

Primary Genetic Linkage Maps of the Ascidian, *Ciona intestinalis*

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For whole-genome analysis in a basal chordate (protochordate), we used F1 pseudo-testcross mapping strategy and amplified fragment length polymorphism (AFLP) markers to construct primary linkage maps of the ascidian tunicate *Ciona intestinalis*. Two genetic maps consisted of 14 linkage groups, in agreement with the haploid chromosome number, and contained 276 and 125 AFLP loci derived from crosses between British and Neapolitan individuals. The two maps covered 4218.9 and 2086.9 cM, respectively, with an average marker interval of 16.1 and 18.9 cM. We observed a high recombinant ratio, ranging from 25 to 49 kb/cM, which can explain the high degree of polymorphism in this species. Some AFLP markers were converted to sequence tagged sites (STSs) by sequence determination, in order to create anchor markers for the fragmental physical map. Our recombination tools provide basic knowledge of genetic status and whole genome organization, and genetic markers to assist positional cloning in *C. intestinalis*.

Key words: genetic mapping, polymorphism, recombination rate, ascidian, AFLP

INTRODUCTION

The ascidian *Ciona intestinalis* is a living descendant of stem chordates, and an excellent model organism to investigate evolution of gene functions and genome structure, with rich genomic and cDNA/EST resources together with an effective system for developmental biology (reviewed by Satoh *et al.*, 2003). For functional analysis, the mutant approach has recently arisen as a powerful means for molecular embryology in *Ciona* species (Sordino *et al.*, 2001; Jiang *et al.*, 2005). While techniques for insertional mutagenesis and enhancer traps are available in ascidians using the *Minos* transposon (Sasakura *et al.*, 2003; Awazu *et al.*, 2004) and *I-SceI* endonuclease (Deschet *et al.*, 2003), spontaneous and chemically induced mutants can provide us with more phenotypes.

Linkage maps are central to positional cloning in *C. intestinalis*, as isolation of genes involved in mutant phenotypes depends on development of genetic infrastructures. However, a high degree of polymorphism and the lack of laboratory strains have caused unstable results during mapping efforts, especially due to primer incompatibility of genetic markers established in non-coding regions such as microsatellites. To solve these issues, the amplified fragment length polymorphism (AFLP; Vos *et al.*, 1995) technique was chosen. The AFLP technique has been widely used to construct primary genetic maps in many organisms, such as medaka (Naruse *et al.*, 2000), Pacific oyster (Li and

Guo, 2004), and plants (*Eucalyptus*: Myburg *et al.*, 2003; *Arabidopsis*: Peters *et al.*, 2004). Since *C. intestinalis* contains high levels of polymorphisms among local populations (Procaccini *et al.*, 2000; Kano *et al.*, 2001; Boffelli *et al.*, 2004), we applied the F1 pseudo-testcross strategy that takes advantage of parental heterozygosity (Grattapaglia and Sederoff, 1994), instead of generating F2 intercrosses or backcrosses. This strategy offered a solution for PCR failure caused by polymorphisms. Recently, pseudo-testcross combined with AFLPs has been applied to aquatic animals, the Pacific oyster (Li and Guo, 2004) and the channel catfish (Liu *et al.*, 2003), allowing quick mapping. Conversion from AFLPs to sequence tagged sites (STSs) may provide us with co-dominant markers (Meksem *et al.*, 2001), which enables researchers to share anonymous markers like AFLPs. This approach can establish entry gates for *in silico* explorations of candidate genes in the *Ciona* genome database and anchor loci for assembly of the genome, which still consists of fragmental scaffolds due to polymorphisms (Dehal *et al.*, 2002). Using genetic variability, we report the first construction of two genome-wide linkage maps of the model species *C. intestinalis* as a new platform to advance *Ciona* genetics and genomics.

MATERIALS AND METHODS

Crossing panel for linkage maps

Individuals derived from British (southern Plymouth, United Kingdom) and Neapolitan (Fusaro lagoon, Naples, Italy) populations were used for crossing panels by virtue of their genetic differences (see Results). F1 progeny were obtained by crossing eggs from a wild British individual with sperm from a wild Neapolitan individual, since this combination showed no significant difference in fertilization ratio if compared with a reciprocal cross. Fifty speci-

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mens were used for the pseudo-testcross F1 mapping. Animals were reared according to Cirino *et al.* (2002) and Sasakura *et al.* (2003), with some modifications.

DNA isolation

Genomic DNA was extracted as previously described (Kano *et al.*, 2001), with minor modifications. Each tissue from the body wall muscle of an adult specimen was lysed for 4 hr in 0.4 M NaCl, 10 mM Tris-HCl pH 8.0, 2 mM EDTA, 2% SDS, and 8 M Urea, and incubated at 95°C for 10 min. After ethanol precipitation, the CTAB procedure was carried out at 37°C instead of 65°C to avoid loss of yield, as suggested by Shahjahan *et al.* (1995). The extracted DNA was of high molecular weight and good for restriction enzymes digestions in the AFLP procedure.

AFLP fingerprints

AFLP fingerprints were generated using the AFLP Analysis System II kit (Invitrogen), *Taq* DNA polymerase (5 units/μl, Roche Applied Science), and a PCR Express DNA thermal cycler (Hybaid) according to manufacturers' instructions. For selective amplifications, 64 primer combinations supplied in the kit were previously tested on a single *C. intestinalis* specimen and AFLP fingerprints visualized with ethidium bromide staining. The efficacy of primer combinations was evaluated based on the sharpness, number, and size of fingerprint bands. Fifteen primer combinations that generated discrete bands ranging from 100 to 500 bp were chosen for the present study (Supplemental Table 1). The preamplification step was performed for 20 cycles as follows: 94°C for 30 sec, 56°C for 60 sec, and 72°C for 60 sec. Prior to PCR, the *EcoRI* primer was end-labeled with [γ -³²P] ATP (3,000 Ci/mmol; Amersham Bioscience), and the selective amplification was performed with a touchdown program for 13 cycles: 94°C for 30 sec, 65°C for 30 sec, and 72°C for 60 sec, with a 0.7°C decrease of annealing temperature each cycle, followed by 23 cycles of amplification at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 60 sec. A total of 4 μl of each sample was loaded on a 6% denaturing polyacrylamide gel and run

for 2 hr at 40 W. AFLP fingerprints were visualized by autoradiography using X-ray film (Kodak Photo Film Co. LTD). PCR was carried out two or three times using the same pre-amplified reactions, and only reproducible markers were used for further analyses. To clearly separate some close markers, electrophoresis was performed for 1.5 hr at 55 W. Markers were manually scored for presence or absence of bands with comparable gel mobility. Each marker was named B (British) or N (Neapolitan), followed by the number of the primer combination and by the product number in decreasing order of fingerprint size. An initial "d" was added to the names of "distorted" markers (see below).

Linkage analyses

Genotyping was performed on each separate data set for the maternal British and the paternal Neapolitan markers. To allow the detection of linkage in repulsion phase, duplicate data sets were prepared (Grattapaglia and Sederoff, 1994). Markers were classified into Mendelian or non-Mendelian (= "distorted") markers using the chi-square test for backcross manner (1:1 ratios) at $P < 0.05$. Extremely distorted markers ($P < 0.01$) were discarded. Only Mendelian markers were used for initial framework maps developed with MAPL98 (Ukai *et al.*, 1991) and Mapmaker II software (Lander *et al.*, 1987). Grouping was carried out manually using the nearest neighboring loci method in MAPL98, with a LOD score of 3.00 and a recombination frequency threshold of 0.26. Unlinked markers below the threshold values were mapped using the TwoPoint/Near and MultiPoint/TRY commands in Mapmaker II with lower thresholds (LOD = 1.44 and recombination frequency = 0.32).

To construct the final maps, non-Mendelian markers were mapped onto the initial framework maps in the same way as for Mendelian markers. Some non-Mendelian markers that disintegrated the initial framework were discarded from further analyses. Genetic distances were estimated using the Kosambi function. The maps derived from the two parents were not merged into an integrated map, by using markers showing intercross manner, because of the limited number of F1 progeny analyzed.

Table 1. Summary of the number of AFLP markers generated from each primer set

	Common Markers			British Markers			Neapolitan Markers			total
	Mono	Mendelian	Distorted	Mono	Mendelian	Distorted	Mono	Mendelian	Distorted	
AFLP#01	1	0	1	2	6	14	3	5	3	35
AFLP#02	2	0	3	1	21	19	3	4	8	61
AFLP#03	1	2	3	1	21	7	6	6	0	47
AFLP#04	3	3	10	1	18	18	3	18	10	84
AFLP#05	1	1	2	2	13	11	8	5	6	49
AFLP#06	1	1	1	1	8	4	2	6	2	26
AFLP#07	2	3	7	1	21	15	3	7	6	65
AFLP#08	1	3	4	0	12	9	1	3	9	42
AFLP#09	1	1	0	3	16	14	3	4	2	44
AFLP#10	0	0	0	1	11	2	2	4	1	21
AFLP#11	1	0	1	0	8	6	4	3	2	25
AFLP#12	5	0	1	7	8	9	5	5	2	42
AFLP#13	3	1	1	4	16	15	6	4	11	61
AFLP#14	2	1	1	2	15	7	6	2	7	43
AFLP#15	4	3	1	1	7	2	2	2	3	25
total	28	19	36	27	201	152	57	78	72	670

Primer combinations for each primer set are indicated in Supplemental Table 1. Mono, Mendelian, and Distorted mean Monomorphic, Mendelian Polymorphic, and Distorted Polymorphic markers, respectively.

Estimation of genome length and coverage

Measures of the total lengths for the initial framework and final maps (*Goi* and *Gof*) were based on the sum of each linkage group. Estimation of genome length (*Ge*) and expected map coverages (*Cei* and *Cef*) were estimated according to Tani *et al.* (2003). A minimum LOD score of 3.08 was chosen to estimate the genome length.

Conversion of AFLP to STS

The target band was cut out from the dried polyacrylamide gel and dissolved in 200 μ l of TE buffer for one hour, followed by incubation at 80°C for 15 min. The mix was centrifuged at 15K rpm for

5 min, and supernatant was placed into another tube. The PCR product was precipitated with 1/50 volume of 5 M NaCl and glycogen in ethanol, and dissolved in 15 μ l of sterile water. Of this, 5 μ l were used for re-amplification with the same touchdown program for AFLP in a total volume of 50 μ l. The PCR product was collected using a low melting point gel and cloned using TOPO TA (Invitrogen). Insert fragments of 14 clones were checked, and four of them were sequenced. If all four clones did not have homologous sequences, the entire process was repeated. Markers that represented multiple sequences, even after double experiments, were discarded from the map and further analyses.

Sequences were analyzed and determined for scaffold location

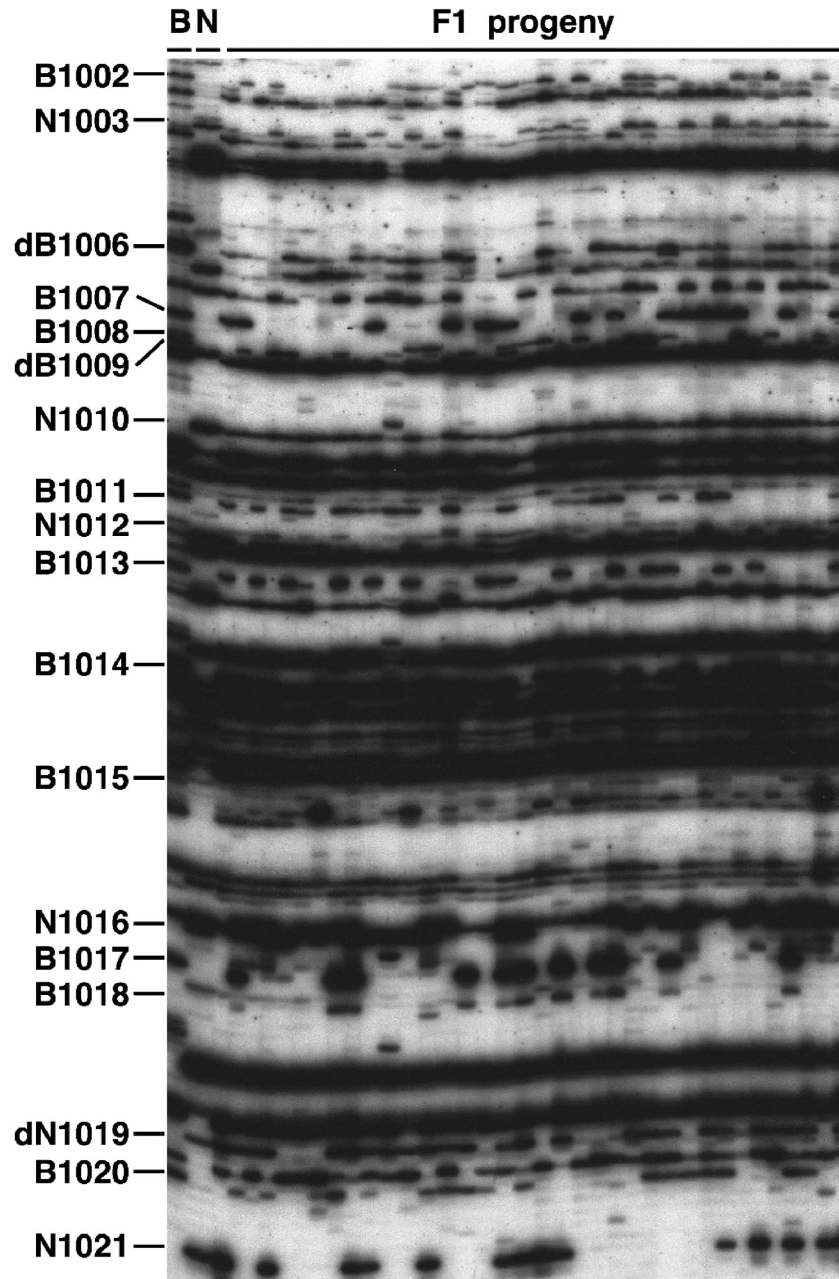


Fig. 1. An example of AFLP gel electrophoresis using primer combination No. 10. The first two lanes represent the two parents (B, British; N, Neapolitan), and the other lanes are fingerprints of F1 progeny. Reproducible markers from several experiments were analyzed, and only polymorphic markers are indicated in this figure, except for one example of a monomorphic marker, N1010. A marker located above the gel image, B1001, is not shown due to size limitation of the figure. One marker, B1018, was analyzed because repeatability was confirmed although the parental band is too weak to be seen in this figure.

using a genome browser, *C. intestinalis* v1.0 by the Joint Genome Institute (JGI; <http://genome.jgi-psf.org/ciona4/>; Dehal *et al.*, 2002). A sequence found in a unique scaffold was presumed to correspond to a single site. Positions on chromosomes for the corre-

sponding scaffolds were confirmed using the Ghost database (<http://hoya.zool.kyoto-u.ac.jp/chromosomeall.html>; Satou *et al.*, 2005). The STS sequences were deposited into GenBank as B607 (AY879054), B608 (AY879055), and B0606d2 (DQ178661).

Table 2. Estimations of genome lengths and map coverages

	the British map	the Neapolitan map
Genome length		
Observed		
<i>Gof</i> (<i>Go</i>) [cM]	4218.9 (2624.5)	2086.9 (980.8)
Estimated		
<i>Ge</i> cM	6199.8	3206.0
Lower bound [cM]	5929.4	2908.2
Higher bound [cM]	6448.5	3457.2
Genome coverage for <i>Cef</i> (<i>Ce</i>) [%]	74.1 (51.7)	69.2 (45.6)

Abbreviations: *Gof*, total observed genome length of the final map; *Go*, total observed genome length of the initial framework map; *Ge*, Estimated genome length; *Cef*, Coverage of estimated genome length of the final map; *Ce*, Coverage of estimated genome length of the initial map.

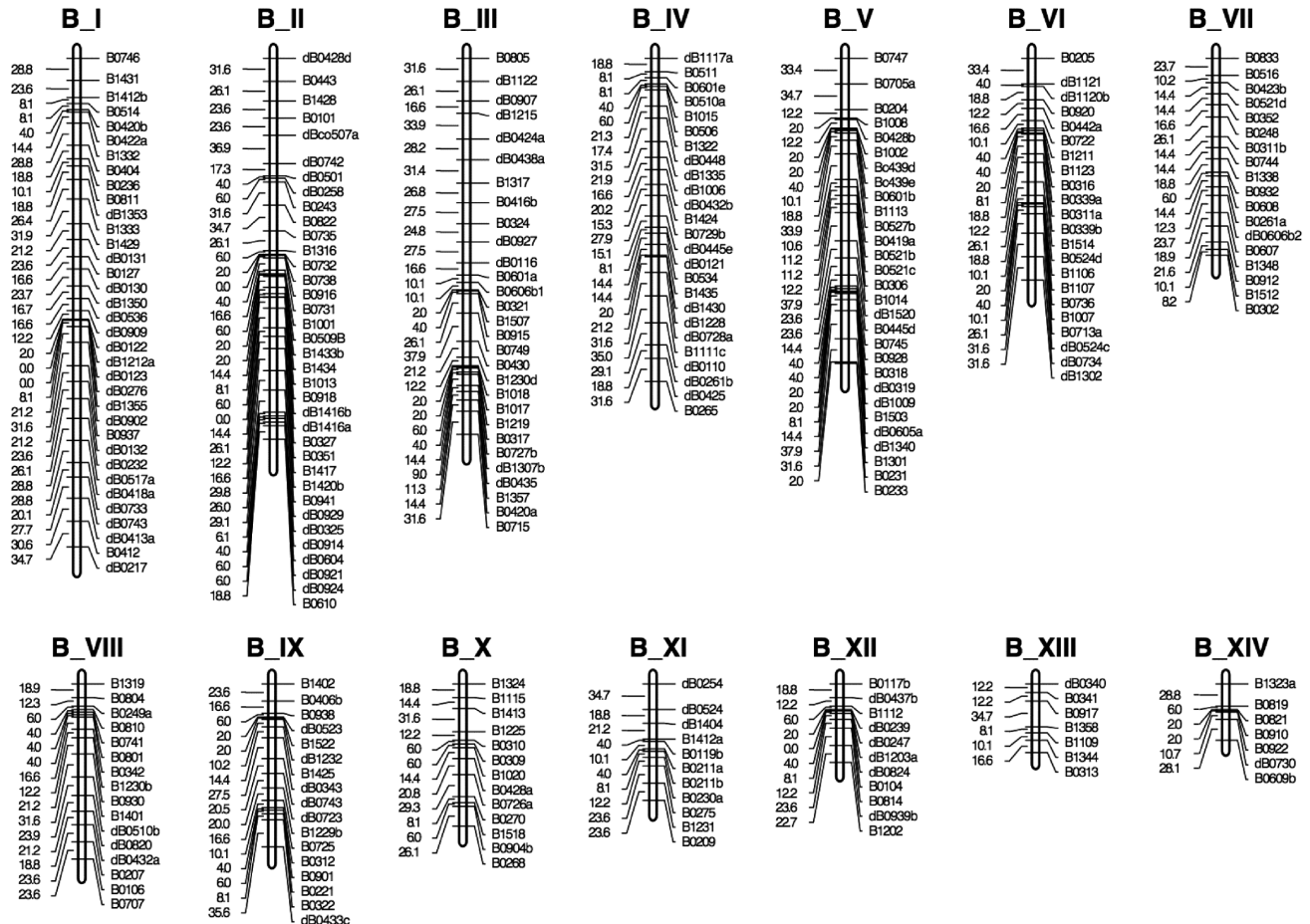


Fig. 2. Genetic linkage map of the ascidian *Ciona intestinalis* derived from the British individual. We mapped 276 AFLPs covering 4218.9 cM (Kosambi mapping function), using 50 F1 progeny. The 14 linkage groups were ordered and numbered on the basis of size. Map distance of each interval is described in centimorgans. Each marker was named B (British), followed by the number of the primer combination and by the product number in decreasing order of fingerprint size. “Distorted” markers are indicated with the initial letter “d”.

RESULTS

Primer combinations and AFLP markers

To begin with, primer screening was performed without

radioisotope labeling, and each combination was evaluated visually as described in Materials and Methods (Supplemental Table 1). Fifteen AFLP primer combinations generated 670 markers in total, ranging in number from 21 to 84 with

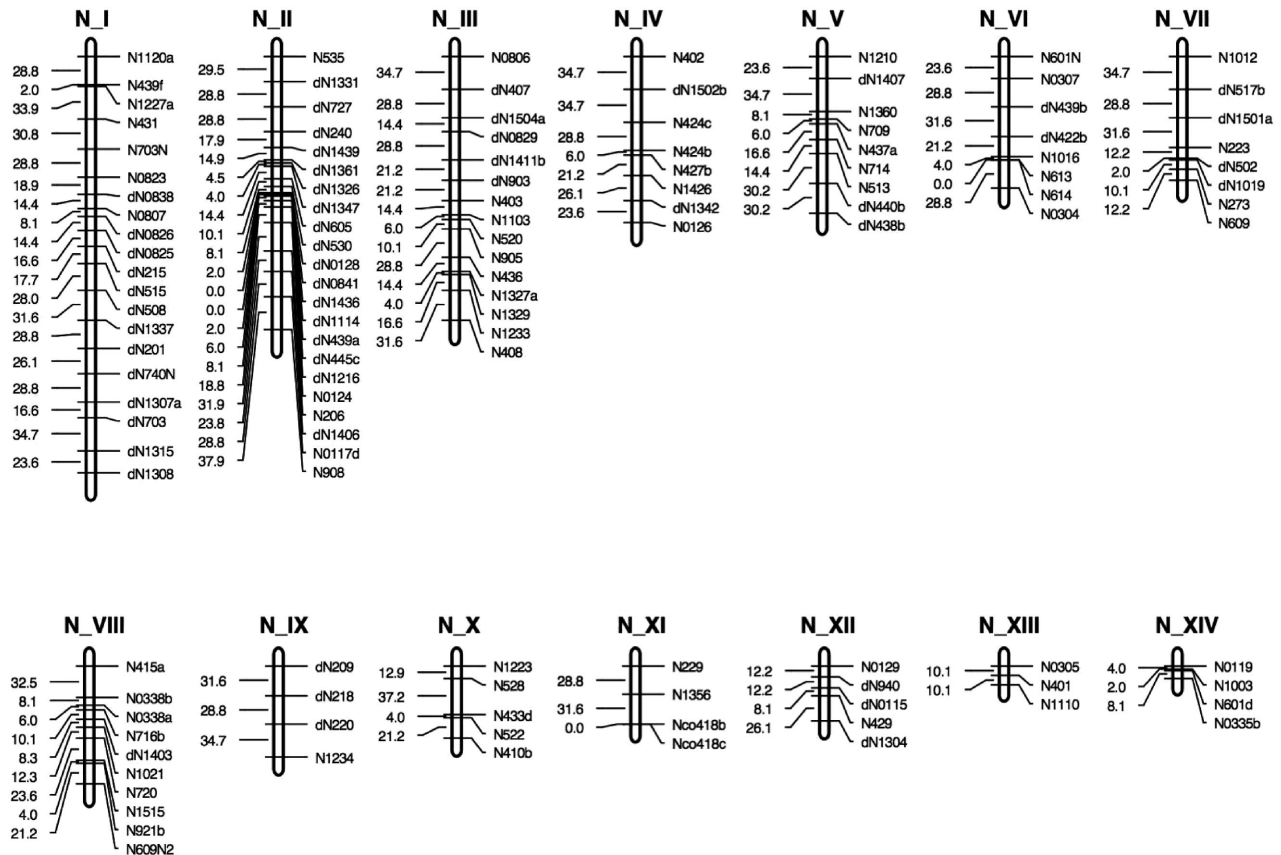


Fig. 3. Genetic linkage map of *C. intestinalis* derived from the Neapolitan individual. A total of 125 AFLPs were mapped, using the same cross panel shown in Fig. 1, covering 2086.9 cM. Each N marker (Neapolitan) was named as in the British map (Fig. 2).

Table 3. Distribution of markers and lengths for each linkage group in the final maps

the British map			the Neapolitan map		
LG	Length (cM)	Total no. loci	LG	Length (cM)	Total no. loci
B_I	657.0	35	N_I	432.7	20
B_II	524.0	36	N_II	320.4	22
B_III	509.0	29	N_III	275.0	15
B_IV	438.5	25	N_IV	175.1	8
B_V	416.2	29	N_V	163.8	9
B_VI	305.1	22	N_VI	138.1	8
B_VII	268.3	18	N_VII	131.7	8
B_VIII	242.0	16	N_VIII	126.1	10
B_IX	223.2	17	N_IX	95.1	4
B_X	193.9	13	N_X	75.3	5
B_XI	160.4	11	N_XI	60.5	4
B_XII	109.8	11	N_XII	58.7	5
B_XIII	93.9	7	N_XIII	20.3	3
B_XIV	77.6	7	N_XIV	14.1	4
total	4218.9	276	total	2086.9	125

an average of 44.2 per primer combination (Table 1). Of these, 380 (56.7%) and 207 (30.9%) markers were derived from the British and Neapolitan specimens, respectively. The remaining 83 (12.4%) AFLP markers were in common, indicating that the genetic difference between the two populations was effective and useful for genetic analyses (Fig. 1). The number of British markers was almost twice that of Neapolitan ones, and this tendency was observed for every primer combination, with statistical significance ($P < 0.01$, chi-square test). Of the parent-specific markers, 303 British and 134 Neapolitan polymorphic markers were considered for linkage analyses after extremely distorted markers were discarded.

Genetic maps

Grouping using the nearest neighboring loci method in MAPL98 provided reasonable results, whereas grouping based only on the LOD threshold and the frequency values resulted in fragmentation of linkage groups. *Ciona intestinalis* is hermaphroditic, with 14 chromosomes per haploid genome (Shoguchi *et al.*, 2005); thus, we expected the markers to be distributed over 14 linkage groups. Using only Mendelian markers in the initial framework maps, British markers grouped into 16 linkage groups, and Neapolitan markers into 8 linkage groups, 2 triplets, and 2 doublets, covering 2624.5 and 980.8 cM, respectively (*Gof* in Table 2). Twelve (6.0%) of 201 British and 14 (17.9%) of 78 Neapolitan Mendelian markers remained unlinked. In total, 85.0% (British) and 67.9% (Neapolitan) of marker linkages showed tight linkage ($\text{LOD} > 3.0$). The average interval in genetic distance was 15.1 cM in the British and 18.5 cM in the Neapolitan initial framework maps. From the partial linkage data of the initial framework maps, we calculated genome length (*Ge*) in genetic distance as 6199.8 cM and 3206.0 cM, respectively, for the British and Neapolitan maps. Comparably, coverage of genome length (*Cef*) was 51.7% (British) and 45.6% (Neapolitan).

In the final maps including non-Mendelian markers (distorted markers; see Materials and Methods), 276 British markers grouped into 14 linkage groups covering 4218.9 cM (*Gof* in Table 2 and Fig. 2). Likewise, 125 Neapolitan markers generated 13 linkage groups and one triplet, corresponding to 2086.9 cM (*Gof* in Table 2 and Fig. 3). While some Mendelian markers were unlinked in the initial maps, they could be assigned to the final maps by increasing the total number of markers with non-Mendelian loci. Only 27 (8.9%) of 303 markers were unlinked in the final British map, and nine (6.7%) of 134 in the Neapolitan one. Lengths of linkage groups in the final maps varied to a large extent, from 77.6 to 657.0 cM (British), and from 14.1 to 432.7 cM (Neapolitan) (Table 3). Coverage (*Cef*) was estimated as 74.1 and 68.7% for the British and Neapolitan total maps, respectively (Table 2). Since any small chromosomes should be at least 50 cM in size due to an obligate chiasma (Henderson, 1963), two linkage groups in the Neapolitan map, LG_N_XIII (37.8 cM) and LG_N_XIV (19.3 cM), can be further expanded. Based on the coverages, approximately 370 British and 200 Neapolitan markers are required to cover the whole genome in the two final maps.

Some loci displayed cluster distributions, with higher marker densities than those for the entire final maps (Fig. 2

and 3). For instance, the average interval size was 5.2 cM in the lower part of LG_B_III and 5.4 cM in a central region of LG_N_II. Some clusters may correspond to centromeres, known to be recombinationally suppressed (Stephan and Mitchell, 1992), such as the region around the marker B0211a in LG_B_XI, which was composed of Mendelian markers at high density. Conversely, clusters of distorted markers were observed in some linkage groups, such as in the lower part of LG_B_II and LG_B_III. Interestingly, distorted markers occurred in the central and slightly upper region of LG_N_II, around markers dN530 and dN1326, and in LG_B_II, around markers B1011 and B0732, suggesting that the two linkage groups could be homologous. Such observations might be associated with biological traits rather than experimental artifacts (see Discussion). However, this hypothesis does not always apply, since distorted markers occurred over broad regions (*e.g.*, LG_B_I, LG_B_IV, and LG_N_I).

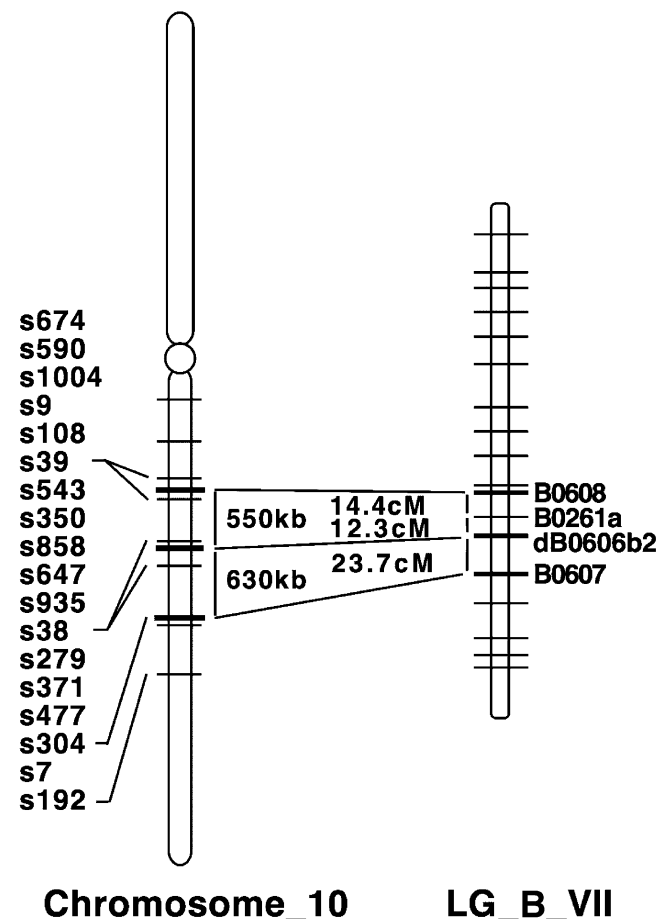


Fig. 4. Correlation between a chromosome No. 10 (referring to the Ghost database) and linkage group B_VII (Fig. 1). Three STS markers, B608, dB606b2, and B607 (Supplemental Table 2), corresponded to three scaffolds (bold lines in chromosome 10) in the same order (bold lines in LG_VII). A marker lying among the three, B0261a, was too short a fragment to convert. Length of the linkage group was adjusted to approximately 60% of the chromosome size, since coverage of the linkage group was estimated at that ratio with the present markers.

Correlation of physical to genetic distance

Considering that the physical genome size is approximately 156 Mb (Dehal *et al.*, 2002), the average ratios of physical to genetic distance were calculated to be 25.2 kb/cM for the British map and 48.7 kb/cM for the Neapolitan map. To directly calculate this correlation, the physical map was compared with the linkage maps through STS markers converted from AFLP markers. Three AFLPs on LG_B_VII, which showed a similar average interval and no clustering in the entire map, were converted to determine corresponding physical scaffolds (Supplemental Table 2). The three corresponding STSs were found to be located on chromosome 10 (*sensu* the Ghost database), indicating that linkage group B_VII corresponds to chromosome 10. The genetic distance corresponded to 50.4 cM, while the physical distance was 1.18 Mb (Fig. 4), leading to a correlation of genetic to physical distance of 23.1~45.2 kb/cM. Based on this range of values and the estimated genome length G_e , the entire genome length was calculated as 137.1~268.3 Mb, which is indeed in agreement with *C. intestinalis* genome length (156 Mb). Hence, the observed recombination values are realistic.

DISCUSSION

Genetic backgrounds and chromosome structures

The AFLP markers obtained were sufficient in number to construct genetic maps, indicating that genetic differences between the two geographically separate populations were sufficient to provide new markers, and that heterozygosities of the parents were high enough to use the F1 pseudo-testcross strategy. We note that the estimated genome size (G_e) of the British map was larger. One possible explanation is that the recombinant ratio varies between female and male gametes. In insects and vertebrates, female gametes show higher recombination than male ones (Yasukochi, 1998; Singer *et al.*, 2002), and a similar phenomenon might occur in *C. intestinalis* as well.

Map analysis revealed some clustered regions consisting of distorted markers, which may be linked with specific biological functions. The genetic backgrounds of the two populations are so distant (as supported by the AFLP data) that genetic incompatibilities could have arisen at various developmental stages. Further, embryos can be negatively selected by environmental factors, for instance, salinity and temperature (Dybern, 1965, 1967). Indeed, British offspring grew poorly in our facility, whereas F1 crosses between British and Neapolitan gametes grew successfully and produced F2 progenies. These observations suggest that the genetic background influences embryonic development and growth, which might be responsible for the observed distortions (Harushima *et al.*, 2001; Myburg *et al.*, 2004). On the other hand, tolerance limits to environmental parameters are so variable that it is hard to define them exactly.

The diploid karyotype of this species contains 20 metacentric and eight submetacentric or subtelocentric chromosomes (Shoguchi *et al.*, 2005). In the present study, we observed several correlations between the karyotype and the linkage groups. Considering that the density of genetic markers likely reveals the presence of a centromere, clusters on LG_B_III, IV, VIII, and IX could reflect submetacentric chromosomes, and LG_B_XI and XIV could be meta-

centric. The very large chromosome No. 1 (*sensu* Shoguchi *et al.*, 2005) might occur in both maps as the largest linkage groups, LG_B_I and LG_N_I, if genetic distance were to reflect physical distance.

Recombination ratio in *Ciona intestinalis*

Linkage analysis of AFLP markers showed that high recombination occurred not in specific genomic sub-regions, but in the entire genome of *C. intestinalis*, indicating that a high recombinant frequency is a normal feature of this genome. The ratio of physical to genetic distance (90 kb/cM) was also low in the *Fu/HC* locus of the tunicate *Botryllus schlosseri* (De Tomaso and Weissman, 2003), suggesting that high recombination may be a common trait of ascidians. It is known that the ratio of physical length to genetic distance has increased during evolution (Catcheside, 1977). For example, this value is 2.32 and 3.84 kb/cM in *E. coli* (Blattner *et al.*, 1997) and yeast (Bussey *et al.*, 1997), and 210, 280, 575, and 770 kb/cM in *Arabidopsis* (Peters *et al.*, 2001), *Drosophila* (Merriam *et al.*, 1991), rice (Harushima *et al.*, 2001) and human (Yu *et al.*, 2001), respectively. Herein, the ratio was estimated to be about 25~49 kb/cM in *C. intestinalis*.

High recombination might be associated with chromosome size and rearrangements. In fact, *C. intestinalis*, with a genome size comparable to that of *Arabidopsis* and *Drosophila*, has 14 pairs of chromosomes, whereas the latter two species have only five and three pairs of chromosomes, respectively (Arabidopsis Genome Initiative, 2000; Adams *et al.*, 2000). Smaller chromosomes should result in a higher average frequency of recombination simply due to chiasma frequency, as in plants (Rees and Durrant, 1986). Thus, the recombinant ratio in *Ciona* should be higher than that in *Arabidopsis* and *Drosophila*, irrespective of overestimation due to dominant markers. Further, evidence of variation in the size of three chromosomes containing ribosomal DNA (rDNA) repeats (Shoguchi *et al.*, 2005) suggests that such repetitive elements are associated with high recombination and genomic mobility.

Together with the effects of large effective population size (Dehal *et al.*, 2002), the recombination rate could also justify the genetic polymorphisms of this species, since crossing-over is known to generate mutations (Lercher and Hurst, 2002; Filatov and Gerrard, 2003). This hypothesis could also explain the persistent polymorphisms occurring in inbreeding lines (Kano *et al.*, 2001).

Applications of the maps in *Ciona* genetics

The main advantage of the F1 pseudo-testcross strategy using AFLP markers was quick construction of genetic maps. The applicative value of co-dominant markers is counterbalanced by efforts required to develop them in cases of a high degree of polymorphism and the absence of laboratory strains, such as in *C. intestinalis*. However, AFLP mapping combined with STS conversion was effective in primary genetic mapping, and then in generating co-dominant markers. Originally derived from the multiallelic polymorphisms due to insertion/deletion and from SNPs at the population and individual levels, STSs can be converted to universal co-dominant markers. In this sense, most marine invertebrates that exhibit a high degree of polymorphism

and large broods, such as the amphioxus *Branchiostoma floridae* and the sea urchin *Strongylocentrotus purpuratus*, carry suitable traits for construction of genetic maps using F1 pseudo-crossing, despite the hurdles of culturing and breeding. Also for these species, whose genome projects are progressing, linkage maps will be crucial for assembling genome fragments, in combination with other physical mapping strategies such as clone-array pooled shotgun sequencing (CAPSS), FISH (Shoguchi *et al.*, 2004), and integrated databases of genomic and cDNA data (*e.g.*, Ghost database; Satou *et al.*, 2005).

In the present study, our genetic maps revealed the genome structure of the ascidian *C. intestinalis* and provided information on genetic loci using widely distributed AFLP markers. The high recombinant ratio may be a trait advantageous for enabling us to carry out fine mapping with a relatively small number of progeny. Information derived from STS markers will contribute to the integration of genetic and physical maps. The cross-panel material gives increasingly numerous AFLP markers, providing a STS resource to study mutant loci. Investigation of distorted genomic regions, which could be associated with physiological events and lethal loci, together with analysis of quantitative trait loci (QTL) associated with growth rate, will help in the establishment of stable laboratory strains.

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