Genet. Res., Camb. (1999), **73**, *pp.* 225–231. *With* 3 *figures.* Printed in the United Kingdom

Male-specific restriction of recombination frequency in the sex chromosomes of the medaka, *Oryzias latipes*

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(*Received 4 August 1998 and in revised form 24 November 1998*)

Summary

In the medaka, *Oryzias latipes*, the mechanism of sex determination (XX/XY) can be revealed by genetic crosses using a body-colour gene, though it does not have cytologically recognizable sex chromosomes. The recombination restriction of sex chromosomes in heterogametic (XY) males has been demonstrated. To elucidate whether the recombination is prevented by the heterogamety of the sex chromosomes or by maleness, we examined the recombination frequencies among three loci located on the sex chromosomes (*r*, *SL1* and *SL2*) in heterogametic males (XY), homogametic males (XX and YY), homogametic females (XX) and heterogametic females (XY). The recombination frequencies between *r–SL1* and *SL1–SL2* were as follows: 0, 0 (XY males); 0, 1·5 (XX males); 1·6% (YY males; 1·2%, 14·4% (XY females); 0·8%, 21·8% (XX females). These results indicate that the recombination restriction of the sex chromosomes in heterogametic males does not result from heterogametic sex chromosomes, but from maleness. Such sex-chromosome-specific recombination restriction in heterogametic sex may have triggered the differentiation of sex chromosomes in vertebrates.

1. Introduction

It is thought that sex chromosomes were originally homomorphic chromosomes and that suppression of recombination between the sex chromosomes caused the heteromorphism (differentiation) of the sex chromosomes (reviewed by Solari, 1994*b*). In mammals and birds, sex chromosomes are morphologically differentiated (heteromorphic) in the heterogametic sex. Furthermore, in mammals, sex chromosomes are functionally differentiated (genes carried on the Y chromosome are different from those on the X chromosome) and the recombination is limited to a cramped region of the XY pair. Although most other vertebrates also have heterogametic sex-determining mechanisms, only a few species have morphologically distinguishable sex chromosomes.

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The medaka, *Oryzias latipes*, is a small fish native to east Asia and has been widely used as an experimental animal in Japan. In medaka, sex is genetically determined and the mechanism of sex determination (XX/XY) can be revealed by genetic crosses using a particular pigment gene, *r* (Aida, 1921). In the d-rR strain of this species, the allele *R* of the *r* locus is located only on the Y chromosome. Therefore, the female X^rX^r results in a white body colour, and the male X^rY^R results in an orange-red body colour. Since, in this strain, the genetical sexes can be identified by their body colour, the medaka has been useful for experiments involving the induction of sex reversal (reviewed by Yamamoto, 1975). In this species, an atypical sex hormone administered prior to and passing through the stage of gonadal sex differentiation induces a complete reversal in sex differentiation. These sex-reversal experiments have revealed that sex-reversed females (XY and YY) lay normal eggs, and sex-reversed males (XX) yield normal sperm. Furthermore, no sex differences could be found in a comparison of karyotypes of males and females in the

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medaka (Uwa & Ojima, 1981). These results have led to the proposal that the sex chromosomes of the medaka are not functionally or morphologically differentiated. Hence, it is assumed that the sex chromosomes of the medaka are in a primitive stage of sex chromosome evolution.

In the medaka, the recombination frequency on sex chromosomes was found to be different in males and females by Yamamoto (1961). He compared recombination frequencies between the sex-determining factor (*SDF*) and the *r* locus in normal XY males and sex-reversed XY females. The recombination frequencies are estimated to be 0·2 % ± 0·01 % (in XY males) and 1·0 % ± 0·4 % (in XY females). This result suggests that recombination of their sex chromosomes was suppressed in heterogametic males. However, it is unclear what prevents such recombination. To elucidate why the recombination is restricted, recombination frequencies of sex chromosome should be studied in homogametic males (XX or YY males). For investigating the recombination frequencies of sex chromosomes in homogametic males, sex-linked DNA markers are necessary. Recently, we have isolated two sex-linked clones: pHO5·5 and pHO5·110 (Matsuda *et al.*, 1997*a*, 1998). The pHO5·5-related sequences are a single copy sequence in the medaka genome. whereas the pHO5·110-related sequences are a tandemly repetitive sequence in the genome. We have designated these sex-linked DNA loci as *SL1* (*S*ex-*L*inked number *1*) and *SL2* (*S*ex-*L*inked *2*), respectively. The genotypes of *SL1* can be assessed by the polymerase chain reaction (PCR) method (Matsuda *et al.*, 1997*a*). The PCR products have shown differences in size between the strains and differences between males and females of the Hd-rR inbred strain. Such an application of PCR is indispensable for genetic mapping in medaka, because this species is a small fish and the DNA quantity obtained from one specimen is insufficient for genetic mapping by Southern blot analysis. Hence, in the present study, we first developed a method of detecting the *SL2* genotypes with PCR. Next, we examined the recombination frequencies of the sex chromosomes in various combinations of sexes and sex chromosome types (XX, XY females and XY, XX, YY males).

2. Materials and methods

(i) *Fish strains and genotypes*

We used four strains of medaka: the HNI, Hd-rR, Hd-rR.Y^{HNI} and Hd-rR.*rr* strains. The HNI and Hd-rR strains are inbred strains. The body colour of the HNI strain is that of the wild-type of (*B R*), and the body colour of the Hd-rR strain is white (*b r*) in females and orange-red (*b R*) in males. The Hd-rR.Y^{HNI} strain is a congenic strain that has been established from Hd-rR females and an HNI male. F1 hybrid males were obtained from crossing an Hd-rR female with an HNI male. An F1 hybrid male was mated to Hd-rR females to obtain backcross progeny (BC1). A BC1 male was further mated to Hd-rR females to obtain a BC2 generation. The progenies produced by the backcrossing to the Hd-rR female were denoted as BC*n*, where *n* is the number of backcross generations. By 1996, the congenic strain had been backcrossed for 11 generations (BC11), that has been designated as the Hd-rR.Y^{HNI} strain. Therefore, the male of the Hd-rR.Y^{HNI} strain as a genetic background derived from the Hd-rR strain, and has only the chromosomal region including the sex-determining factor (also including the *r* locus) from the HNI strain in its Y chromosome. The Hd-rR.*rr* strain is derived from the Hd-rR strain, in which both males (*b r*) and females (*b r*) have a white body colour. We discovered a white male in a stock of the Hd-rR strain. We supposed that the white male was probably a recombinant between the *r* locus and the sex-determining factor.

The genotypes of the four strains for three loci (*r*, *SL1* and *SL2*) in the sex chromosomes are as follows:

the Hd-rR male: XY; $r\ SL1^a\ SL2^d/R\ SL1^b\ SL2^d$;
the Hd-rR female: XX; $r\ SL1^a\ SL2^d/r\ SL1^a\ SL2^d$;
the HNI male: XY; $R\ SL1^n\ SL2^n/R\ SL1^n\ SL2^n$;
the HNI female: XX; $R\ SL1^n\ SL2^n/R\ SL1^n\ SL2^n$;
the Hd-rR.Y^{HNI} male: XY; $r\ SL1^a\ SL2^d/R\ SL1^n SL2^n$;
the Hd-rR.*rr* male: XY; $r\ SL1^a\ SL2^d/r\ SL1^b\ SL2^d$.

The genotypes of the Hd-rR.Y^{HNI} females and Hd-rR.*rr* females are identical to the genotype of the Hd-rR female.

(ii) *Determination of* SL2 *genotype*

The locus *SL2* is a chromosomal region homologous to the insert of the clone pHO5·110. The pHO5·110-related sequences were amplified using PCR primers, pHO5·110-F (5′-GAA TTC AAT TCT TTC GTG ACA CCA C-3′) and pHO5·110-RV (5′-TCC ACA GAG GCA CTA TCT GTC AAG-3′). The reaction mixture for amplification by PCR contained *Ex Taq* Buffer (Takara Shuzo), 0·2 mM dNTPs, 0·2 μM each of the primers, 0·2 μg of template DNA, and 0·6 units of *TaKaRa Ex Taq* polymerase (Takara Shuzo) in a final volume of 25 μl. The thermal cycling conditions were 3 min at 95 °C for 1 cycle; 1 min at 95 °C, 2 min at 55 °C, 1 min at 72 °C for 30 cycles; and 5 min at 72 °C for 1 cycle. Then the reaction mixture was digested with *Dra*I or *Rsa*I, and electrophoresed in a 2% agarose gel.

To confirm that the PCR products were pHO5·110-related sequences, these PCR products were analysed by Southern blotting using the ECL direct labelling and detection Kit (Amersham International) following the manufacturer's instructions. The pHO5·110 clone was used as a probe.

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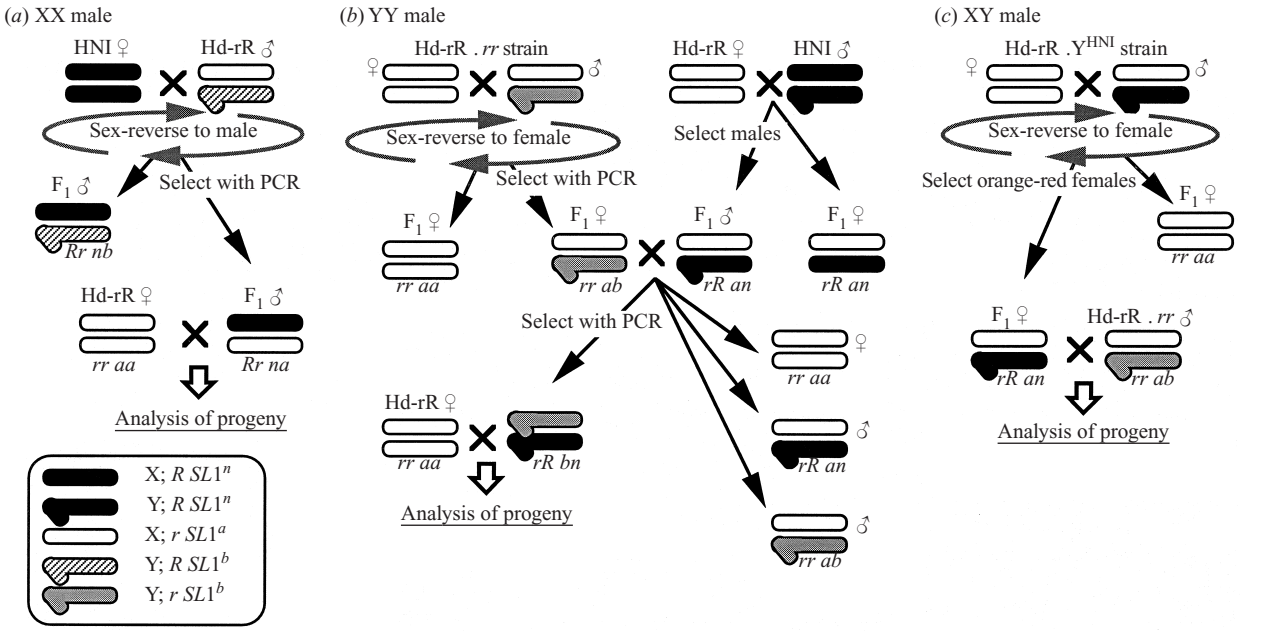

Fig. 1. Mating scheme for production of XX males (*a*), YY males (*b*) and XY females (*c*). X and Y chromosomes with *r* and *SL1* alleles are also shown.

(iii) *Mating experiments*

Five mating experiments for examining recombination frequencies in fish with different types of sex chromosomes were performed.

Experiment I was designed to estimate recombination frequencies in XY males. The XY males were F1 offspring that were obtained from crossing an Hd-rR female with an HNI male. The genotypes of the F1 males were XY; $r\ SL1^a/R\ SL1^n$. The XY males were crossed with Hd-rR females to obtain their progeny.

Experiment II was designed to estimate recombination frequencies in XX females. The XX females were F1 offspring that were obtained from crossing an HNI female with an Hd-rR male. The genotypes of the F1 females were XX; $r\ SL1^a/R\ SL1^n$. The XX females were crossed with Hd-rR.*rr* males.

Experiment III was designed to estimate recombination frequencies in XX males (Fig. 1*a*). The XX males were sex-reversed F1 offspring that were obtained from crossing an HNI female with an Hd-rR male. Sex reversal was induced by a hormone treatment developed by Iwamatsu (1999). In this method, fertilized eggs were soaked in water containing sex hormone (0·08 μg/ml 17-methyltestosterone) until hatching. The sex-reversed F1 males (XX; $r\ SL1^a/R\ SL1^n$) were selected by the *SL1* genotype with PCR. The XX males were crossed with Hd-rR females to obtain their progeny.

Experiment IV was designed to estimate recombination frequencies in YY males (Fig. 1*b*). The YY males were obtained from crossing sex-reversed XY females of the Hd-rR.*rr* strain (XY; $r\ SL1^a/r\ SL1^b$) with F1 males (XY; $r\ SL1^a/R\ SL1^n$) that were obtained from crossing an Hd-rR female with an HNI male. The sex-reversed XY females were induced by hormonal treatment of offspring of the Hd-rR.*rr* strain. Fertilized eggs were soaked in water containing sex hormone (0·2 μg/ml β-estradiol) until hatching. The sex-reversed females were selected by the *SL1* genotype with PCR. The YY males (YY; $r\ SL1^a/R\ SL1^n$) were also selected by the genotype of the *SL1*. The YY males were crossed with Hd-rR females to obtain their progeny.

Experiment V was designed to estimate recombination frequencies in XY females (Fig. 1*c*). The XY females were sex-reversed offspring of the Hd-rR.Y^{HNI} strain. Fertilized eggs were soaked in water containing sex hormone (0·6 μg/ml β-estradiol) until hatching. The sex-reversed F1 females were selected by their body colour. The genotype of the sex-reversed F1 females was XY; $r\ SL1^a/R\ SL1^n$. The XY females were crossed with Hd-rR.*rr* males to obtain their progeny.

(iv) *Estimation of genotypes*

To measure recombination frequencies, we used three loci on the sex chromosomes and two loci on a pair of autosomes (*Bf/C2* (Kuroda *et al*., 1996) and *HSC70* (Arai *et al*., 1995)). The autosomal loci were examined in progenies of XX females, XY males and XX males. The alleles of the *r* gene were simply detected by their body colour. The alleles of other four DNA loci were

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Table 1. *Map distances calculated by recombination frequencies*

(Experiment no.)		*r*–*SL1*					*SL1*–*SL2*					*Bf/c2*–*HSC70*				
Parent type	n^a	X^b	Map	SE	95% CI[c]	LOD	X^b	Map	SE	95% CI[c]	LOD	X^b	Map	SE	95% CI[c]	LOD
(I) XY male	98	0	0·00	0·00	0–3·7	29·5	0	0·00	0·00	0–3·7	19·5	15	15·31	3·64	8·8–24·0	11·3
(II) XX female	124	1	0·81	0·80	0–4·5	34·8	27	21·77	3·71	14·5–29·0	9·1	28	22·58	3·75	15·2–29·9	8·6
(III) XX male	68	0	0·00	0·00	0–5·3	20·5	1	1·47	1·46	0–7·9	18·2	7	10·29	3·69	4·2–20·1	10·7
(IV) YY male	63	0	0·00	0·00	0–5·7	19·0	1	1·59	1·57	0–8·5	16·7	—	—	—	—	—
(V) XY female	167	2	1·20	0·84	0·1–4·3	45·6	23	13·77	2·67	8·5–19·0	21·2	—	—	—	—	—

[a] Number of progeny examined.
[b] Number of recombinants.
[c] 95% confidence limits.

examined by PCR. For PCR amplification, total DNAs were prepared as described in Matsuda *et al.* (1997*b*). The genotypes of the *SL1* and *HSC70* loci were identified by electrophoresis of PCR products using a 1% agarose gel. The genotypes of the *SL2* and *Bf/C2* loci were identified by the PCR-RFLP method. Restriction enzymes *Rsa*I and *Dra*I were used for the detection of the *SL2* and *Bf/C2* loci, respectively. For selection of sex-reversed XX males in experiment III, and of sex-reversed F1 females and YY males in experiment IV, the genotypes of the *SL1* were identified by 8% polyacrylamide gel electrophoresis (Matsuda *et al.*, 1997*a*).

Sexes of all progeny obtained from experiment I (XY male parent) and 111 progeny obtained from experiment V (XY female parent) were checked by histologically inspecting gonads or secondary sex characteristics.

(v) *Data analysis*

Map distance, standard error, 95% confidence limit and LOD score were computed by the computer program Map Manager version 2·5.

Statistics on the difference of recombination frequencies among five examinations were estimated using 2×2 contingency table (chi-squared test for independence or Fisher's exact probability test which was used when cells of expectation values were less than five).

3. RESULTS

(i) *PCR identification of the* SL2 *genotype*

The pHO5·110-related sequences (the DNA locus *SL2*) are tandemly repetitive sequences on the sex chromosomes of the medaka (Matsuda *et al.*, 1998). The lengths of the PCR products seemed to be the same (Fig. 2). The PCR products were digested with *Dra*I and *Rsa*I. The *Dra*I-digested products demonstrated two bands that were peculiar to the Hd-rR strain, whereas the *Rsa*I-digested products demonstrated a band peculiar to the HNI strain. In this study, genotypes of the *SL2* locus in backcross progenies were detected by the *Rsa*I-digested PCR products.

(ii) *Selection of parents*

To select sex-reversed XX males for experiment III (Fig. 1*a*), we used the *SL1* genotypes in male offspring that were obtained from crossing the HNI female with the Hd-rR male. Nineteen male offspring were obtained and two of these had the genotype $SL1^a/SL1^n$. The two sex-reversed males were used for a mating experiment to estimate the recombination frequency in XX males.

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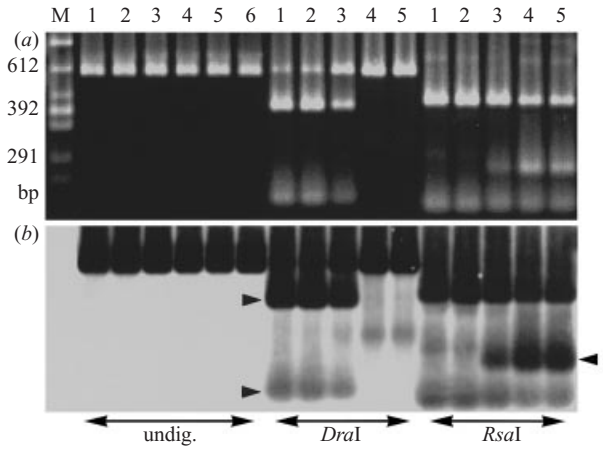

Fig. 2. *SL2* genotypes. (*a*) PCR amplification of pHO5·110-related sequences. Numbers above lanes refer to the template DNA (M, DNA size marker (ϕX174 *Hin*cII digest): 1, Hd-rR male; 2, Hd-rR female; 3, Hd-rR.Y^{HNI}male; 4, HNI male; 5, HNI female; 6, clone pHO5·110. Lanes 2–6, undigested DNA; lanes 8–12, DNA digested with *Dra*I; lanes 13–17, DNA digested with *Rsa*I. (*b*) Southern hybridization of (*a*), using the pHO5·110 clone as a probe. PCR products were pHO5·110-related sequences. The *Dra*I-digested products show two bands that were peculiar to the Hd-rR strain (black arrowhead), whereas the *Rsa*I-digested products show a band peculiar to the HNI strain (grey arrowhead).

To obtain YY males (Fig. 1*b*), we selected sex-reversed XY females from offspring of the Hd-rR.*rr* strain. From the offspring with hormone treatment, we obtained 13 females. Of these 13 females, eight were sex-reversed XY females with *SL1* genotypes of $SL1^a/SL1^b$. The sex-reversed females were used for the next mating of F1 males (Hd-rR × HNI). White body colour males and all female offspring were discarded, and the *SL1* genotypes of 13 orange-red males obtained were checked with PCR. Then, we selected four males as YY males. We used two of these males for a mating experiment to estimate recombination frequency in YY males.

To select XY females (Fig. 1*c*), orange-red offspring with hormone treatment were obtained from the Hd-rR.Y^{HNI} strains. Of the 10 offspring, five were sex-reversed females. Two sex-reversed females were used for a mating experiment to estimate recombination frequency in XY females.

(iii) *Recombination frequencies*

The recombination frequencies obtained from this study are summarized in Table 1. With regard to sex-linked loci, in 98 progeny from XY males no recombinants were obtained. In 124 offspring from XX females, one recombinant between *r* and *SL1* and 27 recombinants between *SL1* and *SL2* were obtained. In 68 and 63 progenies from XX and YY males, only one recombinant between *SL1* and *SL2* was obtained from each progeny. In 167 offspring from XY females, two recombinants between *r* and *SL1* and 24 recombinants between *SL1* and *SL2* were obtained. Furthermore, the sexes of 111 specimens out of 167 offspring from experiment V were checked, and 52 of these inherited the X chromosome ($SL1^a$) from the Hd-rR.*rr* male. These 52 offspring were orange-red males or white females, and were not recombinant between *r* and *SDF*. The sexes of 98 offspring from experiment I were also checked, and none were recombinants among sex-linked loci.

On the other hand, in autosomal loci, 15, 28 and 7 recombinants were obtained from the progenies of XY males, XX females and XX males, respectively (Table 1).

Highly significant sex difference was observed between sex-linked loci *SL1* and *SL2* ($P < 10^{-9}$, $\chi^2 = 37{\cdot}87$), whereas no significant sex difference between autosomal loci *Bf*/*c2* and *HSC70* ($P = 0{\cdot}0820$, $\chi^2 = 3{\cdot}02$). In pairwise comparisons within females (experiment II and V) or males (experiment I, III and IV), no significant differences at the 5% level were observed between sex-linked loci *SL1* and *SL2* ($P = 0{\cdot}0736$ in females, $P > 0{\cdot}39$ in males).

4. Discussion

Figure 3 shows genetic maps of the sex chromosomes of the medaka deduced from the present study. The genetic maps in male demonstrate short map distances, regardless of whether their sex chromosome type is

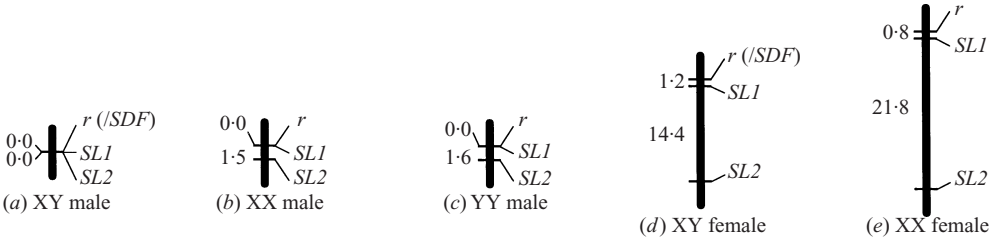

Fig. 3. Sex difference of linkage maps of the medaka sex chromosomes. Map distances between two marker loci are indicated. An extremely high frequency of recombination during female meiosis was observed in the XX and XY females.

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homogematic (XX, YY) or heterogametic (XY). These results indicate that the recombination restriction in heterogametic males does not result from heterogametic sex chromosomes, but from maleness.

With regard to the difference in recombination frequencies between sex-linked DNA loci in XX females (experiment II) and those in XY females (experiment V), the probability is not significant at the 5% level. However, the *P* value was near the boundary of significance ($P = 0{\cdot}0596$). We assumed that the differences were caused primarily by the genetic background of these parents. The XX female in experiment II was an F1 offspring, all of which were heterozygous in all loci, and the XY female in experiment V was derived from the Hd-rR.Y^{HNI} strain, which had only the Y chromosome derived from the HNI strain in the Hd-rR genetic background. These results suggest an autosomal gene effect on the recombination frequencies of the sex chromosomes. On the other hand, the differences in recombination frequencies between heterogametic and homogametic sex chromosome types in males are small. The map distance of 1·5 cM between *r*/*SL1*–*SL2* in the XX male as contrasted with the distance 0·0 cM in the XY male could suggest a heterogametic effect on recombination suppression.

An extreme example of a sex difference in recombination frequency is in *Drosophila*, in which there is no recombination in males. Our results also indicated a sex difference in recombination frequency. However, in the medaka the sex difference on sex chromosome loci is more noticeable than that on autosomal loci. In addition, regular recombination of autosomal loci in medaka males has also been demonstrated using random amplified polymorphic DNA (RAPD) markers (Wada *et al*., 1995). These results propose that there is a sex-chromosome-specific recombination restriction in males of the medaka, which have morphologically and genetically undifferentiated sex chromosomes. In vertebrates, such sex-chromosome-specific recombination restriction in the heterogametic sex may have triggered the evolution of sex chromosomes.

What is the mechanism of recombination restriction of sex chromosomes during meiosis in medaka males? Two hypotheses are considered: extinction of recombinant sperm and existence of a sex-chromosome-specific mechanism reducing chiasmata in prophase I of meiosis in the testes. Since YY females and XX males have a normal phenotype (Yamamoto, 1961, 1963), it is assumed there is no significant difference in genes (including genes controlling gametogenesis) located on the X and Y chromosomes except for the sex-determining gene. Therefore, it seems more probable that sperm recombining the X and Y chromosomes remain viable. Hence, we assumed that the second hypothesis is plausible, because it is also well known that there is a marked sex difference in recombination frequency in certain regions of human and mouse autosomes (reviewed by Solari, 1994*a*). One attractive interpretation for sex differences is that there exist sex-dependent hotspots at which recombinations take place preferentially, either during female or male meiosis (reviewed by Shiroishi *et al*., 1993). Progress in understanding the sex chromosomes of the medaka will provide valuable clues to clarifying the mechanisms responsible for differences in recombination frequencies between the sexes.

The order of the genes including *SDF* on the Y chromosome can be estimated from the genetic map produced in this study and from the genotype of the Hd-rR.*rr* strain. In this study, the *SL1* locus was closely linked to the *r* locus, the two being approximately 1 cM apart in females (Fig. 3). *SDF* mapped to the same chromosomal region of the *r* locus in the progeny of the XY male and XY female. The alleles of the Y chromosome in Hd-rR.*rr* males are Y; $rSL1^b$ (described in Section 2), suggesting recombination between *r* and *SDF*/*SL1*. Hence, the gene order may be *r*, *SDF*, *SL1* and *SL2*, with *SL2* being located far away from the others. If the gene order is correct, the *SL1* locus would be closely linked to *SDF* (within 1 cM).

In vertebrates, a sex-determining gene is identified only in mammals (Gubbay *et al*., 1990; Sinclair *et al*., 1990). In mammals, this gene is referred to as *SRY* or *Sry*. In other, non-mammalian vertebrates *Sry*-related sequences have not been found to be sex-linked. We suppose that the positional cloning method is useful for identifying the sex-determining genes in non-mammalian species. To apply this approach to a species, high-resolution mapping of their sex chromosomes is needed. The present study is the first step in the molecular cloning of the sex-determining factor of the medaka. In order to construct a high-resolution map of the sex chromosomes of the medaka and to find the sex-determining factor, many recombinant progenies from XY females and many DNA markers on the sex chromosomes are needed. These projects are now in progress.

We are grateful to Dr K. Naruse (University of Tokyo) for access to unpublished data of linkage between *HSC70* and *Bf*/*C2* and for providing us with PCR primers of *HSC70*. We also thank Dr M. Nonaka (University of Tokyo) for providing PCR primers of *Bf*/*C2*.

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