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Differences in recombination frequencies during female and male meioses of the sex chromosomes of the medaka, *Oryzias latipes*

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Summary

In the medaka, *Oryzias latipes*, sex is determined chromosomally. The sex chromosomes differ from those of mammals in that the X and Y chromosomes are highly homologous. Using backcross panels for linkage analysis, we mapped 21 sequence tagged site (STS) markers on the sex chromosomes (linkage group 1). The genetic map of the sex chromosome was established using male and female meioses. The genetic length of the sex chromosome was shorter in male than in female meioses. The region where male recombination is suppressed is the region close to the sex-determining gene y, while female recombination was suppressed in both the telomeric regions. The restriction in recombination does not occur uniformly on the sex chromosome, as the genetic map distances of the markers are not proportional in male and female recombination. Thus, this observation seems to support the hypothesis that the heterogeneous sex chromosomes were derived from suppression of recombination between autosomal chromosomes. In two of the markers, Yc-2 and Casp6, which were expressed sequence-tagged (EST) sites, polymorphisms of both X and Y chromosomes were detected. The alleles of the X and Y chromosomes were also detected in *O. curvinotus*, a species related to the medaka. These markers could be used for genotyping the sex chromosomes in the medaka and other species, and could be used in other studies on sex chromosomes.

1. Introduction

The medaka, *Oryzias latipes*, is a small freshwater teleost, native to Asian countries such as Japan, Korea and China (Yamamoto, 1975; Naruse *et al*., 1994). This fish has been widely used as an experimental animal because of its rather compact genome, availability of mutant strains, ease of maintenance and degree of genetic variation. Importantly, the shapes of the dorsal and ventral fins of males differ from those of females, allowing one to distinguish males from females on the basis of fin morphology. The medaka was the first fish in which Mendel's laws were shown to be valid and in which Y-linked inheritance was demonstrated (reviewed in King & Stansfield, 1997), indicating the feasibility of using this species for studies on sex-chromosome-associated matters. As early as 1921, Aida showed that the male-determining factor gene (Y) was linked with a pigment gene R and suggested the existence of X and Y sex chromosomes. Later, in 1961, Yamamoto showed crossover between the X and Y chromosomes. The genetic map of the sex chromosome can be obtained using backcross panels (Wada *et al*., 1995; Naruse *et al*., 2000), and indicates that these two sex chromosomes should have a highly homologous region.

Although the determination of sex in the medaka is chromosomal, as in mammals, the medaka sex chromosome differs from those of mammals in that the X and Y chromosomes are not morphologically recognizable. In other fish, some species such as platyfish (*Xiphophorus maculatus*) and goldfish (*Carassius auratus*) have an XX/XY sex-determining system, whereas others such as swordtail (*Xiphophorus alverezi*) have a ZZ/ZW system. The sex-determining system is unknown in zebrafish (*Danio rerio*), and is

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not genetically determined. Therefore, the medaka would be a very useful model animal for studying the mechanism of sex determination as well as the evolution of sex chromosomes.

It has been proposed that the X and Y chromosomes may be derived from an ancestral autosomal pair (Ohno, 1967; reviewed in Charlesworth, 1991). In this hypothesis, the divergence is thought to result from suppression of recombination between these chromosomes. Thus, suppression in recombination frequency of autosomal as well as sex chromosomes is an interesting conjecture for the evolution of sex chromosomes. Studies on the recombination frequencies of sex chromosomes of medaka indicated that recombination may be suppressed in males (Yamamoto, 1961; Matsuda *et al*., 1999). However, because very few alleles were used as markers in those studies, this finding might apply only to a restricted region of the sex chromosome. The present study was done to elucidate to what extent recombination may be suppressed, by collecting a large number of DNA markers to scan the sex chromosomes. This study also documents markers that were not aligned, owing to the small number of backcross progenies used in previous study (Naruse *et al*., 2000).

2. Materials and methods

(i) *Strains and genetic crosses*

The AA2 (Shimada & Shima, 1998) and HNI (Hyodo-Taguchi & Sakaizumi, 1993) strains are inbred strains, established from the southern and northern wild populations of the Japanese medaka, *Oryzias latipes*, respectively. These two populations are genetically divergent, and polymorphisms in DNA sequences between them are easily identified (Naruse *et al*., 1994). By crossing AA2 females and (AA2 female × HNI male) F1 males, backcross progeny were obtained allowing us to observe recombination frequencies in meioses of XY males. Thirty-nine cell lines from this backcross series were established (Naruse *et al*., 2000). Similarly, by crossing AA2 males and (AA2 female × HNI male) F1 females, backcross progeny were obtained to permit the study of recombination frequencies in meioses of XX females.

An HNI Y congenic strain was generated as follows. The male progeny from crossing an AA2 female and an (AA2 female × HNI male) F1 male was designated as a BC1 male. This was then crossed with an AA2 female. The male progeny of this cross was selected and designated as BC2. By consecutive crossing(s) of a BC*n* (*n* = number of backcross generation) male to an AA2 female, a congenic strain was established which has an AA2 background, with only a region containing the male sex-determining gene (y) derived from the HNI strain.

(ii) *Sex reversal*

Congenic BC11 male fish were crossed to AA2 females, and the progeny were treated with β-oestradiol at a concentration of 0·1 or 0·5 μg/ml as described by Iwamatsu (1999). XY female fish were then chosen from the treated progeny on the basis of the existence of the HNI allele of the two DNA markers Yc-2 and Casp6, and the phenotypic marker *lf* (leucophore-free). These markers were used as the index to detect the congenic HNI Y chromosome. The XY congenic female fish were crossed to normal AA2 males to produce backcross progeny to allow us to observe recombination frequencies in XY females.

(iii) *Genomic DNA extraction*

Genomic DNA was extracted from the cultured backcross cell lines as described previously (Naruse *et al*., 2000). Genomic DNA from the caudal fin of adult fish or larvae was extracted using either GenomicPrep DNA Extraction Kit (Amersham-Pharmacia Biotech, Piscataway, NJ) or Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) according to the manufacturers' protocols.

(iv) *Markers and linkage analysis*

A total of 47 DNA markers and two phenotypic markers – *lf* (leucophore-free) and *Y* (male sex-determining gene) – were previously mapped on the sex chromosome (linkage group 1, LG1; Naruse *et al*., 2000). Of the previously mapped markers, six AFLP markers and one RAPD marker were converted into sequence-tagged site (STS) markers. Eight supplementary STS markers were mapped. The markers obtained in the present study are as described in Table 1.

Segregation of the markers was analysed by the MAPMAKER Macintosh version 2 software (Lander *et al*., 1987). Thirty-nine to 100 backcross progeny were used for segregation analysis.

(v) *Polymorphism between alleles on the X and Y sex chromosomes*

The EST markers Yc-2 and Casp6 were amplified by PCR using genomic DNA from both female and male fish of either HNI or AA2 strain as templates. All the PCR products were sequenced either directly or after cloning into plasmid vectors. PCR products with or without restriction enzyme digestion were electrophoresed on 12% acrylamide gels to detect the polymorphisms.

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Table 1. *STS markers used in this study*

Gene	Forward primer		Reverse primer		Polymorphism	GenBank Accession No.	Reference
C3-1[a]	C3-1f	GCAGAGATGCTTTGAAT	C3-1r	ATTTTATTCTGCACACATCTTTTACCA	*Hinf* I	AB025575	Naruse *et al.* (2000)
C3-2[a]	C3-1f	GCAGAGATGCTTTGAAT	C3-2r	TTTCCACTGGGCCTTGGTAAAAT	*Hph*I	AB025576	Naruse *et al.* (2000)
Casp3B[a]	Casp3B-f	CAGATTAAACAGCTTTTGACTGATGGT	Casp3B-r	TGACTCAAAAGGATGCAGACGA	in/del	AB032608	Naruse *et al.* (2000)
Casp6[a,c]	casp6-intf 2	TAGCACTTTCACATTTCCAAGC	Medac6R	CGTCTCTCGATGAGAATAGAAACC	*Taq*I	AB047771-4	
dd048[a]	48f	ACGGTTCTTCTCCTGTGGTCTA	48r2	AACAATACAAACTGAGACCCTGC	allele-specific	AB033206	Naruse *et al.* (2000)
ER-1[c]	10-4E	GAATTCAAGTACATCTGCTGCA	10-4M	TTAACACCACCCATCATCTGA	in/del	AB047779	
ER-2[c]	ER2-f	TTAACAACAGTGCCCTCTTGTGG	ER2-r	GAATTCACCAACCTTGTCAAACATG	*Pvu*II	AB047780	
ER-3[c]	ER3-f	TGTGGAGGAGAAATGAATAATAGCG	ER3-r	GAATTCACGAGCTCATGACATCA	*Hae*III	AB047781	
SL1[c]	pHO5.5F	CCTGCAATGGGAAATTATTCTGCTC	pHO5.5RV	CTTTTGTGTCTTTGGTTATGAAACGATG	in/del	AB005037	Matsuda *et al.* (1997)
SL2[c]	pHO5.110F	GCATCTTGAGGTAGCCCATT	pHO5.110RV	CCTGAAATCCACAGAGGCAC	*Alu*I	AB010892	Matsuda *et al.* (1998)
stEM14-6[b]	14-6E	GAATTCAAGCACACATCGTTAGAAC	14-6M	TTAACTCTAAATAGTGCTGCACAATAAATG	in/del	AB047768	
stEM18-2[b]	18-2E	GAATTCACAGTAGATGAGAAAGTGA	18-2r	AGAGATGCCCAGTTTAGCTTC	allele-specific	AB047777	
stEM32-5L[b]	32-5LE	GAATTCACTTGCACTGCAAAG	32-5LM	TTAACTTTGAAAACTGGCCG	allele-specific	AB047770	
stEM47-3[b]	47-3E	GAATTCACGAGCTCATGACATCAC	47-3M	CTGTGGAGGAGAAATGAATAATAGCG	*Nru*I (*Bsp*68I)	AB047775	
stEM8-1[b]	8-1E	GAATTCAACCAGCGTTTTCAAG	8-1M	TAACTTACACAACTGGAGCTGAAGC	in/del	AB047769	
stEM8-8[b]	8-8E	GAATTCAACCTGTTGTCATTC	8-8M	TTAACTTATCTAAATCTACAACTACATATGT	allele-specific	AB047776	
stLCF-2[b]	LCF-2f	CAAATGCCAAGACAAACTCAAA	LCF-2r	ACGGGAGTCCGAAATATTACAA	*Hinc*II	AB047778	
stsOPQ05-1[c]	Q05-1F	AATCTGCCAGGATCCAGTCA	Q05-1R	CCTACGGAGCGGTCATTTCTGTAG	*Hinf*I	AB030363	Ohtsuka *et al.* (1999)
stsOPR04-1[c]	stsOPR04-1F	GCAGGCATCATTCATAATGC	stsOPR04-1R	GAGTTGCTGCAAGGTCAAAG	*Alu*I	AB030364	Ohtsuka *et al.* (1999)
Yc-1[a]	Yc1-f	GGAAAAGGCTTTAAGCGGTAAT	Yc1-r3	TACTGAGCTCAACCAGAGGACA	*Bgl*II	AB033606	Naruse *et al.* (2000)
Yc-2[a]	Yc2-f 3	CGTACACTTTATTCCATCAACTGCG	Yc2-r3	GTAAAAATAGAATCAGGAGAAGGC	in/del	AB033607	Naruse *et al.* (2000)

[a] EST markers; [b] markers converted to STSs (previously reported as AFLP or RAPD markers); [c] markers newly added to LG1.

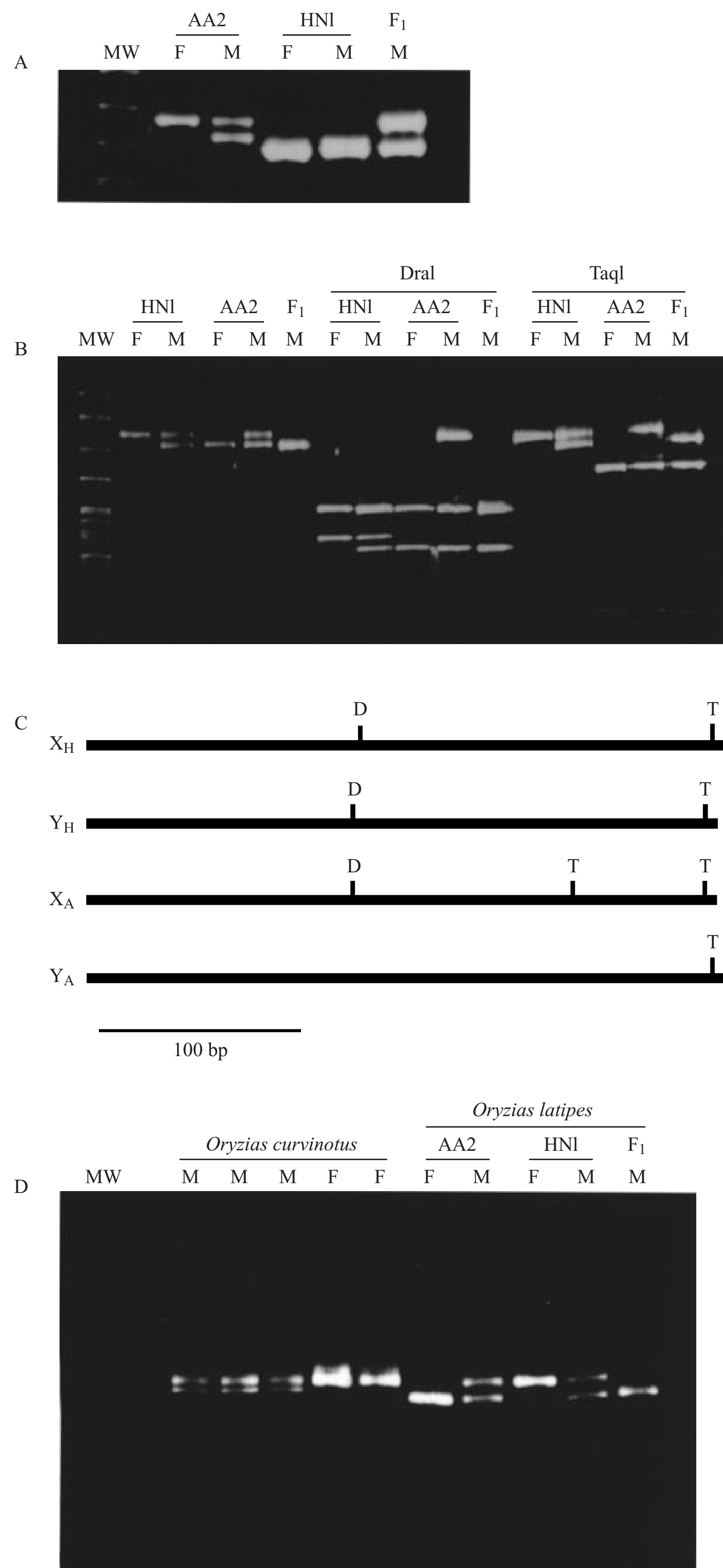

Fig. 1. Dimorphic DNA markers on the sex chromosome. (*A*), (*B*) Polyacrylamide gel electrophoresis of the Yc-2 (*A*) and Casp6 (*B*) PCR products. The templates were genomic DNA from female (F) and male (M) fish of AA2 or HNI, or (AA2 female × HNI male) male F1. The products in (*B*) were either undigested or digested with *Dra*I or *Taq*I. (*C*) Position of *Dra*I (D) and *Taq*I (T) sites in Casp6 PCR products. X_H, product from the X allele of HNI; Y_H, product from the Y allele of HNI; X_A, product from the X allele of AA2; Y_A, product from the Y allele of AA2. (*D*)

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(vi) *Gene-centromere mapping*

The genetic map distance between DNA markers and the centromere of the sex chromosome was estimated as described by Naruse *et al*. (1988). The genetic map distance can be estimated from the number and the fraction of heterozygous diploid progeny.

3. Results

(i) *Sequence-tagged Site* (*STS*) *markers on the sex chromosome*

To the previously established genetic map of the sex chromosome (LG1; Naruse *et al*., 2000) on which 49 markers were mapped, eight supplementary markers were added. Six amplified fragment length polymorphism (AFLP) markers and one random amplification of polymorphic DNA (RAPD) marker, reported previously, were converted to STS markers (Table 1). Thus, 57 markers are now mapped on LG1, among which 21 markers are STSs and used for further analysis. The markers Yc-1, Yc-2, Casp6, Casp3B, C3-1, C3-2 and dd048 are expressed sequence-tagged (EST) sites.

(ii) *Dimorphic DNA markers on the sex chromosomes*

Two EST markers, Yc-2 and Casp6, were amplified from genomic DNA of male and female fish of both the AA2 and HNI strains. Two bands were amplified from AA2 male DNA using primers for Yc-2, while only one band was generated from DNA of female AA2 or both sexes of HNI (Fig. 1*A*). Casp6 primers amplified two bands from both the HNI and AA2 male DNA, while only one band was generated from DNA of females (Fig. 1*B*). The polymorphisms of the Casp6 PCR products were distinguishable by digestion with either *Taq*I or *Dra*I (Fig. 1*B*, *C*). From linkage analysis, these dimorphic products were defined to come from alleles on either the X or Y chromosomes. Therefore, the Yc-2 marker distinguishes between the X and Y chromosomes of AA2, and the Casp6 marker distinguishes between X and Y chromosomes of both HNI and AA2.

To see whether alleles on the X and Y chromosomes of other species related to medaka can be detected, Casp6 was amplified by polymerase chain reaction (PCR) from DNA of three male and two female *O. curvinotus* fish. Two bands were amplified from male DNA, whereas only a single band was amplified from female DNA (Fig. 1*D*), showing that the Casp6 marker is linked to sex dimorphism.

(iii) *Selection of sex reversed XY females and construction of a backcross panel*

Male fish treated with oestrogen to cause sex reversal were reared until maturity. Their sex chromosome genotypes were checked by carrying out PCR to amplify two markers, Yc-2 and Casp6, from DNA extracted from tail fins. Fish that carried the HNI alleles for these two markers were judged to have the Y chromosome containing the sex-determining region from the HNI strain. The fish thus selected were mated with AA2 male fish for producing backcross progeny, to determine the recombination frequency in sex-reversed XY females. After the progeny thus produced had been sexed on the basis of their gonadal morphology, DNA was extracted and genotyped by PCR for the marker Yc-2. This marker was used first as the index to detect the Y chromosome of AA2. As expected, the female-to-male ratio was 1:3, and about half of all the progeny carried the Y chromosome of the AA2 strain (data not shown). In the fish carrying the Y chromosome of the AA2, sex determination should be under the control of the sex-determining gene of the AA2, even though they might carry the y gene from the HNI allele. These fish were excluded from typing for the sex phenotype, but other DNA markers were checked.

(iv) *Comparison of genetic maps established by female or male recombination*

A total of 21 STSs and two phenotypic markers (*lf* and *Y*) were used for linkage analysis. The genetic maps produced from the backcross progeny of XY males or XX females are as shown in Fig. 2*A* and 2*B*, respectively. The genetic map distance between markers stsOPQ05-1 and ER-1 was 42·6 cM in males and 57·4 cM in females.

To elucidate the linear order of the markers flanking the *Y* locus, a genetic map was produced from the backcross progeny of XY congenic sex-reversed females (Fig. 2*C*). The markers telomeric to either stsOPR04-1 or *lf* were not analysed in progeny of XY congenic sex-reversed females, as those regions closer to the telomere originated from the AA2. The sex-determining gene y is between the markers Yc-2 and Casp3B, but as no recombination was observed among *Y*, Casp6 and SL1, the position could not be determined.

Polyacrylamide gel electrophoresis of the Casp6 PCR products. The templates used were genomic DNA from *O. curvinotus* or *O. latipes* fish. MW, DNA size markers.

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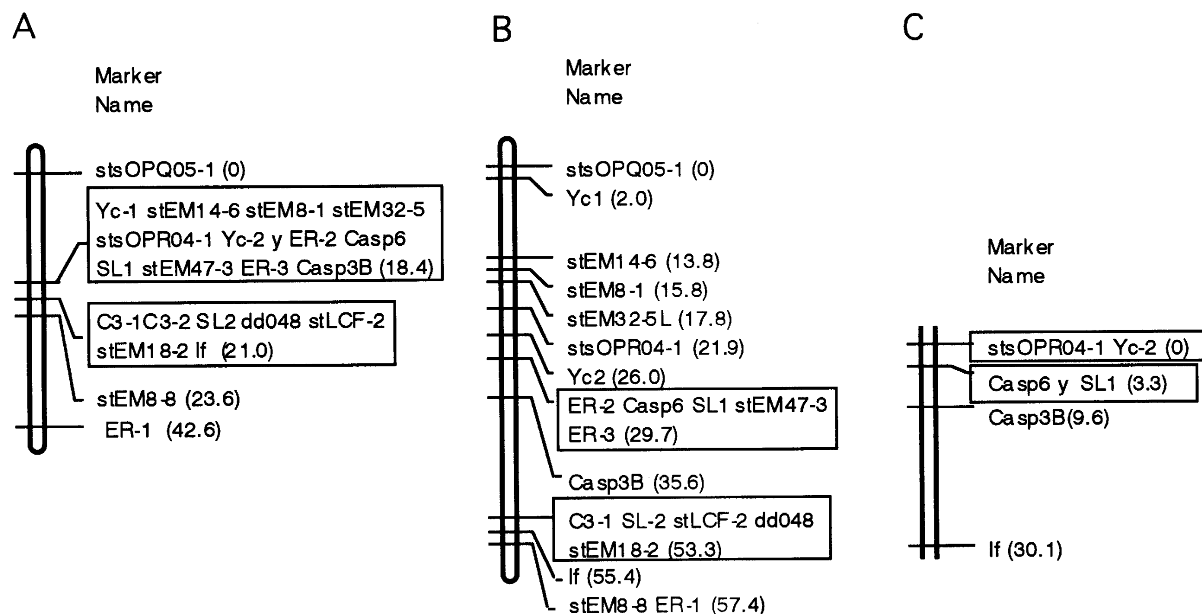

Fig. 2. Genetic linkage map of the sex chromosome of medaka. Numbers in parentheses indicate map distance from the top locus in terms of centimorgans (Kosambi mapping function). Maps are based on recombination in (*A*) male meiosis, (*B*) female meiosis and (*C*) XY sex-reversed female meiosis. Markers in the box showed no recombination.

Table 2. *Progeny phenotypes in gynogenetic diploid medaka and gene–centromere distances*

	No. of progeny			
Locus	Homozygous for AA2	Heterozygous	Homozygous for HNI	Gene–centromere distance (cM)
Casp3B	13	5	9	9·3
stLCF-2	14	4	8	7·1
dd048[a]	16	4–6	8–6	7·1–10·7
ER-1	12	10	6	17·9

[a] dd048 is an allele-specific marker.

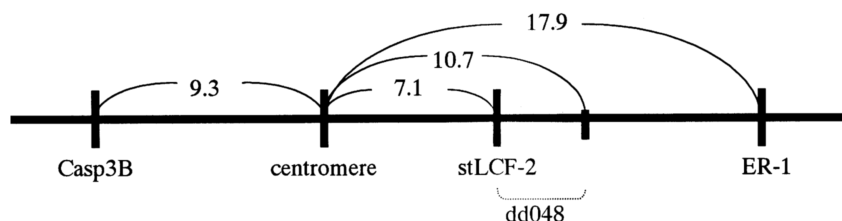

Fig. 3. Location of the centromere of the sex chromosome of the medaka. Genetic distances in centimorgans are shown. The centromere lies between Casp3B and stLCF-2.

(v) *Location of the centromere of the sex chromosome*

We tested four DNA markers to determine the location of the centromere of the sex chromosome. Using the gynogenetic diploid panel, the genetic map distance was deduced (Table 2). It was impossible to distinguish the heterozygotes from the homozygotes of dd048 because it is an allele-specific marker. The dd048 genotypes were determined based on results of markers flanking dd048. Compared with the genetic map order of the female medaka genetic map, the heterozygous patterns of diploid progeny indicate the location of the centromere between Casp3B and stLCF-2 (Fig. 3). The genetic map distance derived from this panel was 16·4 cM between Casp3B and stLCF-2, and 16·4–21·0 cM between Casp3B and dd048. These values were in agreement with the

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genetic map distance generated by female meiotic recombination, which should be 17·7 and 19·8 cM, respectively. The ratio of genetic map distance between the centromere to Casp3B and of stLCF-2 is 9·3 : 7·1; thus, the centromere on the female meiosis map is calculated to be 10·0 cM from Casp3B and 7·7 cM from stLCF-2.

4. Discussion

We report here a comparison of the male and female genetic maps of medaka sex chromosomes. In this study, we mapped new markers on LG1, and the map distance between those markers was studied in detail. Until now, 57 markers have been mapped on the sex chromosome, and the two markers stsQ05-1 and ER-1 are thought to be the closest to the telomeres. As no recombination was detected between the AFLP marker EM47-7 (Naruse *et al.*, 2000) and ER-1, EM47-7 may be the most distal marker. Except for that, no other marker has been found to be closer to the telomere. Thus, we think that most of the sex chromosome of the medaka is between these two markers and the genetic map distance between these markers should approximate the total length of the medaka sex chromosome.

Previously, the total genetic map length of the medaka chromosome was reported to be 1776 cM (Ohtsuka *et al.*, 1999) using female meiosis, and 1354·5 cM (Naruse *et al.*, 2000) using male meiosis. The sex chromosome length was reported to be 56·9 cM in females (Ohtsuka *et al.*, 1999) and 44·2 cM in males (Naruse *et al.*, 2000). In this study, as shown in Fig. 1, the length obtained was 57·4 and 42·6 cM in females and males, respectively.

The difference in genetic lengths of the sex chromosome between females and males reflects the difference in recombination frequency. Sex-specific differences in recombination frequency were observed, with male recombination being more prevalent in the telomeric regions and female recombination dominating in the more centromeric regions of the chromosome. The map distance between the two markers stEM8-8 and Yc-1 is 5·2 cM in male meiosis, whereas it is 55·4 cM in female meiosis. On the contrary, the region between stsOPQ05-1 and Yc-1, stEM8-8 and ER-1 was restricted for recombination in females but not males. The region where male recombination is suppressed is the region close to the sex-determining gene y. This restriction in recombination does not occur uniformly on the sex chromosome, as the genetic map distances of the markers are not proportional in male and female recombination. The difference in recombination frequency of the sex chromosomes in females and males has already been reported using the r and sex-differential loci (Yamamoto, 1961). In addition, from studies using several marker loci of the medaka sex chromosome, it has been reported that the map distance is shorter in males (Matsuda *et al.*, 1999). Nevertheless, with the smaller number of markers employed in the earlier studies the recombination suppression and/or enhancement of the whole sex chromosome could not readily be detected.

As previously reported, the medaka sex chromosome is one of the largest chromosomes (Matsuda *et al.*, 1998). However, no difference in morphology between the X and Y chromosomes has been observed. It has been considered that the sex chromosomes are highly homologous in structure and in nucleotide sequence, and that the difference lies only in the sex determining region. In addition, functioning genes have been found to exist on the sex chromosome. Thus, most regions of the sex chromosome may be called pseudoautosomal. This report shows that suppression of recombination between X and Y occurs close to the region of sex determination. This observation seems to support Ohno's hypothesis that the diverse sex chromosomes were derived by suppression of recombination between autosomal chromosomes.

The molecular nature of suppression in recombination is not yet clear. Sakamoto *et al.* (2000) reported that, in rainbow trout, recombination in the male genome is suppressed compared with that in females. This difference may be attributable to the quadrivalent formation that occurs exclusively in males during meiosis. However, there is no evidence that this occurs also in the medaka, as no pseudolinked regions are observed in the medaka linkage map. Thus, the proposed mechanism for the rainbow trout would not be valid for the medaka.

Our study shows the expected location of the centromere to lie between Casp3B and C3-1. It is generally accepted that recombination near the centromere is restricted in proportion to chromosomal length. Restriction in recombination of this region was detected only in male meioses. This restriction could have been masked by the restriction in the sex-determining region. The reason why we failed to detect the restriction in the centromere in female meiosis may be that the number of markers in this region was too small, and unevenly spaced. If more markers could be available and randomly spaced, recombination frequency around the centromere could be studied in detail.

As already reported, the X and Y chromosomes of the medaka cannot be distinguished by their morphology. In the present study, however, we were able to find DNA markers corresponding to alleles on the sex chromosomes and these markers could be used in studies that require the genotyping of sex chromosomes. Alleles of the SL1 locus of the X and Y chromosomes have been reported previously, but in only the *O. latipes* Hd-rR strain (Matsuda *et al.*,

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1997). The dimorphic DNA markers Yc-2 and Casp6 generated in the present study are ESTs, which means that these markers may serve as markers to identify not only the sex-determining region of other strains of the medaka, but also the corresponding regions of other species, such as zebrafish or *Fugu* sp. Though the genomes of these two species are being extensively studied, the sex chromosome has not yet been identified. We confirmed that the marker Casp6 could be used for distinguishing the sex chromosomes of *O. curvinotus*, which is a closely related species to *O. latipes*. This provides a good tool to study and compare the sex chromosomes of other species, especially for the evaluation of synteny or the evolutionary rearrangement of genes.

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