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ORIGINAL ARTICLE

Heritable artificial sex chromosomes in the medaka, *Oryzias latipes*

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Chromosomal sex determination is widely used by vertebrates, however, only two genes have been identified as master sex-determining genes: *SRY*/*Sry* in mammals and *DMY* in the teleost medaka. Transfer of both genes into genetically female (XX) individuals can induce male development. However, transgenic strains have not been established in both cases because of infertility of the transgenic founders in mammals and low germline transmission rates in medaka. In this study, we used a BAC clone containing *DMY* in a 117 kb genomic region and two types of fluorescent marker to establish two *DMY*-transgenic medaka strains. In these strains, exogenous *DMY* is completely linked to a male phenotype and early gonadal development is not different from that of the wild-type strain. Sex-linkage analysis showed that the exogenous *DMY* was located on linkage group (LG) 23 in one strain and on LG 5 in the other strain, whereas the sex chromosome in medaka is on LG 1. Real-time PCR analysis indicated that these strains have multiple copies of *DMY* and higher *DMY* expression levels than the wild-type strain. These results showed that LGs 23 and 5 function as sex chromosomes in the two strains, respectively. This is not only the first example of the artificial generation of heritable sex chromosomes in vertebrates but also the first evidence showing plasticity of homomorphic sex chromosomes. This plasticity appears to be a characteristic of lower vertebrates and the underlying cause of frequent sex chromosome switching, recently reported in several fish and frog species.
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Introduction

Genetic sex determination, based on sex chromosomes, is widely used by many vertebrates. The sex chromosome can be defined as the chromosome containing a single master sex-determining gene that initiates sexual development; X and Y in a male heterogametic (XX/XY) system or Z and W in a female heterogametic (ZZ/ZW) system. To date, only two genes have been identified as master sex-determining gene in vertebrates: *SRY*/*Sry* in mammals (Gubbay *et al*., 1990; Sinclair *et al*., 1990) and *DMY* (the DM-domain gene on the Y chromosome) in the teleost medaka, *Oryzias latipes* (Matsuda *et al*., 2002; Nanda *et al*., 2002).

The X and Y chromosomes in mammals are highly differentiated. Crossovers occur only in a small homologous region, designated the pseudoautosomal region. *SRY* is located on the degenerated Y chromosome, which is small, gene poor, repetitive and half heterochromatic. The gene content of the Y varies between species, but all groups of therian mammals possess an *SRY* gene on the Y chromosome (Waters *et al*., 2007). On the other hand, X and Y chromosomes are homomorphic in medaka. Sex chromosomal crossovers occur over almost the entire chromosome, whereas *DMY* is located in a very small Y-specific region. In contrast to the widespread distribution of *SRY*/*Sry* in mammals, *DMY* is found only in medaka and in *O. curvinotus*, the closest relative to medaka (Matsuda *et al*., 2003; Tanaka *et al*., 2007). *DMY* encodes a putative transcription factor belonging to the family of DM-domain proteins. All members of this protein family have a conserved DNA-binding motif originally found in two proteins, DSX in *Drosophila melanogaster* and MAB-3 in *Caenorhabditis elegans* (Raymond *et al*., 1998). One of these DM-domain genes, *DMRT1* (DM-related transcription factor 1), has been implicated in male sexual development in vertebrates (Zarkower, 2001). The cDNA sequences of medaka *DMY* and *DMRT1* show a high similarity (about 80%), and comparative analysis of X and Y chromosomes indicated that *DMY* arose through a duplication event of the autosomal *DMRT1* gene (Kondo *et al*., 2006). This *DMRT1* duplication event is estimated to have occurred about 10 million years ago, suggesting that the medaka Y chromosome is one of the youngest male sex-determining systems in vertebrates.

The male-determining functions of both *SRY*/*Sry* and *DMY* were shown by transgenic experiments that showed testis differentiation and male development of chromosomally female (XX) individuals (Koopman *et al*., 1991; Matsuda *et al*., 2007). However, no transgenic strains, in any species, have been established. *Sry*-transgenic mice showed normal copulatory behavior

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but were sterile because germ cells are prevented from progressing beyond prospermatogonia. In medaka, although transgenic fish injected with a BAC clone containing *DMY* in a 117 kb genomic region show testis differentiation with spermatogenesis, low germline transmission rates have prevented the establishment of transgenic strains. Here, with the use of two types of fluorescent marker integrated into the BAC clone, we established two *DMY*-transgenic medaka strains, in which the exogenous *DMY* gene determined the sex of each individual. We show that linkage groups (LGs) 23 and 5 function as sex chromosomes in the two strains, respectively. This is the first example of the generation of artificial sex chromosomes in vertebrates.

Our results also highlight the property of homomorphic sex chromosomes. Many species of fish and amphibians, including medaka, have homomorphic sex chromosomes. Recently, several comparative studies have provided evidence that different sex chromosomes and sex-determining systems have evolved in closely related fish species (Woram *et al*., 2003; Peichel *et al*., 2004; Takehana *et al*., 2007) and in the same species of frog (Ogata *et al*., 2003). The present results show that in vertebrates possessing homomorphic sex chromosomes, autosomes can easily become sex chromosomes. The frequent switching of sex chromosomes appears to be based on the plasticity of homomorphic sex chromosomes. This plasticity is characteristic of lower vertebrates and is not found in higher vertebrates, such as mammals and birds.

Methods

Strains and sexing

We used the Hd-rR inbred strain of medaka, which was derived from the Southern population. In this strain, because a sex-linked pigment gene, *R*, is located on the Y chromosome, the female X^rX^r has a white body color and the male X^rY^R has an orange-red body (Aida, 1921). The genetic sex of each individual was confirmed by genomic PCR of fin clip DNA in accordance with an earlier method (Shinomiya *et al*., 2004). As *DMY* in the transgene construct was derived from the HNI inbred strain, which is derived from the Northern population, the exogenous *DMY* and the host *DMY* could be distinguished by a polymorphic genomic PCR product.

F0 XX males with germline transmission of the exogenous *DMY* were mated with Hd-rR strain female. To maintain the transgenic strains, exogenous *DMY*-positive males were outcrossed to a female of Hd-rR strain in the subsequent generations.

BAC modification

We inserted two fluorescent reporter genes into the *DMY* BAC: a germ-cell-specific *olvas* promoter driving green fluorescent protein (GFP) and a crystal-lens-specific *crystallin-γM2* promoter driving red fluorescent protein (RFP). The two fluorescent reporters were inserted into the BAC vector using a Quick and Easy BAC Modification kit (Gene Bridges GmbH, Dresden, Germany), which relies on homologous recombination in *Escherichia coli*.

Microinjection

Fertilized eggs were collected within 15 min of spawning. The attached filaments were scraped off the chorion onto a piece of paper. Eggs were microinjected as described earlier (Kinoshita *et al*., 1996). We used DNA at 10 ng μl^{-1} in Yamamoto's solution (133 mM NaCl, 2.7 mM KCl, 2.1 mM $CaCl_2$, 0.2 mM $NaHCO_3$, pH 7.3). The injected eggs were incubated at 27 °C.

In situ hybridization

Testes were dissected from adult males, fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C overnight, embedded in paraffin and cut into 5 μm cross-sections. Digoxigenin-labeled antisense RNA probes were generated by *in vitro* transcription with DIG RNA labeling kit (Roche, Basal, Switzerland) from a *DMY* cDNA plasmid as described earlier (Kobayashi *et al*., 2004). Sections were deparaffinized, hydrated, treated with proteinase K (10 μg ml^{-1}) at 37 °C for 7 min and hybridized with the DIG-labeled antisense RNA probes at 57 °C for 18–24 h. Hybridization signals were detected using an alkaline phosphatase-conjugated anti-DIG antibody (Roche) with NBT/BCIP (Roche) as the chromogen.

Histological analysis

For histological analysis and the counting of germ-cell numbers, F_3 transgenic-strain embryos were dissected into head and body segments. The body portions were fixed overnight in Bouin's fixative solution and then embedded in paraffin. Each dissected head was used to confirm the presence or absence of exogenous *DMY* by PCR; 5 μm cross-sections of gonad were cut serially and, after H&E staining, all of the germ cells in each of the fry were counted. After cell counting, the mean and standard error were calculated for each of group *DMY*-positive and -negative groups. Differences between groups were evaluated statistically using the paired *t*-test.

Transgene mapping

We used M-marker (Kimura *et al*., 2004) to search for the nearest DNA marker to the exogenous *DMY*. M-marker is a set of 48 expressed sequence tag markers (two expressed sequence tags on each of 24 chromosome pairs); each DNA marker shows a length polymorphism between the Southern and Northern strains. As *DMY*-transgenic strains were derived from a Southern inbred strain (Hd-rR), *DMY*-transgenic F_2 males were mated with a Northern inbred strain, Kaga. F_3 transgene-positive male progeny were mated with Hd-rR strain females to obtain the backcross generation. The exogenous *DMY* was expected to be located in the Hd-rR homozygous region of transgene-positive fish or in the Hd-rR/Kaga heterozygous region of transgene-negative fish in the backcross generation. We calculated the concordance rate by adding together the ratio of Hd-rR homozygous fish to transgene-positive fish and that of Hd-rR/Kaga heterozygous fish to transgene-negative fish for each M-marker (Supplementary Figure 1). We used 28 and 27 backcross individuals for mapping in transgenic strains 1 and 2, respectively.

Quantification of transgene copy number and of DMY expression levels

Copy numbers were quantified using genomic DNA from the tail muscle. Dissected tail muscle was placed in

700 µl of 100 mM EDTA, 50 mM Tris (pH 8.0), 100 mM NaCl, 1% sodium dodecyl sulfate and 100 µg ml^{-1} of proteinase K, and homogenized. The homogenate was incubated at 55 °C for 3 h. The lysate was purified using phenol–chloroform and DNA was precipitated with isopropyl alcohol, as described earlier (Shinomiya *et al.*, 1999). The purity and concentration of the DNA were determined with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, MA, USA). The DNA concentrations of all samples were adjusted to give a total of 20 ng for each real-time PCR assay. Primers were designed to amplify a 99 bp fragment of *DMY* intron 4 (forward 5′-GGGTTTGCTTTTCTGCTTTCTGATTTC-3′ and reverse 5′-AACTATTTTTACAGGCCCAGAATTAA-3′) and a 128 bp fragment of β-actin (forward 5′-CTAC GTAGGTGATGAAGCCCAGAG-3′ and reverse 5′-TCAGCTCATTGTAGAAGGTGTGGT-3′). SYBR Green PCR master mix (Takara Bio, Otsu, Japan) was used in accordance with the manufacturer's protocol in an ABI 7000 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). The PCR conditions were 1 min at 94 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 65 °C. To confirm that the amplified PCR products were specific to *DMY*, we determined their melting temperatures from the dissociation curves. The fact that the melting temperature was the same for each product indicated that the same product was amplified from all the DNA samples. Direct sequencing confirmed that each product was amplified from the *DMY* gene. *DMY* copy number was determined by the comparative Ct method (Livak and Schmittgen, 2001; Pfaffl, 2001) as a relative value to the Hd-rR.Y^{HNI} congenic strain (Matsuda *et al.*, 1998). Real-time PCR efficiencies of target gene (*DMY*) and reference gene (β-actin) are same (Supplementary Figure 2a). The male of the Hd-rR.Y^{HNI} strain has a genetic background derived from the Hd-rR strain, and has only the chromosomal region including *DMY* from the HNI strain in its Y chromosome. Thus, Ct values from the Hd-rR.Y^{HNI} strain were set to 1, and sample copy numbers were calculated as $2^{-\Delta Ct}$.

Expression levels were quantified using RNA from the body trunks of fry on hatching day. The purity and concentration of RNAs were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific), and concentrations were adjusted to give a total of 20 ng for each real-time assay. Primers were designed to amplify a 217 bp fragment of *DMY* exons 2 and 3 (forward 5′-CGGAACCACAGCTTGAAGACC-3′ and reverse 5′-CTCCGGCTTCATTCTTCACCAC-3′) and a 130 bp fragment of *elongation factor 1* (*Elf1a*) (forward 5′-CACCGGTCACCTGATCTACA-3′ and reverse 5′-GCT CAGCCTTGAGTTTGTCC-3′). A One Step SYBR PrimeScript RT-PCR kit (Takara Bio) was used in accordance with the protocol of the manufacturer of the ABI 7000. The PCR conditions were 5 min at 42 °C, 10 s at 95 °C, then 40 cycles of 5 s at 95 °C and 31 s at 65 °C. The melting temperature and direct sequence analysis confirmed that each product had been amplified from *DMY^{HNI}*. Ct values from the Hd-rR.Y^{HNI} strain were set to 1 and the relative expression levels (as $2^{-\Delta\Delta CT}$) were calculated after correction (as $2^{-\Delta CT}$) against the Ct value for *Elf1a*. Real-time PCR efficiencies of target gene (*DMY*) and reference gene (*Elf1a*) are same (Supplementary Figure 2b). All real-time quantitative PCR experiments were performed three times.

Results

Establishment of DMY-transgenic strains

An earlier study showed that a *DMY* BAC clone containing a 60 kb upstream region, a 56 kb coding region and a 1.4 kb downstream region could mimic the expression pattern of *DMY* during the period of sex determination in medaka (Matsuda *et al.*, 2007). To facilitate the screening of transgene-positive fish, we integrated genes encoding GFP and RFP into this BAC clone (Figure 1a). GFP was regulated by the *olvas* promoter, a medaka *vasa* homolog (Shinomiya *et al.*, 2000). Olvas-GFP signals were observed in the gonad on hatching day (Figure 1b). The usefulness of olvas-GFP for isolating candidate founders with germline integration capability in the F_0 generation has been described earlier (Tanaka *et al.*, 2001). RFP was regulated by a *γM2-crystallin* homolog promoter (M Matsuda, unpublished

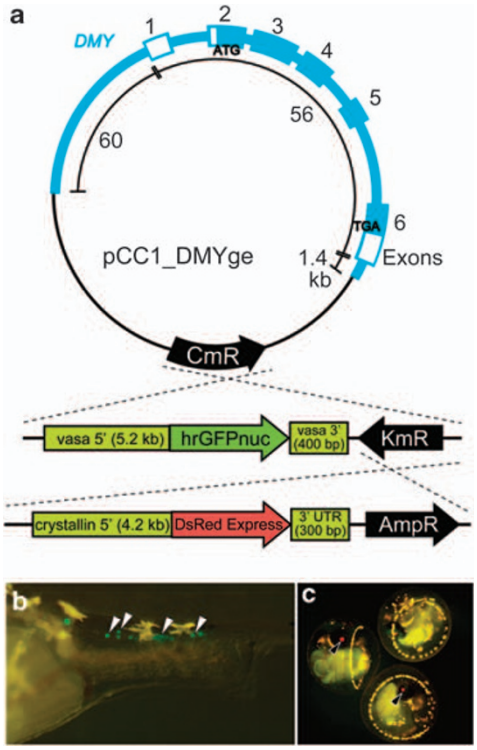

Figure 1 Transgene construct and expression of fluorescent markers in the stable transgenic lines. (**a**) Structure of the BAC clone containing the DMY genomic region. DMY gene comprises six exons and the translation start (ATG) and termination (TGA) site are located in exons 2 and 6, respectively. The numbers inside the circle indicate the length of upstream, gene coding and downstream region of the DMY gene contained in the BAC construct. A fragment encoding olvas-GFP and a kanamycin resistance gene was integrated into the ORF of the chloramphenicol resistance gene of the BAC construct. A fragment encoding crystallin-RFP and an ampicillin resistance gene was then integrated into the ORF of the kanamycin resistance gene. (**b**, **c**) Representative fluorescence microscopy images at 7 days after hatching (**b**) and 6 days after fertilization (**c**). (**b**) Lateral view, anterior is on the left and the dorsal side is up. The gonad lies dorsal to the gut. In the DMY-transgenic lines, green and red signals were observed in the gonad (**b**) and the eye (**c**). GFP and RFP proteins were localized specifically in the germ cells (indicated by white arrowheads in **b**) and the lens (indicated by black arrowheads in **c**), respectively.

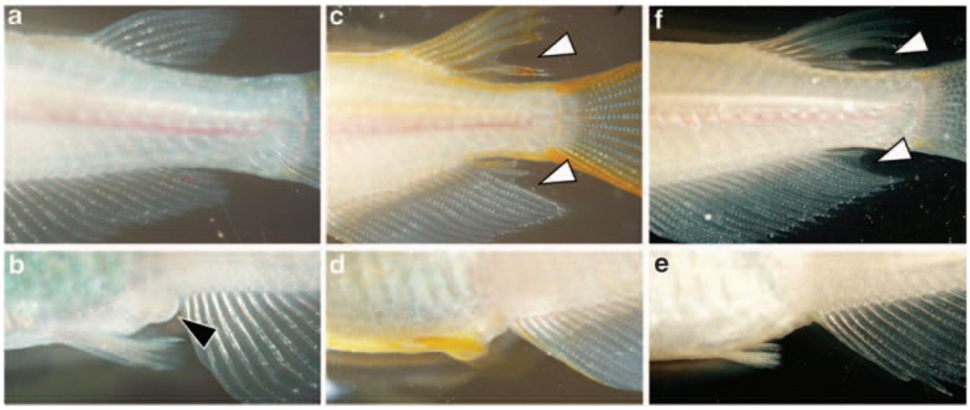

Figure 2 XX sex-reversed males observed in F0-transgenic fish. Representative images of the secondary sex characteristics of normal XX female (**a**, **b**), normal XY male (**c**, **d**), and DMY-injected XX male (**e**, **f**). Both dorsal and anal fins of males are sharp and have a slit (indicated by white arrowheads), whereas rounded anal fins and developed urogenital papillae (indicated by black arrowhead) are characteristic of females.

data). Crystallin-RFP signals were clearly observed in the eye from 3 days after fertilization (Figure 1c). The high intensity of these signals indicated that crystallin-RFP was highly effective for screening F_1 generation progeny that had the heritable transgene. The modified BAC construct was injected into 897 one-cell-stage embryos of the inbred strain Hd-rR. On hatching day, we screened for fry that had green signals in the gonad and were therefore considered strong candidates for germline-transmitting founders. We obtained 57 olvas-GFP-positive fry. After they had grown to adulthood, 5 of the 15 XX fish had male secondary sex characteristics (Figure 2). The XX males were mated with Hd-rR females, and all matings produced fertilized eggs, showing that the XX fish were functional males. Two (ID Nos. 1 and 4) out of five F0 XX males produced embryos that had red signals in the eye, indicating that these progeny had inherited the transgene (Table 1). Genomic PCR analysis revealed that all F_1 crystallin-RFP-positive fish had the exogenous *DMY* gene, and they all developed into male adults. In subsequent generations, the male phenotype was completely linked to the presence of the exogenous *DMY* gene in both lines (Table 2). These results show the establishment of two *DMY*-transgenic strains in which exogenous *DMY* determined the sex of individuals. The strains derived from ID Nos. 1 and 4 XX males are hereafter referred to as strains 1 and 2, respectively.

Table 1 Screening of germline transmission

ID no. of F0 XX male	*Number of F_1 embryos*	
	Crystallin-RFP (−)	*Crystallin-RFP (+)*
1	158	60
2	149	0
3	39	0
4	>50	10
5	>50	0

Abbreviation: RFP, red fluorescent protein.

Table 2 Phenotypic sex of transgenic strains

ID no. of XX male	*Generation*	*Exogenous DMY (−)*		*Exogenous DMY (+)*	
		Female	*Male*	*Female*	*Male*
1	F_1	14	0	0	11
(Strain 1)	F_2	19	0	0	6
	F_3	14	0	0	11
	F_4	6	0	0	6
	F_5	ND		0	20
4	F_1	ND		0	10
(Strain 2)	F_2	12	0	0	22
	F_3	9	0	0	25

Abbreviation: ND, not determined.

Characterization of DMY-transgenic strains

We then examined the genetic and developmental characteristics of the *DMY*-transgenic strains. To validate the expression patterns of the exogenous *DMY*, we performed RT-PCR on several tissues and *in situ* hybridization on adult testes. The exogenous *DMY* were expressed only in the testes (Figure 3a) and *DMY* transcripts were localized in the somatic cells surrounding germ cells in both *DMY*-transgenic strains (Figure 3b). These results show that the exogenous *DMY* recapitulate the expression patterns of the endogenous *DMY*. Next, to examine early gonadal development, we performed histological observations on hatching day. In medaka, it is well known that the first indication of morphological sex differentiation is the difference in the number of germ cells between the sexes at hatching. The germ cells in females continue to proliferate and then enter meiosis, whereas the male germ cells arrest in mitosis just before hatching (Satoh and Egami, 1972). Therefore, germ cells in XX individuals outnumber those in XY individuals at hatching (Kobayashi *et al*., 2004). We counted the number of germ cells at hatching in the two *DMY*-transgenic strains. In both strains, transgene-negative fish had significantly more germ cells than transgene-positive fish—the same

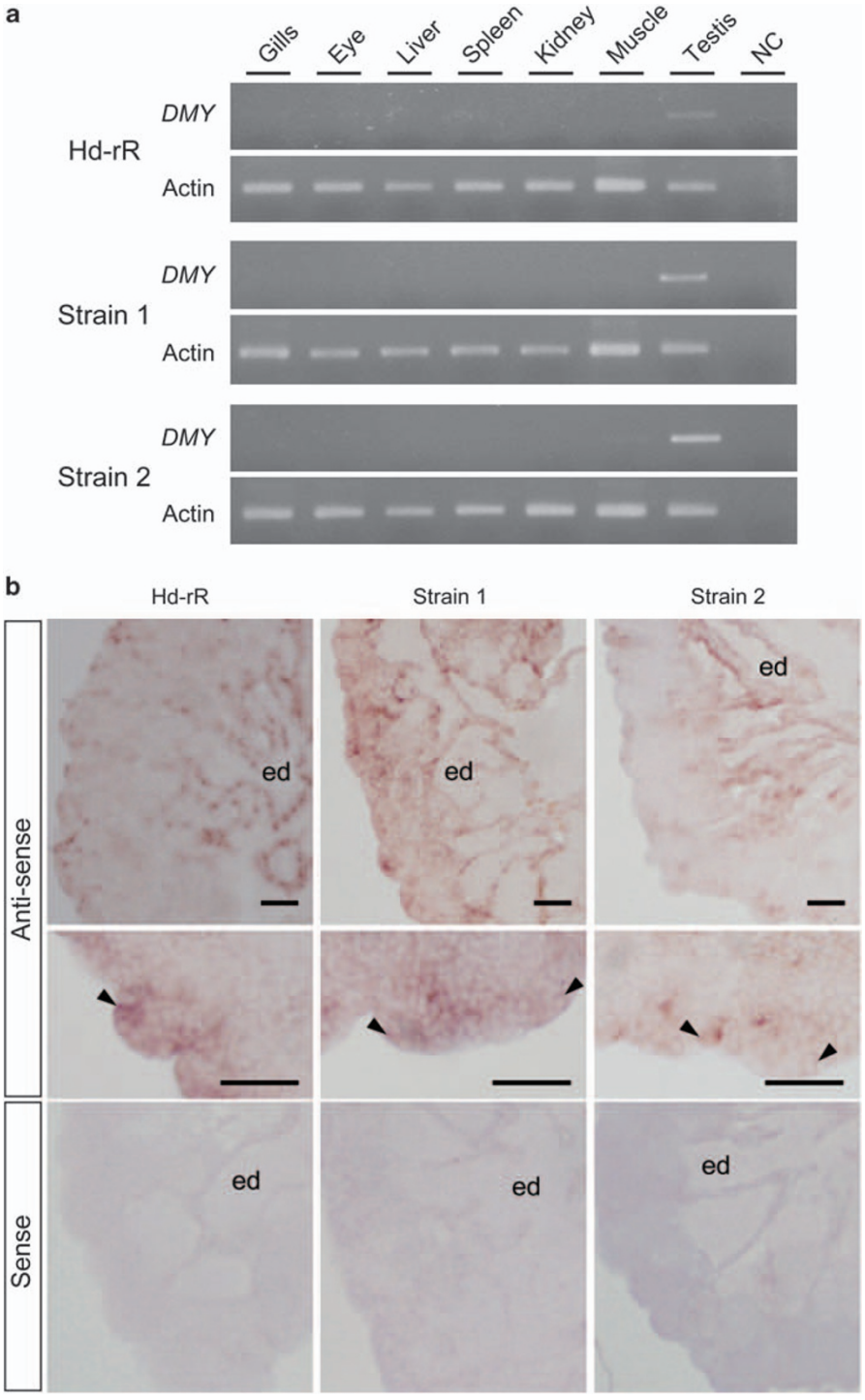

Figure 3 Spatial expression patterns of the exogenous DMY. (**a**) RT-PCR analysis using total RNA extracted from various tissues. A 404-bp DMY product was amplified. As a control, the same RNA extracts were used to amplify a 322-bp product of the ubiquitously expressed β-actin gene. (**b**) *In situ* hybridization for DMY in the adult testes of Hd-rR inbred strain, DMY-transgenic strains 1 and 2. Arrowheads, germ cell-supporting cells; ed, efferent duct; Scale bar, 20 μm.

pattern as in Hd-rR XX and XY individuals (Figure 4). We also examined early gonadal development according to the presence or absence of diplotene oocytes at 10 days after hatching. In this period, the female germ cells are recognizable as oocytes. The gonads of female fry contain germ cells in mitosis and in meiotic prophase (Figure 5a), whereas those of male fry contain only gonia (Figure 5b). In both strains, transgene-negative fish (5 in each strain) had diplotene oocytes (Figures 5c and e), whereas transgene-positive fish (13 in each strain) had no oocytes (Figures 5d and f). No morphological differences were observed between normal males and transgenic-strain

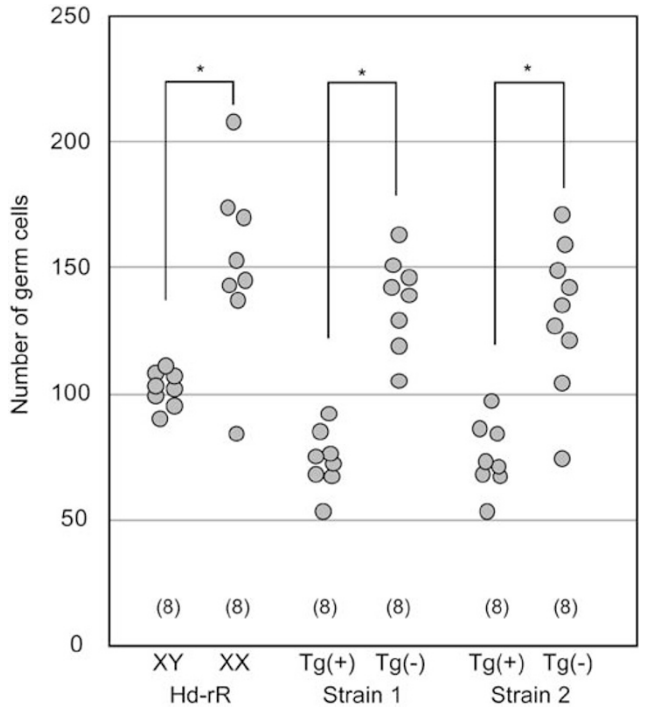

Figure 4 Germ cell numbers in the Hd-rR inbred strain and in the DMY-transgenic strains on hatching day. Numbers of samples are shown in parentheses. There were significant differences between the germ cell numbers in XX and XY fish of the Hd-rR strain and the transgene-positive and -negative fish in both DMY-transgenic strains (*$P<0.01$).

males at 10days after hatching (Figures 5b, d, e). These results show that early gonadal development in these strains did not differ from that of the wild-type strain, suggesting that the exogenous *DMY* gene functioned identically to the wild-type *DMY*. In addition, two observations, there is no morphological differences between normal males and transgenic-strain males in the histological examination of adult testes (Supplementary Figure 3) and transgenic-strain males are fully fertile at least in the laboratory condition, indicate that transgene-positive male fish is functionally equivalent to wild-type males.

The karyotype of medaka consists of 24 chromosome pairs, and *DMY* is located on LG 1. We performed a linkage analysis to reveal the locus of the exogenous *DMY*. In strain 1, a marker on LG 23, *MF01SSA036A04*, had a 100% concordance rate with the exogenous *DMY* (Figure 6a). To narrow down the candidate locus, we examined three additional markers (*MF01FSA018C03*, *OLb1905f* and *OLe1111g*). *OLb1905f* had a concordance rate of 96.2%, and that of *OLe1111g* was 100%, indicating that exogenous *DMY* was located on the telomeric side of *OLb1905f* on LG 23. In strain 2, two markers on LG 5, *Mf01SSA017E11* and *MF01SSA102C03*, had high concordance rates (both 92.6%) (Figure 6b). To obtain a completely linked marker, we examined two additional markers (*MF01SSA013C06* and *MF01SSA035D11*). These markers had respective concordance rates of 96.3 and 100%, indicating that exogenous *DMY* was located between *MF01SSA013C06* and *MF01SSA102C03* on LG 5. In addition, no markers had high concordance rates with other LGs in either strain, suggesting that in both strains exogenous *DMY* was integrated into one locus.

Next, we used real-time PCR to examine the copy number and expression levels of exogenous *DMY* during the period of sex determination. The comparative Ct method revealed that the copy number of strain 1 was 2.0 and that of strain 2 was 10.9 relative to that in the Hd-rR.Y^{HNI} strain (Table 3). The expression level of exogenous *DMY* relative to that in Hd-rR.Y^{HNI} on hatching day was 1.6 in strain 1 and 10.6 in strain 2. This suggests that strain 1 had 2 copies of exogenous *DMY* and strain 2 had 11 copies, and the expression levels were proportional to the copy numbers, suggesting that there was no negative feedback mechanism in the transcriptional regulation of *DMY*.

Discussion

Artificial sex chromosomes in vertebrates

Present results show that two *DMY*-transgenic strains, in which the exogenous *DMY* gene determines the sex of individuals, were established. Our results also show that LGs 23 and 5 functioned as sex chromosomes in *DMY*-transgenic strains 1 and 2, respectively. Transgenic insertion of *DMY* has made an autosome into a sex chromosome in other words, the *DMY*-transgenic strains each had an artificial Y chromosome.

Chromosomal sex determination is widely used by vertebrates such as many fish and reptiles, all amphibians, birds and mammals. Although the origin of sex chromosomes may differ among taxa, it is hypothesized that sex chromosomes are derived from autosomes (Charlesworth *et al*., 2005). The most studied sex chromosomes are X and Y in mammals and Z and W in birds and snakes. These sex chromosome pairs are morphologically distinct and contain several chromosome-specific genes. According to standard models for sex chromosome evolution, heteromorphic sex chromosomes are thought to arise through suppression of recombination around the sex-determining gene, maintaining the non-recombining region in a constantly heterozygous state and leading to numerous deletions of functional genes and the accumulation of repetitive sequences. This degenerative process spreads the sex-specific region over almost the entire chromosome, and several genes with sex-specific advantages accumulate. In the mouse, besides *Sry*, two male-specific genes, an RNA-binding motif (*Rbm*) gene and the ubiquitin-activating enzyme gene (*Ube1y*), are located on the Y chromosome (Kay *et al*., 1991; Mitchell *et al*., 1991; Elliott *et al*., 1996). These genes are expressed in spermatogonia and spermatids (Odorisio *et al*., 1996; Mahadevaiah *et al*., 1998); a deletion mutant of the Y chromosome that had lost these genes had a high incidence of sperm abnormalities (Mahadevaiah *et al*., 1998). These results indicate that *Rbm* and *Ube1y* are required for spermatogenesis, and that the lack of these genes is the cause of the sterility observed in the *Sry*-transgenic mouse (Koopman *et al*., 1991). Thus, a high level of differentiation of sex chromosomes is likely to act as a barrier to the production of artificial sex chromosomes in mammals. In contrast to mammals, sex chromosomes are homomorphic in medaka. The sex-determining region on the Y chromosome and the corresponding region on the X

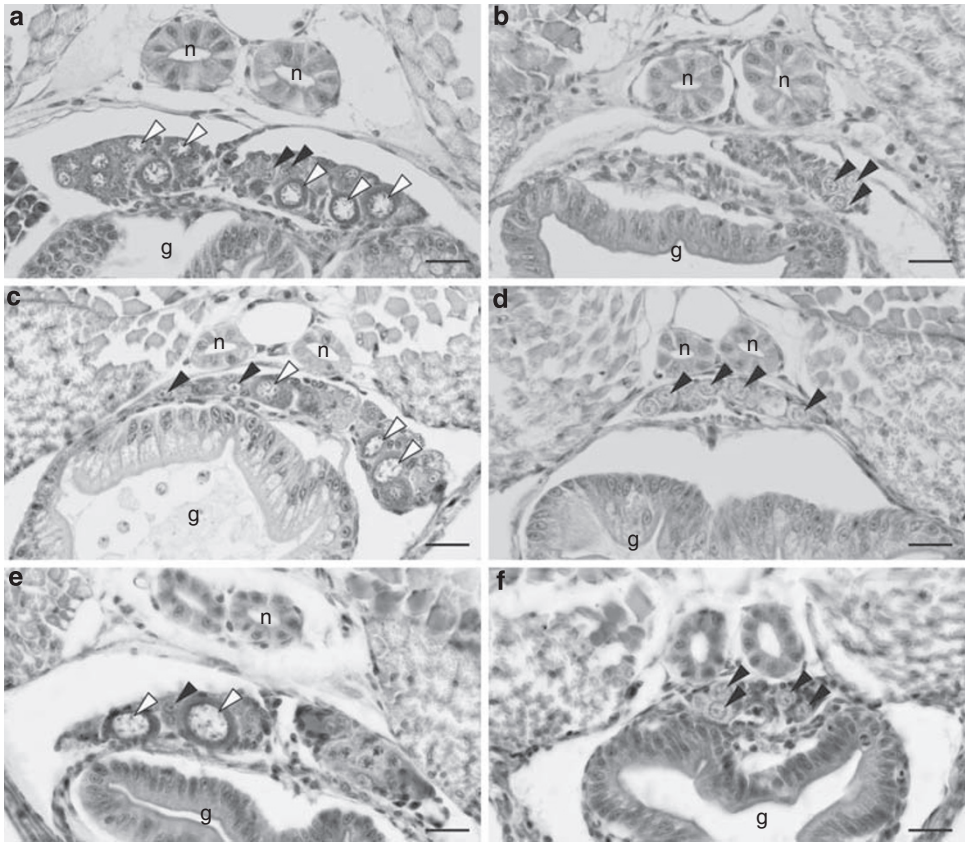

Figure 5 Gonadal sex differentiation in the DMY-transgenic strains at 10 days after hatching . Representative gonadal histologies of normal XX female (**a**), normal XY male (**b**), transgene-negative (**c**), and transgene-positive (**d**) fish in strain 1 and transgene-negative (**e**) and transgene-positive (**f**) fish in strain 2. Black arrowheads indicate gonia and white arrowheads indicate diplotene-stage oocytes. n, nephric duct; g, gut. Bars, 20 μm.

chromosome were recently fully sequenced. The Y-specific region proved to be 258 kb long and *DMY* appeared to be the only functional gene in this region (Kondo *et al*., 2006). In addition, we found a wild medaka population in which Y chromosomes bearing a loss-of-function *DMY* mutation were present in one-third of females (Otake *et al*., 2008). In this population, the Y chromosome, which has lost its male-determining function, behaves as an X chromosome. These results reveal that in medaka, the difference between the X and Y chromosomes is defined only by the absence or presence of *DMY*. Here, we show that the introduction of *DMY* is sufficient to produce an artificial sex chromosome in this species. Furthermore, unlike *Sry*, *DMY* appears to have arisen from duplication of the autosomal *DMRT1* gene and its insertion into LG 1 (Kondo *et al*., 2006). This event suggests that any chromosome could have become the sex chromosome in the ancestor of medaka.

Plasticity of sex chromosomes in lower vertebrates

The mammalian XY sex-determining system appears to be at least 150 million years old, since *SRY* orthologs have been identified on the Y chromosome in both branches of therian mammals, placentals and marsupials (Waters *et al*., 2007), except for a few exceptional rodents that have recently lost their Y chromosome (Just *et al*., 1995). The avian ZW sex-determining system is likely to have appeared between 60 and 100 million years ago according to fossil, cytogenetic and molecular data (Harlid *et al*., 1997; Shetty *et al*., 1999). Hence, higher vertebrate species seem to have conserved their sex chromosomes in their long lineage.

On the other hand, no consistent sex-specific chromosomes have been observed in fish and amphibians with genetic sex determination that would suggest conservation of sex chromosomes (Schmid and Steinlein, 2001; Devlin and Nagahama, 2002). Recent studies have shown that the sex chromosomes in two medaka-related species, *O. luzonensis* and *O. dancena*, are homologous to different autosomes in medaka (Takehana *et al*., 2007; Tanaka *et al*., 2007). Similar phenomena have been observed in salmonids and sticklebacks, suggesting that sex chromosomes have changed frequently in fish (Woram *et al*., 2003; Peichel *et al*., 2004; Phillips *et al*., 2007). In the Japanese frog, *Rana rugosa*, two distinct sex chromosome types, XX/XY and ZZ/ZW, were observed in the same species (Miura *et al*., 1998). In addition, the change of heterogametic sex from male to female appears to have independently occurred twice during frog speciation (Ogata *et al*., 2008). These results suggest that the sex chromosomes are generally not conserved in lower vertebrates. Our results show that switching of sex chromosomes can occur easily through the generation of new master sex-determining genes in vertebrates that possess homomorphic sex chromosomes. This plasticity

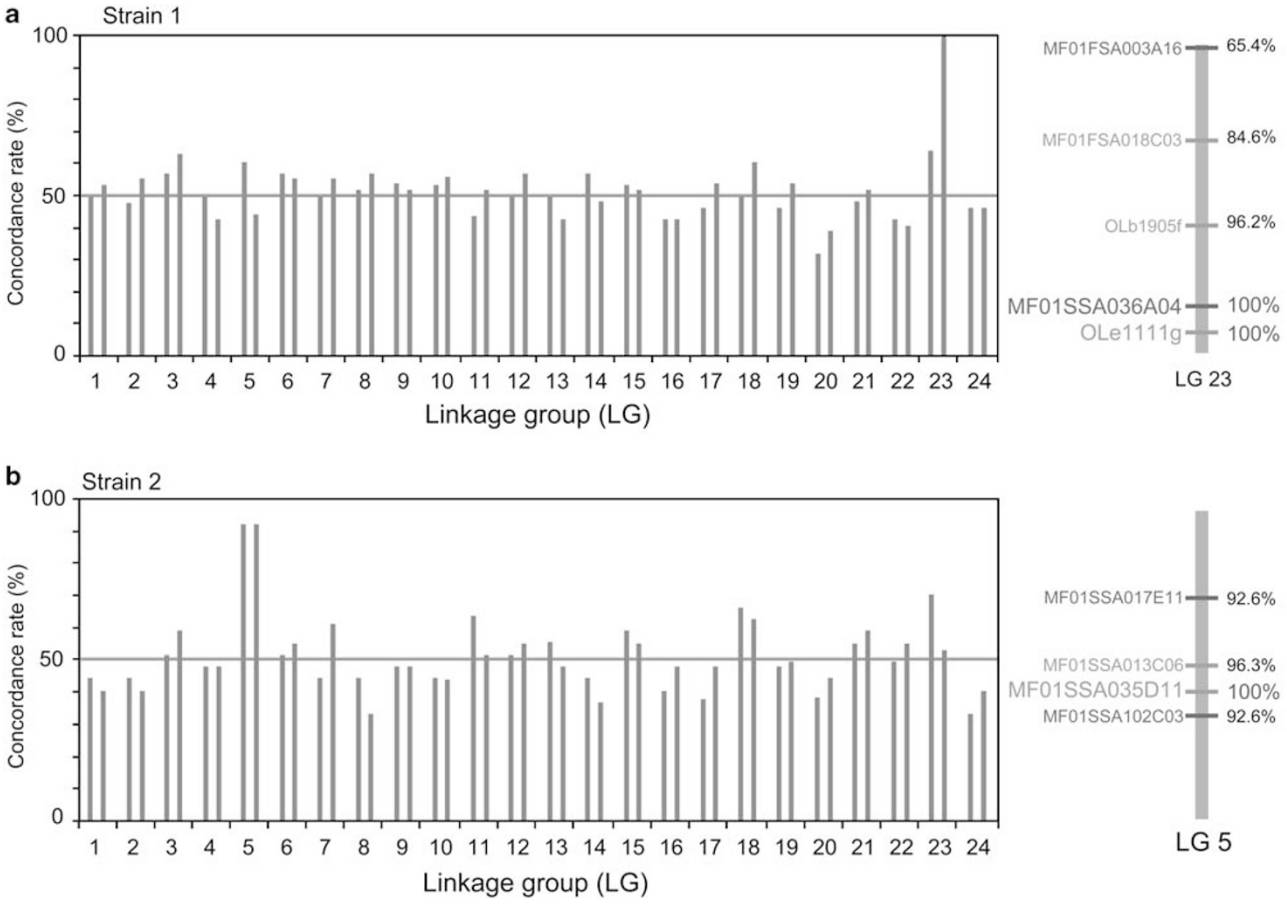

Figure 6 Mapping of the transgene in DMY-transgenic strains. The concordance rates of each M-marker are shown by solid bars for strain 1 (**a**) and strain 2 (**b**) in the graphs on the left. Positions of the markers that showed high concordance rates are represented on the right. Markers with 100% concordance rates were found in linkage group 23 in strain 1 and in linkage group 5 in strain 2.

Table 3 Copy number and expression level of exogenous *DMY* in the transgenic strains

Strain	*Copy number*			*Expression level*				
	Average Ct (DMY)	*ΔCt*	*Relative to Hd-rR.Y^{HNI}*	*Average Ct (DMY)*	*Average Ct (EF-1a)*	*ΔCt*	*ΔΔCt*	*Relative to Hd-rR.Y^{HNI}*
Hd-rR.Y^{HNI}	24.63 ± 0.03	0	1	29.41 ± 0.39	18.24 ± 0.16	11.17 ± 0.49	0	1
Strain 1	23.63 ± 0.07	1.00 ± 0.09	2.0 (1.9–2.1)	28.92 ± 0.58	18.42 ± 0.13	10.5 ± 0.69	−0.67 ± 0.69	1.59 (0.99–2.57)
Strain 2	21.18 ± 0.13	3.45 ± 0.15	10.9 (9.8–12.1)	25.79 ± 0.38	18.03 ± 0.18	7.76 ± 0.49	−3.41 ± 0.49	10.63 (14.93–7.57)

appears to underlie the frequent switching of sex chromosomes observed in fish and frogs. As many lower vertebrate species have morphologically homomorphic sex chromosomes (Schmid and Steinlein, 2001; Devlin and Nagahama, 2002), this plasticity may be a characteristic of lower vertebrates that is not found in higher vertebrates.

Conflict of interest

The authors declare no conflict of interest.

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