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The Y Chromosome That Lost the Male-Determining Function Behaves as an X Chromosome in the Medaka Fish, *Oryzias latipes*

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ABSTRACT

The medaka, *Oryzias latipes*, has an XX/XY sex-determination system, and a Y-linked DM-domain gene, *DMY*, is the sex-determining gene in this species. Since *DMY* appears to have arisen from a duplicated copy of the autosomal *DMRT1* gene ~10 million years ago, the medaka Y chromosome is considered to be one of the youngest male-determining chromosomes in vertebrates. In the screening process of sex-reversal mutants from wild populations, we found a population that contained a number of XY females. PCR, direct sequencing, and RT–PCR analyses revealed two different null *DMY* mutations in this population. One mutation caused loss of expression during the sex-determining period, while the other comprised a large deletion in putative functional domains. YY females with the mutant-type *DMY* genes on their Y chromosomes were fully fertile, indicating that the X and Y chromosomes were functionally the same except for the male-determining function. In addition, we investigated the frequencies of the sex chromosome types in this population over four successive generations. The Y chromosomes bearing the mutant-type *DMY* genes were detected every year with no significant differences in their frequencies. These results demonstrate that aberrant Y chromosomes behaving as X chromosomes have been maintained in this population.

In vertebrates, only two genes have been identified as master sex-determining genes, namely *SRY/Sry* in mammals (Gubbay *et al.* 1990; Sinclair *et al.* 1990; Koopman *et al.* 1991) and *DMY* (DM-domain gene on the Y chromosome) in the medaka, *Oryzias latipes* (Matsuda *et al.* 2002, 2007; Nanda *et al.* 2002). It has been assumed that *SRY/Sry* evolved before the eutherian radiation ~80 million years ago (MYA), since humans and rodents, and all other orders of eutherian mammals examined so far, have an *SRY/Sry* gene (Graves 2002). In contrast to the widespread distribution of *SRY/Sry* in mammals, *DMY* is found only in the medaka and *O. curvinotus*, the closest relative to the medaka (Kondo *et al.* 2003; Matsuda *et al.* 2003; Tanaka *et al.* 2007). The *DMY* gene appears to have arisen from a duplicated copy of the autosomal *DMRT1* gene (Nanda *et al.* 2002; Kondo *et al.* 2006) and this *DMRT1* duplication event is estimated to have occurred ~10 MYA in a common ancestor of *O. latipes* and *O. curvinotus* (Kondo *et al.* 2004).

In mammals, sex chromosomes are highly dimorphic. The large gene-rich X and small heterochromatic Y chromosomes are almost completely differentiated. The human X chromosome bears ~1000 genes with a variety of general and specialized functions (Ross *et al.* 2005), whereas the human Y chromosome encodes only 45 unique proteins (Skaletsky *et al.* 2003). In addition, crossovers occur only in a small homologous region, designated the pseudoautosomal region. On the other hand, both X and Y chromosomes are homomorphic in the medaka. Sex chromosomal crossovers occur over almost the entire length of the corresponding linkage groups. In fact, the Y chromosome-specific region is ~260 kb in length (Kondo *et al.* 2006). In addition, the genotypic sex can easily be reverted by sex hormone or high-temperature treatments (Kobayashi and Iwamatsu 2005; Sato *et al.* 2005; Iwamatsu *et al.* 2006; Hattori *et al.* 2007). These results indicate that the medaka sex chromosomes are quite undifferentiated and suggest that sex determination in the medaka is at an early stage of evolution and has not reached a similar stability to that in other vertebrates like birds and mammals.

Since the identification of *DMY*, we have screened sex-reversal mutants from wild populations of the medaka to reveal the molecular functions of *DMY* and identify other genes involved in sex differentiation. In the process of screening, we found a population in O-bu (Aichi prefecture) that contained a number of XY sex-reversed females. In the present study, we demonstrate that there are two types of Y chromosomes that have loss-of-function-type *DMY* mutations in this population and found that these Y chromosomes were stably maintained for at least four successive generations. These

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TABLE 1

Phenotypic and genotypic sexes of the wild medaka in the O-bu population (2003)

	Female		Male	
Total	XX	XY	XX	XY
113	35	17	0	61

facts imply that in organisms like the medaka with undifferentiated sex chromosomes, the Y chromosome that has lost the male-determining function can behave as an X chromosome.

MATERIALS AND METHODS

Sexing of wild fish: Phenotypic sex was judged from secondary sex characters, namely the shapes of the dorsal and anal fins and the papillary processes on the male anal fin rays. Genotypic sex, XY or XX, was determined by the presence or absence of the *DMY* gene using PCR amplification of caudal fin clip DNA extracted according to Shinomiya *et al.* (1999). PCR was performed with the primer set PG17.5 and PG17.6 for *DMY* and *DMRT1* at an annealing temperature of 55°. This primer set amplified a 982-bp *DMY* fragment and a 1245-bp *DMRT1* fragment. The PCR products were analyzed by 1% agarose gel electrophoresis. Individuals with only the *DMRT1* fragment were judged to be XX, while those with both the *DMY* and *DMRT1* fragments were judged to be XY.

Progeny test: The *DMY* gene of the northern population had 21 nucleotide deletions in intron 2 compared to that of the southern population (Shinomiya *et al.* 2004). In order to distinguish between Y chromosomes derived from XY females and that derived from inbred strain XY males, O-bu XY females were mated with XY Hd-rR.Y^{HNI} males, which had the HNI (northern population)-derived *DMY* gene on the genetic background of Hd-rR (southern population) (Matsuda *et al.* 1998), since the O-bu population belongs to the southern population. Four genotypes were distinguished in the F_1 progeny (XX, XY^m, XY^p, and Y^mY^p; where Y^m and Y^p are maternal and paternal Y chromosomes, respectively) by separating the *DMY* PCR products in 10% vertical polyacrylamide slab gels according to a previous report (Shinomiya *et al.* 2004).

PCR and direct sequencing: To screen for mutations in the amino acid coding sequence of *DMY*, exons 2–6 of *DMY* were PCR amplified from caudal fin clip DNA using the following primer sets: exon 1: PG17_103S, 5′-GGA AAC AAT TTT GCC TTG GA-3′ and PG17_102U, 5′-ACA CAA CGC ACG CAT AAA AA-3′; exon 2: PG17ex2.1, 5′-GGA GTC ACG TGA CCC TCT TTC TTG GG-3′ and PG17ex2.2, 5′-TTT CGG GTG AAC TCA CAT GGT TGT CG-3′; exon 3: PG17ex3.1, 5′-GCA ACA GAG AGT TGG ATT TAC GTC TCA-3′ and PG17ex3.2, 5′-CTT TTG ACT TCA GTT TGA CAC ATC AAT G-3′; exon 4: PG17ex4.1, 5′-CTC AGG TTT GAC TTG GAT GCT GAC CTG A-3′ and PG17ex4.2, 5′-CAA ACC AGG CCA TGA CCA TTC CGA-3′; exon 5: PG17ex5.1, 5′-CCG ATT CTA GCG GAT GAT GCC ACC-3′ and PG17ex5.2, 5′-GGG AGC CAA AAA TGC GCC ACA TAA-3′; and exon 6: PG17ex6.1, 5′-GTC ATT AAC ACA ACG CAC AAC AAC TT-3′ and PG17ex6.2, 5′-AAA AAC CAG AAG ACC CGA GAG GAA G-3′ (Figure 1). The PCR products were sequenced directly in an ABI Prism 310 automated sequencer. Two additional primers in intron 1, upDMY_D_31 (5′-GAG TGT GTG TGA GCG CAA GT-3′) and upDMY_D_33 (5′-TTG AAA TCC GAG CTT CTG AAA-3′), were used for PCR and direct sequencing of mutant *DMY* genes.

RNA extraction and RT–PCR: Total RNA was extracted from embryos or fry at 9.5 days after fertilization in the F_1 generation using an RNeasy Mini kit (QIAGEN) and subjected to RT–PCR using a OneStep RT–PCR kit (QIAGEN). Aliquots (20 ng) of the total RNA samples were used as templates in 25-μl reaction volumes. The PCR conditions were 30 min at 55°; 15 min at 95°; 35 cycles of 20 sec at 96°, 30 sec at 55°, and 60 sec at 72°; and 5 min at 72°. The primers for *DMY* (DMYspe, 5′-TGC CGG AAC CAC AGC TTG AAG ACC-3′ and 48U, 5′-GGC TGG TAG AAG TTG TAG TAG GAG GTT T-3′) amplified a 404-bp DNA fragment. The primers for β-actin (3b, 5′-CMG TCA GGA TCT TCA TSA GG-3′, and 4, 5′-CAC ACC TTC TAC AAT GAG CTG A-3′) amplified a 322-bp DNA fragment. A primer in exon 1, PG17_61S (5′-CGT CTG GCT TCA CCG TTG GA-3′), was used to amplify the mutant-type *DMY* of Y^{wObu2}.

Gene copy number quantification using real-time PCR: Wild medaka DNA was extracted according to Shinomiya *et al.* (1999). The purity and concentration of the DNA were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The DNA concentrations of all samples were adjusted to give a total of 20 ng for each real-time quantitative PCR assay. Primers were designed to amplify a 106-bp fragment of *DMY* exon 4 (DMY_RT_4-1, 5′-GAG GAA GCG TCT GAC TGC-3′ and DMY_RT_4-2, 5′-CCT GGT ACT GCT GGT AGT TGT G-3′). SYBR Green PCR master mix (Takara) was used according to the manufacturer's protocol with an ABI 7000 real-time PCR instrument (Applied Biosystems). The PCR conditions were 1 min at 94°; 40 cycles of 5 sec at 95°, and 30 sec at 65°. To confirm that the amplified PCR products were specific for the *DMY* gene, their melting temperatures were determined from dissociation curves generated by the real-time quantitative PCR instrument. 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 (data not shown). Quantification of the copy number of *DMY* was achieved by comparison of the real-time quantitative PCR results to a standard curve generated from

TABLE 2

Progeny test of XY females in the O-bu population

ID no. of XY female	Total	XX		XY^m		XY^p		Y^mY^p	
		Female	Male	Female	Male	Female	Male	Female	Male
01	54	11	0	10	0	0	23	0	10
02	34	11	0	7	0	0	8	0	8
03	22	5	0	4	0	0	5	0	8

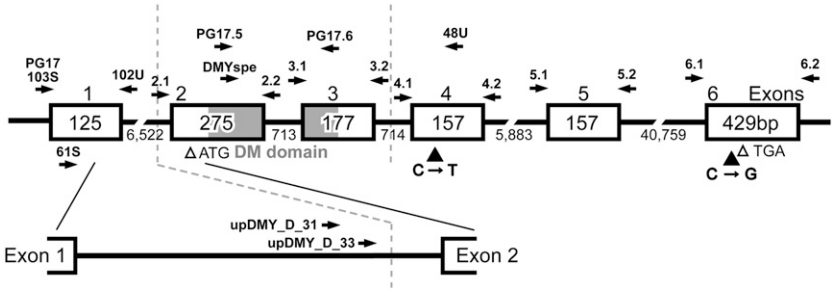

FIGURE 1.—*DMY* structure of the Hd-rR.Y^{HNI} congenic strain. Open boxes, shaded boxes, and the horizontal line indicate exons, the DM domain, and introns, respectively. Open arrowheads indicate the translation start site, ATG, and stop site, TGA. The numbers represent the nucleotide sequence lengths (bp). Solid arrows indicate the primer positions. Solid arrowheads indicate the positions of substitutions in the *DMY* of Y^{wObu1}. The region between the two dotted vertical lines was deleted in the *DMY* of Y^{wObu2}.

the inbred strain Hd-rR. Hd-rR DNA was serially diluted by 1:2 from 80 ng to 5 ng. The real-time quantitative PCR instrument software was used to plot the threshold cycle (cycle number of each sample that intersected the threshold line) of each sample on the standard curve of Hd-rR DNA and thereby determine the quantity. Since each sample had an identical amount of DNA as the initial template (20 ng), the *DMY* gene copy number could be calculated as a ratio of the quantity of each sample to that of Hd-rR. The real-time quantitative PCR experiments were repeated three times.

Statistical analysis: The significance of differences among the frequencies of sex chromosomes was tested by the *G*-test.

RESULTS AND DISCUSSION

Frequent appearance of XY females: Previously, we performed genotypic sexing of 2274 wild-caught medaka from 40 localities and found 12 XY females from 8 localities (SHINOMIYA *et al.* 2004). The average frequency of XY sex-reversed females was 1.1% (12 of 1089 XY individuals). In 2003, we performed genotypic sexing of 113 fishes in the O-bu population (Aichi prefecture) and found 17 XY females (Table 1). The frequency of XY sex-reversed females in this population was 21.8% (17 of 78 XY individuals). This value was significantly higher than those in the other populations examined in our previous study.

Y chromosomes bearing *DMY* with no expression during the sex-determining period: To clarify the cause of the XY sex reversals, three XY females were mated with XY males of Hd-rR.Y^{HNI}, and the genotypic and phenotypic sexes of the F1 progeny were analyzed (Table 2). All XX individuals in the F1 progeny were female and all XY^p and Y^mY^p individuals were male, while all XY^m individuals were female (where Y^m and Y^p are maternal and paternal Y chromosomes, respectively). In other words, the occurrence of XY sex reversals in the F_1 progeny was perfectly linked to the presence of the maternal Y chromosome. Since *DMY* is considered to be the sole functional gene in the Y-specific region (KONDO *et al.* 2006), this finding suggests that the sex-reversal mutants had a mutation at *DMY* or a gene tightly linked to *DMY*. A previous study demonstrated that all XY sex-reversal mutants in wild populations were associated with defective *DMY* and could be classified into two types. One had mutations in the amino acid coding sequence of *DMY*, while the other had a normal coding sequence but exhibited depressed or no *DMY* expression during the sex-determining period (OTAKE *et al.* 2006). Therefore, we sequenced exons 2–6 of *DMY* to identify possible mutations in the amino acid coding sequence. We found a C-to-T substitution in exon 4 and a C-to-G substitution in exon 6 (Figure 1), both of which were synonymous substitutions. Next, to examine *DMY* expression during the sex-determining period, we performed RT–PCR on the hatching day. *DMY* transcripts were not detected in F_1 XY^m progeny (Figure 2), suggesting that the cause of the sex reversal in the XY

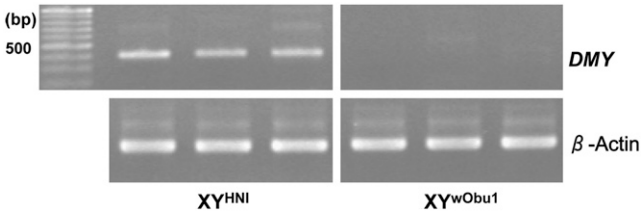

FIGURE 2.—RT–PCR analysis of *DMY* in XY^{wObu1} on the hatching day. The *DMY* PCR products obtained using the DMYspe and 48U primer set were subjected to 1% agarose gel electrophoresis. β-actin served as a positive control.

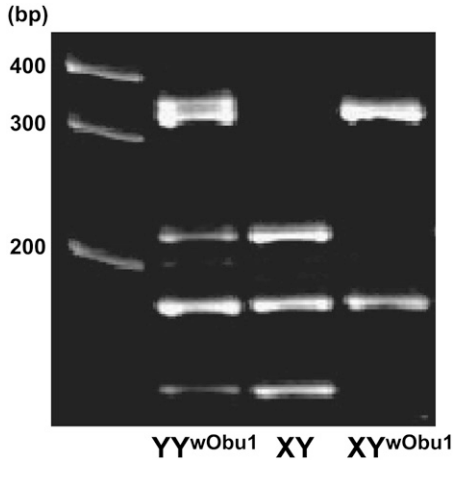

FIGURE 3.—Electrophoretic patterns of PCR–RFLP products for identifying XY^{wObu1}. *DMY* PCR products digested with *Hpy*8I were subjected to 10% polyacrylamide gel electrophoresis. The *DMY* fragments of Hd-rR and XY^{wObu1} have one and two recognition sites, respectively.

TABLE 3

Progeny test of YY^{wObu1} females

	XY^{wObu1}		XY	
Total	Female	Male	Female	Male
21	13	0	8	0

females was severe suppression or elimination of *DMY* expression during the sex-determining period, similar to the case for sex-reversal mutants from Oura (Otake *et al.* 2006). We designated this Y chromosome Y^{wObu1}. This mutated *DMY* may contain regulatory mutations in the flanking region of this gene.

Spread of mutant-type Y chromosomes: To investigate the frequency of Y^{wObu1}, we performed a PCR–RFLP analysis for all 78 XY individuals in the O-bu population. Since the mutant-type *DMY* on Y^{wObu1} had a C-to-T substitution in exon 4, PCR amplification using the Ex4.1 and Ex4.2 primer set (Figure 1) and restriction enzyme digestion with *Hpy*8I produced three bands for wild-type *DMY*, two bands for mutant-type *DMY*, and four bands for wild-type and mutant-type heterozygote *DMY* (Figure 3). We found 16 individuals with Y^{wObu1} and 4 individuals with both wild-type Y and Y^{wobu1}. These results demonstrated that Y^{wObu1} existed at a high frequency in the O-bu population and raised the possibility that there were $Y^{wObu1}Y^{wObu1}$ individuals in this population since XY^{wObu1} females were fully fertile. Since the PCR–RFLP method cannot distinguish between XY^{wObu1} and $Y^{wObu1}Y^{wObu1}$ individuals, we performed real-time PCR for quantification of the copy number of mutant-type *DMY*. By comparison of the threshold cycle of each sample with the standard curve of Hd-rR genomic DNA, which contains one copy of the *DMY* gene, we determined the copy number (see materials and methods). We examined 16 individuals with Y^{wObu1} and concluded that 14 individuals had a single copy and 2 individuals had two copies of the mutant-type *DMY*. As a result, PCR–RFLP and real-time PCR analyses could detect 14 XY^{wObu1}, 2 $Y^{wObu1}Y^{wObu1}$ and 3 YY^{wObu1} males, and interestingly one YY^{wObu1} female in this population.

Y chromosome with a second *DMY* mutation: Our previous study showed that all YY individuals with wild-type and mutation-bearing Y chromosomes with low *DMY* expression developed as males (Otake *et al.* 2006). In fact, the normal Y chromosome could induce male development in three YY^{wObu1} individuals in this population. These findings raised the possibility that the YY^{wObu1} female had another null mutation at the *DMY* locus. Therefore, we mated the YY^{wObu1} female with XX males of an Hd-rR inbred strain induced by high-temperature treatment (Sato *et al.* 2005; Hattori *et al.* 2007). Not only the F_1 progeny with Y^{wObu1} but also the progeny with the other Y chromosome grew as females (Table 3). These results demonstrated that the other Y chromosome had also lost the male-determining function and bore another *DMY* mutation. We designated this new deficient Y chromosome Y^{wObu2}. Next, we carried out PCR and direct sequencing analyses of the *DMY* gene using the F_1 XY^{wObu2} female progeny. The sequences of exons 1 and 4–6 were normal. However, the primer sets for exons 2 and 3 resulted in no amplification. Thus, we designed a new primer, upDMY_D_31, in intron 1 and performed PCR with 48U in exon 4 (Figure 1). This primer set amplified an ~2-kb fragment in XY^{wObu2} females in contrast to a 5.5-kb fragment in normal XY males (Figure 4A). Sequencing analysis of these PCR fragments revealed that an ~3.5-kb genomic region including exons 2 and 3 was deleted in the *DMY* gene of Y^{wObu2}. RT–PCR and direct sequencing analyses of embryos on the hatching day demonstrated that exons 2 and 3 were deleted in the transcripts (Figure 4B). Exons 2 and 3 of *DMY* contain the DM domain, a putative functional DNA-binding motif found in DSX of *Drosophila melanogaster* and MAB-3 of *Caenorhabditis elegans* (Raymond *et al.* 1998). Since all the F_1 XY^{wObu2} progeny were females, the *DMY* transcripts lacking the DM domain appeared to be nonfunctional. In addition, the fact that the *DMY* gene

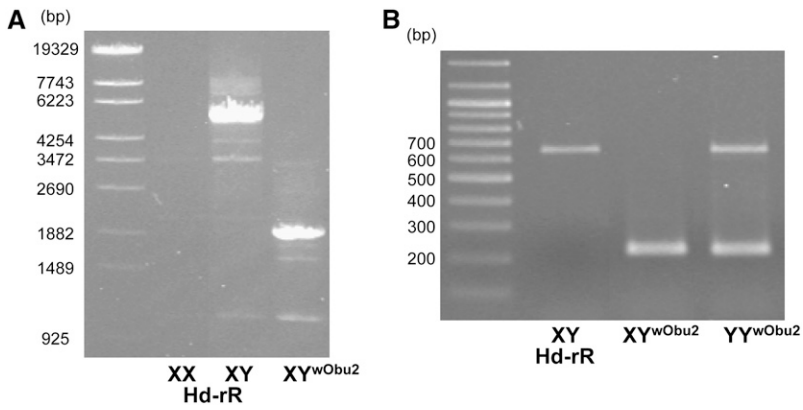

Figure 4.—Genomic and RT–PCR analyses of *DMY* in XY^{wObu2}. (A) The *DMY* PCR products obtained using the upDMY_D_33 and 48U primer set were subjected to 1% agarose gel electrophoresis. (B) The *DMY* RT–PCR products obtained using the 61S and 48U primer set were subjected to 1% agarose gel electrophoresis.

TABLE 4

Phenotypic sex and sex chromosome types in the O-bu population

		Female					Male		
Year	Total	XX	XY^{wObu1}	XY^{wObu2}	$Y^{wOub1}Y^{wObu1}$	$Y^{wObu1}Y^{wObu2}$	XY	YY^{wObu1}	YY^{wObu2}
2003	113	34	14	1	2	1	58	3	0
2004	131	46	12	0	0	0	59	14	0
2005	55	10	14	0	1	0	27	3	0
2006	113	52	9	0	0	0	36	13	3

on Y^{wObu2} did not have a C-to-T substitution in exon 4 (data not shown) suggested that Y^{wObu1} and Y^{wObu2} were generated independently. The *DMY* gene in Y^{wObu2} carries a deletion of the region bound by the primers for genotyping (PG17.5 and PG 17.6), thereby raising the possibility that XY^{wObu2} individuals had been judged as XX. To explore this possibility, we examined all 35 individuals judged as XX using the upDMY_D_33 and 48U primer set (Figure 1), which amplified a 1013-bp fragment of the *DMY* gene in Y^{wObu2}. As a result, we found one XY^{wObu2} female. Collectively, seven combinations of sex chromosomes were found: XX, XY^{wObu1}, $Y^{wObu1}Y^{wObu1}$, XY^{wObu2}, $Y^{wObu1}Y^{wObu2}$, XY, and YY^{wObu1} in the 2003 O-bu population (Table 4).

Stability of mutant-type Y chromosomes: Four types of sex chromosomes, namely two aberrant Y chromosomes with loss-of-function *DMY* mutations and normal X and Y chromosomes, were observed in the O-bu population. To explore whether the mutant-type Y chromosomes were maintained in subsequent generations, we analyzed the frequency of each sex chromosome type in this population over four successive generations (Table 4). We found the Y^{wObu1} chromosome in each year and the Y^{wObu2} chromosome in 2006. The differences in the frequencies of each sex chromosome over 4 years were not significant (*G*-test, Gadj = 12.542, d.f. = 9, $P = 0.1844$; Table 5). These results demonstrated that the mutant-type Y chromosomes have been maintained in this population. In addition, the facts that XY^{wObu1}, $Y^{wObu1}Y^{wObu1}$, XY^{wObu2}, and $Y^{wObu1}Y^{wObu2}$ are viable in the wild and XY^{wObu1} and $Y^{wObu1}Y^{wObu2}$ females are fully fertile in the laboratory condition suggest that X and mutant-type Y chromosomes were functionally the same and these Y chromosomes appear selectively neutral.

The frequency of XY sex-reversed females in the O-bu population was considerably high compared with the average frequency of other wild populations (~1%) described in Shinomiya *et al.* (2004). High frequency of XY females has been observed in Goshogawara (Aomori prefecture). We found 14 XY females (18.2%) of 77 XY individuals from this population in 2005 (M. Sakaizumi, unpublished data). All these XY females had a single nucleotide insertion in the poly (C) tract in exon 3 of *DMY* identical to the case for XY sex-reversal mutants from Awara (Matsuda *et al.* 2002; Otake *et al.* 2006). Both populations appear to have small population sizes and to be isolated from other populations. These facts imply that the mutant-type Y chromosomes could have become frequent by means of genetic drift due to population bottlenecks.

On the other hand, the following three results indicated that the XX/XY genetic sex-determination system controlled by the *DMY* gene appeared to be active. First, all male individuals had a normal Y chromosome (Table 4). Second, no significant deviation from the expected 1:1 ratio was found for the sex ratio in each year ($\chi^2 = 0.72$ in 2003, 1.72 in 2004, 0.45 in 2005, and 1.1 in 2006). Third, the frequency of the normal Y chromosome was ~25% (Table 5).

Taken together, our data demonstrated that the Y chromosome lacking the male-determining function behaved as an X chromosome in this population. This phenomenon appears to be characteristic of organisms like the medaka that have quite undifferentiated sex chromosomes.

TABLE 5

Estimated number and frequency of each sex chromosome in the O-bu population

Year	Total	X	Y^{wObu1}	Y^{wObu2}	Y
2003	226	141 (0.624)	22 (0.097)	2 (0.009)	61 (0.270)
2004	262	163 (0.622)	26 (0.099)	0 (0.000)	73 (0.279)
2005	110	61 (0.555)	19 (0.173)	0 (0.000)	30 (0.273)
2006	226	149 (0.659)	22 (0.097)	3 (0.013)	52 (0.230)

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