the legend to Figure 1.

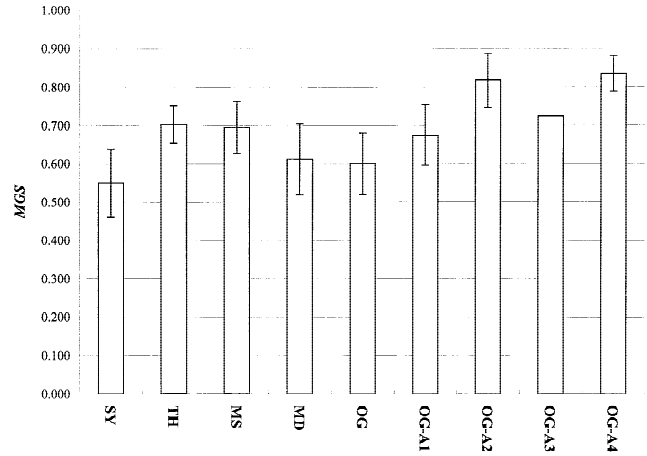

Figure 5. Genetic similarity within each local population and each generation of OG-A during successive inbreeding. Abbreviations for local populations are described in the legend to Figure 1. Abbreviations for the inbred line are described in the legend to Figure 2. As for OG-A3, the data should be taken as approximate because only 2 specimens were analyzed. The KS and SK populations were not surveyed because of the scarcity of specimens. Bars indicate standard deviations.

netic markers, including rapid molecular clocks like mitochondrial DNA, with specimens collected from foreign locations.

Effective Combinations of Local Populations for Outbreeding

Genetic differences between 2 individuals, such as those derived from the local specificities, can be exploited for outbreeding. To examine genetic differences among the 7 local populations, genetic similarities were estimated using 377 RAPD loci by the bulked RAPD analyses. The bulking for 10 individuals merged all markers derived from every individual, and all the markers corresponded to the whole set of loci derived from over 20 individuals (data not shown).

The genetic similarities obtained from the *MGS* values ranged from 0.778 to 0.900 (Table 2). About 40% of RAPD loci on average were distinguishable between individuals of different localities (Table 1). It is likely that the bulked RAPD analysis obscures local specificities, although the method makes it possible to compare large numbers of specimens (Yu and Pauls, 1993). The relatedness between the OG and TH populations was the most distant among the combinations of the 7 populations. The analyses at the individual level also indicated the relatively large distance between the 2 populations (Table 1). This indicates that the OG and TH populations are the best candidates for outbreeding.

Genetic Status of the Wild Populations

To examine the genetic status of wild populations, genetic similarities were estimated for the OG, SY, TH, MS, and MD populations, although their *MGS* values ranged from 0.551 to 0.703 (Figure 5). An average of about 216 RAPD markers were obtained from the 5 wild populations. The *MGS* within the TH population was the highest among them, although there was no significant difference between the *MGS*s within the TH and MS populations. Similarly, there was no significant difference between the *MGS*s within the OG and MD populations. The differences between the other combinations of 2 populations were significant ($P < .001$). The findings suggest that the TH and MS populations were less variable genetically than the other populations, and that the SY population was the most variable among the 5 populations. Using the TH or MS population as founders, it is expected that the establishment of strains would be relatively fast because characterization of the genetic constitution would be relatively easy.

Genetic Status of the Inbred Line

Pilot inbreeding was carried out to establish an inbred strain. Some individuals from Onagawa were used as the founders for the following reasons. As mentioned above, the OG population was one of the candidates for outbreeding. A steady supply of specimens can be collected from Onagawa in every season. Furthermore, individuals from On-

agawa produce more zygotes than those from the other sites because of their bigger body size. In the present study, a pedigree, which was named "OG-A," was inbred for 5 generations (Figure 2).

F_2 progeny of OG-A were easy to produce. However, most F_3 embryos were lethal. Only two F_3 individuals produced many F_4 progeny, and the F_4 progeny seldom had matured oocytes. Finally, the OG-A line was terminated. Self-fertilization is said to cause various developmental defects. In the present study, most defects seen in lethal embryos were failures of the morphogenesis of the head or tail of the larvae. In addition, some abnormal phenotypes were observed in adults: decrease in the number of ocellusli of the siphon, a coiled body, closed spermiduct, etc.

These observations correspond to the theoretical genetic status. When the initial heterozygosity is 1.000, the inbreeding coefficient (F) value with self-mating is calculated as 0.500, 0.750, 0.875, 0.938, and 0.959 at F_1, F_2, F_3, F_4, and F_5, respectively. The true F value must be higher because the actual heterozygosity must be lower in wild populations. For an example, the average heterozygosity of 15 isozyme loci was 0.319 in wild specimens from Helgoland (Schmidtke and Engel, 1980). On the basis of this value, the F value is calculated as 0.841, 0.920, 0.960, 0.980, and 0.990 at F_1, F_2, F_3, F_4, and F_5, respectively. These estimates suggest that recessive loci are likely to appear as phenotypes starting in the third generations. Thus some recessive lethal loci might have caused the inbreeding depression in the present study.

The genetic status of each generation of the inbred line was monitored with RAPD markers. The fingerprints among individuals of OG-A4 were more similar than those among wild specimens, although some polymorphic loci still remained at the fourth generation (Figure 6). The number of RAPD loci of the inbred line decreased during inbreeding (Table 3). This suggests that dominant negative homozygotic loci, which might be segregated from heterozygotic loci, increase during inbreeding. The ratio of monomorphic markers of the pedigree ranged from 6.0% to 14.5%. These markers might indicate homozygotic loci ($P < .01$). In particular, the RAPD markers that were monomorphic throughout the 4 generations are considered to be homozygotic loci fixed in the inbred line.

Genetic similarities of each generation of the inbred line were monitored during the inbreeding and compared with those of 5 wild populations (Figure 5). The results indicated that the genetic similarity of the inbred line became higher than those of the wild populations. The *MGS*

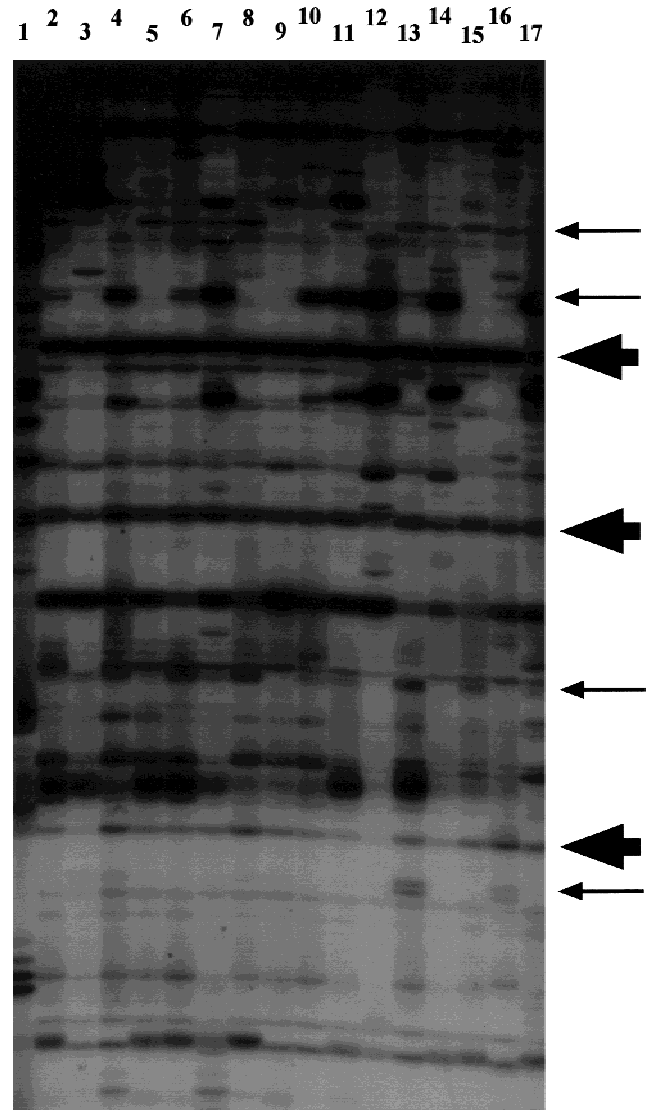

Figure 6. AP-PCR fingerprint of OG-A line. Arrowheads indicate some examples of monomorphic markers throughout 4 generations, which could imply homozygotic loci. Arrows indicate polymorphic markers. Lane 1, OG-A0 (the founder of the OG-A line); lane 2, OG-A1 (the parent of OG-A2); lane 3, OG-A2 (the parent of OG-A3); lane 4, OG-A3 (the parent of OG-A4); lanes 5–17, 13 specimens of OG-A4.

value of OG-A1 was significantly higher than those of all wild populations, including the OG population ($P < .001$). The *MGS* of OG-A2 was greater than that of OG-A1 ($P < .001$); however, no significant difference was recognized between the *MGS* of OG-A2 and that of OG-A4. This might have been caused by a slower rate of increase in the inbreeding coefficient after the F_2 generation. These data suggest that the inbreeding by self-mating rapidly increases genetic homogeneity.

Table 3. The Number and Ratio of Monomorphic Loci Revealed by AP-PCR

	Number of monomorphic loci	Percentage of monomorphic loci	Total number of loci	*n*
OG-wild	13	6.0	215	20
OG-A1	20	9.2	217	18
OG-A2	14	9.1	154	24
OG-A3	59	53.6	110	2
OG-A4	21	14.5	145	13

Prospects for the Establishment of Strains and Genetic Approaches

More successive inbreeding would be necessary to establish an inbred strain; however, most pedigrees were in fact terminated at F_4 or F_5. For establishment of stable strains without inbreeding depression, some lethal loci must be excluded. Large-scale breeding is one means of achieving this purpose, although it requires a large amount of labor and a place to breed. Practically, not only inbreeding but also closed breeding can be used to establish strains after the genetic homogeneity is increased to that of the fourth generation. The genetic difference between the OG and TH populations is sufficient to distinguish them from each other for genetic analysis. Using these 2 populations, we are in the process of developing polymorphic lines that will be used for future genetic mapping. Such RAPD markers as those identified in the present study are useful for the purposes mentioned above.

Ascidians have stable cell lineages that show no differences among individuals. Thus most ascidian embryologists have cared little about the genetic status of the animals. We emphasize that the "genetic polymorphisms" of *C. intestinalis* will be useful for developmental genetics of ascidians in the near future.

Acknowledgments

We thank the Education and Research Center of Marine Bio-resources, Tohoku University, Mukaishima Marine Biological Laboratory, Hiroshima University, and Misaki Marine Biological Station (MMBS), Tokyo University, for collecting wild ascidian specimens. We thank the Ootsuchi Marine Research Center, Tokyo University, for providing us with a laboratory to breed ascidians. We are grateful to Mr. M. Sekifuji (MMBS) and Mr. N. Sensui (Ryukyu University) for useful advice on how to breed *C. intestinalis*. We also thank Dr. Hironori Wada (The Institute of Physical and Chemical Research) for his helpful discussions and for technical help with the AP-PCR technique and Dr. William Bates (Okanagan University College, Canada) for critical reading of the manuscript.

References

Aljanabi, S.M., and Martinez, I. (1997). Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucleic Acids Res* 25:4692–4693.

Di Gregorio, A., and Levine, M. (1998). Ascidian embryogenesis and the origins of the chordate body plan. *Curr Opin Genet Dev* 8:457–463.

Felsenstein, J. (1993). PHYLIP: Phylogeny Inference Package, 3.5c, Seattle, University of Washington.

Holland, P., Garcia-Fernàndez, J., Williams, N., and Sidow, A. (1994). Gene duplications and the origins of vertebrate development. *Development Suppl* 125–133.

Hoshino, Z., and Nishikawa, T. (1985). Taxonomic studies of *Ciona intestinalis* (L.) and its allies. *Publ Seto Mar Biol Lab* 30:61–79.

Kawamura, K., Fujita, H., and Nakauchi, M. (1987). Cytological characterization of self incompatibility in gametes of the ascidian, *Ciona intestinalis. Dev Growth Differ* 29:627–642.

Matsumoto, M., Shimada, T., and Hoshi, M. (1992). Genetical variability in rRNA gene in *Ciona. Zool Sci* 9:1304.

Morgan, T.H. (1904). Self-fertilization induced by artificial means. *J Exp Zool* 1:135–178.

Nei, M., and Li, W.H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc Natl Acad Sci USA* 76:5269–5273.

Satoh, N. (1994). *Developmental Biology of Ascidians.* New York: Cambridge University Press.

Satoh, N., and Jeffery, W.R. (1995). Chasing tails in ascidians: developmental insights into the origin and evolution of chordates. *Trends Genet* 11:354–359.

Schmidtke, J., and Engel, W. (1980). Gene diversity in tunicate populations. *Biochem Genet* 18:503–508.

Scott, M.P., Haymes, K.M., and Williams, S.M. (1992). Parentage analysis using RAPD PCR. *Nucleic Acids Res* 20:5493.

Simmen, M.W., Leitgeb, S., Clark, V.H., Jones, S.J.M., and Bird, A. (1998). Gene number in an invertebrate chordate, *Ciona intestinalis. Proc Natl Acad Sci USA* 95:4437–4440.

Sordino, P., Heisenberg, C.P., Carino, P., Toscano, A., Giuliano, P., Marino, R., Pinto, M.R., and De Santis, R. (2000). A mutational approach to studying development of the protochordate *Ciona intestinalis* (Tunicata, Chordata) *SARSIA* 85:173–176.

Swofford, D.L., Olsen, G.J., Waddell, P.J., and Hills, D.M. (1996). *Phylogenetic Inference. Molecular Systematics, 2nd ed.* Sunderland, Mass.: Sinauer Associates, Inc.

Taylor, K.M. (1967). The chromosomes of some lower chordates. *Chromosoma* 21:181–188.

Wada, H., Naruse, K., Shimada, A., and Shima, A. (1995). Genetic linkage map of a fish, the Japanese medaka *Oryzias latipes. Mol Mar Biol Biotechnol* 4:269–274.

Welsh, J., and McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrarily primers. *Nucleic Acids Res* 18:7213–7218.

Williams, J.G.K., Hanafey, M.K., Rafalski, J.A., and Tingey, S.V. (1993). Genetic analysis using random amplified polymorphic DNA markers. *Methods Enzymol* 218:704–740.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., and Tingey, S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* 18:6531–6535.

Yu, K., and Pauls, K.P. (1993). Rapid estimation of genetic relatedness among heterogeneous populations of alfalfa by random amplification of bulked genomic DNA samples. *Theor Appl Genet* 86:788–794.