DOI: 10.1534/genetics.107.075598

Evidence for Different Origins of Sex Chromosomes in Closely Related Oryzias Fishes: Substitution of the Master Sex-Determining Gene

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Manuscript received May 10, 2007
Accepted for publication September 17, 2007

ABSTRACT

The medaka *Oryzias latipes* and its two sister species, *O. curvinotus* and *O. luzonensis*, possess an XX–XY sex-determination system. The medaka sex-determining gene *DMY* has been identified on the orthologous Y chromosome [*O. latipes* linkage group 1 (LG1)] of *O. curvinotus*. However, *DMY* has not been discovered in other Oryzias species. These results and molecular phylogeny suggest that *DMY* was generated recently [~10 million years ago (MYA)] by gene duplication of *DMRT1* in a common ancestor of *O. latipes* and *O. curvinotus*. We identified seven sex-linked markers from *O. luzonensis* (sister species of *O. curvinotus*) and constructed a sex-linkage map. Surprisingly, all seven sex-linked markers were located on an autosomal linkage group (LG12) of *O. latipes*. As suggested by the phylogenetic tree, the sex chromosomes of *O. luzonensis* should be "younger" than those of *O. latipes*. In the lineage leading to *O. luzonensis* after separation from *O. curvinotus* ~5 MYA, a novel sex-determining gene may have arisen and substituted for *DMY*. Oryzias species should provide a useful model for evolution of the master sex-determining gene and differentiation of sex chromosomes from autosomes.

MAMMALS and birds have genetic sex determination with cytogenetically well-differentiated sex chromosomes. By contrast, various sex-determination mechanisms have evolved independently in fishes, and most species with genetic sex determination have undifferentiated sex chromosomes (Solari 1994; Devlin and Nagahama 2002). Recent studies have shown that different sex chromosomes have evolved even among closely related fishes (Woram *et al.* 2003; Takehana *et al.* 2007) or among intraspecific populations (Volff and Schartl 2001), but the mechanisms for these changes are unknown.

A phylogenetic tree of the medaka, *Oryzias latipes*, and its relatives is available in Takehana *et al.* (2003) and has been redrawn here in Figure 1 with the species' sex-determining system. *O. latipes* and a sister-species pair, *O. curvinotus* and *O. luzonensis*, have an XX–XY genetic sex-determination system (Aida 1921; Matsuda *et al.* 2003; Hamaguchi *et al.* 2004). Like other fishes, these Oryzias species have no heteromorphic sex chromosomes (Uwa and Ojima 1981; Matsuda *et al.* 1998), and their sex chromosomes can be regarded as being in a primitive stage of differentiation.

A DM-domain gene, *DMY*, has been identified in the medaka *O. latipes* as the first nonmammalian sex-determining gene (Matsuda *et al.* 2002, 2007). *DMY* was conserved among other populations of *O. latipes* (Shinomiya *et al.* 2004). *O. curvinotus* also have *DMY* on the Y chromosome, which is orthologous to that of *O. latipes* (Matsuda *et al.* 2003) (see Figure 1). However, *DMY* has not been detected in any other fishes, such as guppy, tilapia, zebrafish, or even in the Oryzias species *O. celebensis* and *O. mekongensis* (Kondo *et al.* 2003).

These results suggest that *DMY* is not the universal primary sex-determining gene in fishes, in contrast to the mammalian *SRY/Sry* (Volff *et al.* 2003), which is well conserved among placental mammals and marsupials (Gubbay *et al.* 1990; Sinclair *et al.* 1990; Foster *et al.* 1992), with the exception of some species (Just *et al.* 1995; Soullier *et al.* 1998). Analysis of the Y-specific region of the *O. latipes* sex chromosome has demonstrated that *DMY* arose from a duplicated copy of the autosomal *DMRT1* gene (Nanda *et al.* 2002; Kondo *et al.* 2006). This *DMRT1* duplication event is estimated to have occurred ~10 million years ago (MYA) in a common ancestor of *O. latipes* and *O. curvinotus*. However, in *O. luzonensis*, no functional duplicated copy of *DMRT1* has been detected, although there is a pseudogene, *Oludmrt1p* (Kondo *et al.* 2004). The evolution of the sex-determining system in these closely related species, including the origin of this pseudogene, remains a mystery.

Here, we identified seven sex-linked sequences of *O. luzonensis* and constructed a recombination map. The map demonstrated that the sex chromosome of

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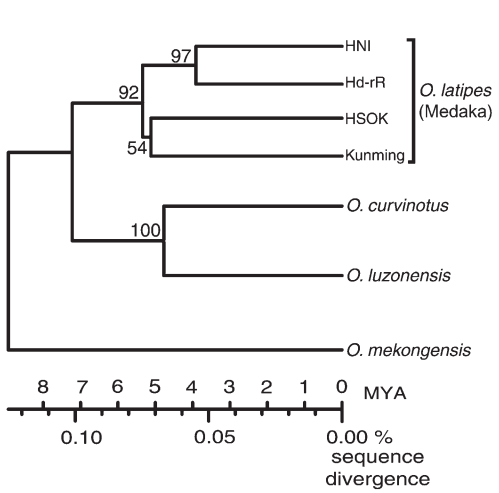

	Sex-determination System	*DMY*
O. latipes (Medaka)	XX-XY (Aida, 1921)	Present (Matsuda *et al.* 2002; Shinomiya *et al.* 2004)
O. curvinotus	XX-XY (Matsuda *et al.* 2003)	Present (Matsuda *et al.* 2003)
O. luzonensis	XX-XY (Hamaguchi *et al.* 2004)	Not detected (This study and Kondo *et al.* 2004)
O. mekongensis	XX-XY (Hamaguchi *et al.* 2004)	Not detected (Kondo *et al.* 2003)

Figure 1.—Phylogenetic relationship of Oryzias species based on mitochondrial DNA sequences. Using cytochrome *b* gene sequences from Takehana *et al.* (2003), we redrew a linearized neighbor-joining tree. The separation of *O. luzonensis* from *O. curvinotus* was estimated to have occurred ~5 MYA according to a divergence rate of 2.8%/MY. *DMY* has been identified only in *O. latipes* and *O. curvinotus*.

O. luzonensis is orthologous to an *O. latipes* autosome (LG12) and, unlike in *O. latipes*, it does not show recombination suppression around the sex-determining region. On the basis of the draft genomic sequence of *O. latipes*, the sex-determining region of *O. luzonensis* is calculated to be <860 kbp. These results suggest that *O. luzonensis* has "younger" sex chromosomes than *O. latipes* and that the master sex-determining gene has changed at least twice in 10 million years (MY) during diversification of this species group.

MATERIALS AND METHODS

Fishes: *O. luzonensis* was collected by M. J. Formacion and H. Uwa in 1982 at Solsona, Ilocos Norte, Luzon, Philippines (Formacion and Uwa 1985). *O. curvinotus* was collected by D. Dudgeon and H. Uwa in 1986 at Sam A. Tsuen, Plover Cove Country Park, Hong Kong (Uwa 1991). These species have been maintained as a closed colony. An inbred strain, Hd-rR, was established from the Southern population of *O. latipes* (Hyodo-Taguchi and Sakaizumi 1993). These fishes were supplied by a subcenter (Niigata University) of the National BioResource Project (medaka) supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan. Wild *O. latipes* were collected at Niitsu, Niigata Prefecture (northern population) in 2004.

Hormonal sex reversal: Fertilized eggs of *O. luzonensis* were treated with either 0.025 μg/ml methyl testosterone (Sigma Chemical, St. Louis) or 0.2 μg/ml 17β-estradiol (Sigma Chemical) until hatching. They were then reared in aged tap water until sexual maturation.

Genetic crosses: Three *O. luzonensis* families (Lz1–Lz3) were prepared: XX female × XY male [Lz1; number of progeny (n) = 190], XX female × XY male (Lz2; n = 93), and a sex-reversed XY female × sex reversed XX male (Lz3; n = 48). Two BC_1 progeny of *O. latipes* were produced: (Niitsu♀ × Hd-rR♂) F_1♂ × Hd-rR♀ (ND1; n = 94) and (Niitsu♀ × Hd-rR♂) F_1♀ × Hd-rR♂ (ND2; n = 94). Interspecific BC_1 offspring were obtained from an (*O. luzonensis* × *O. curvinotus*) F_1 female crossed with an *O. curvinotus* male (CL1; n = 43).

Search for X–Y polymorphisms of *O. luzonensis* and linkage analysis: To find polymorphisms between the X and Y of *O. luzonensis*, we randomly selected 250 expressed sequence tag (EST) markers from the medaka expressed sequence tag databases (http://mbase.bioweb.ne.jp/~dclust/medaka_top.html/ and http://medaka.lab.nig.ac.jp/). ESTs were amplified using previously published primers designed for *O. latipes* (Naruse *et al.* 2004). PCR amplification was performed as follows: 33 cycles at 95° for 30 sec, 55° for 30 sec, and 72° for 3 min. Polymerase chain reaction (PCR) products were electrophoresed on polyacrylamide gels as described by Kimura *et al.* (2004). We adopted the PCR direct-sequencing method using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA). An EST, OLb24.08a, was sequenced after TA cloning because its Y sequence contained a 39-bp deletion.

Linkage maps were constructed by using MAPL97 for Windows (Ukai *et al.* 1995). Both male and female recombination data were merged for consensus marker ordering. Each amplified marker was genotyped by restriction fragment length polymorphism (RFLP) analysis on polyacrylamide gels or single nucleotide polymorphism (SNP) analysis with the ABI PRISM 310 genetic analyzer.

Fluorescence *in situ* hybridization: A bacterial artificial chromosome (BAC) genomic library, constructed from the Hd-rR strain of *O. latipes* (Matsuda *et al.* 2001), was screened, and three clones—Md0173J11 (containing *SL1*), Md0172B19 (containing *DMRT1*), and Md0171M23 (containing a body-color gene, *b*)—were used as probes. These BAC clones were located on the sex chromosomes (LG1) and on autosomes (LG9 and LG12) in *O. latipes*, respectively.

Metaphase cells from cultured caudal fins were prepared by standard cytogenetic methods (Uwa and Ojima 1981; Matsuda *et al.* 1998). Fluorescence *in situ* hybridization (FISH) was performed as described by Matsuda and Chapman (1995) and Takehana *et al.* (2007).

RESULTS AND DISCUSSION

***O. latipes* sex-determining gene *DMY* is absent in *O. luzonensis*:** Kondo *et al.* (2004) did not detect a sex-linked *DMRT1* gene in *O. luzonensis* by Southern hybridization analysis. To confirm the absence of *DMY*, we searched *O. luzonensis* genomic DNA for the *DMY* gene by PCR with 10 primers (five forward and five reverse) designed for *O. latipes DMY* (see supplemental Table 1 at http://www.genetics.org/supplemental/; Figure 2A). Seven of the primer pairs (supplemental Table 2) produced a male-specific band in *O. latipes* and *O. curvinotus*

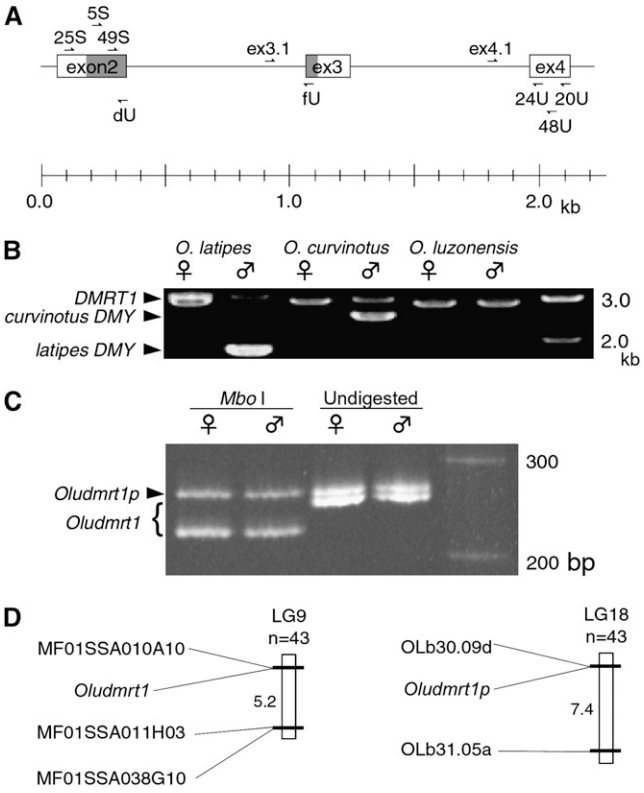

Figure 2.—Search for the *DMY* gene and mapping of *DMY*-related genes. (A) *DMY* structure of the *O. latipes* and positions of primers used in this study. Open boxes, shaded boxes, and horizontal lines indicate exons, the DM domain, and introns, respectively. (B) Agarose gel electrophoresis (1%) of PCR products using the 49S and 48U primers. Only *O. latipes* and *O. curvinotus* gave a male-specific band (*DMY*). (C) Polyacrylamide gel electrophoresis (9%) of PCR products using the ex4.1 and 48U primers. *Mbo*I-digested and undigested samples were loaded on the gel. The lower band of digested samples was judged as *Oludmrt1*, which has an *Sau*3AI (isochizomer of *Mbo*I) restriction site (Kondo *et al.* 2004). (D) Linkage analysis using CL1 cross. Map distances between markers are shown in centimorgans.

(Figure 2B). In contrast, they did not amplify male-specific fragments in *O. luzonensis*. We also checked amplification of other genes, such as *tyrosonase* and *b/AIM1*, and obtained PCR products of the expected sizes in each of the three species (data not shown). For these genes, the synonymous substitution rate between *O. latipes* and *O. luzonensis* was similar to that between *O. latipes* and *O. curvinotus* (supplemental Table 3). Furthermore, a recent PCR survey of 47 loci, which were used for a genomewide SNP analysis (Kasahara *et al.* 2007), showed that 39 of these loci were successfully amplified in *O. latipes*, *O. luzonensis*, and *O. curvitnotus*, although the other 6 and 2 loci were not amplified in both *O. luzonensis* and *O. curvinotus* and in neither *O. luzonensis* nor *O. curvinotus*, respectively (Narita 2007). These results suggest that the lack of *DMY* amplification in *O. luzonensis* is not due to the large sequence divergence, but to the absence of *DMY* from the *O. luzonensis* genome.

When the *DMRT1* primers ex4.1 and 48U were used, two bands were obtained from both sexes in *O. luzonensis*. By RFLP analysis as described by Kondo *et al.* (2004), the lower bands were judged to represent the *O. luzonensis DMRT1* (*Oludmrt1*) and the upper band to represent a pseudogene, *Oludmrt1p* (Figure 2C). Kondo *et al.* (2004) argued that this pseudogene might be either a degenerate version of a copy from the initial gene duplication or the result of another independent duplication of *DMRT1*. Our linkage analysis using an interspecific cross between *O. curvinotus* and *O. luzonensis* (CL1 cross) demonstrated that *O. luzonensis DMRT1* (*Oludmrt1*) and *Oludmrt1p* are linked to markers belonging to *O. latipes* LG9 and LG18, respectively (Figure 2D). These data supported the second hypothesis of Kondo *et al.* (2004). Because no *DMRT1*-related genes on LG18 have been reported in *O. latipes*, the pseudogene may not have originated as a degenerate copy of the initial gene duplication but as an independent duplicate specific to *O. luzonensis* of the *DMRT1*(LG9). However, another possibility—that a degenerate copy of *DMY* on LG1 has been transposed to LG18—cannot be excluded. *DMY* and *Oludmrt1p* may thus have different origins, and *DMY* (LG1) may have been lost in *O. luzonensis*.

The sex-linkage group of *O. luzonensis* is orthologous to a medaka autosome (LG12): We found that three *O. latipes* ESTs (AU167284, MF01SSA025F03, and OLb24.08a) yielded male-specific banding patterns in *O. luzonensis* (Figure 3A). Sequencing analyses (Figure 3B) suggested that these patterns could be due to DNA heteroduplex formation (Hauser *et al.* 1998). These differences in electrophoretic mobility result from the heteroduplex DNA conformation of the mismatches. These heterogametic patterns were passed from father to son, confirming that *O. luzonensis* has an XX–XY sex-determination system, as indicated previously (Hamaguchi *et al.* 2004).

Because the markers AU167284 and OLb24.08a were already described as located on *O. latipes* LG12 (Naruse *et al.* 2004), other sequences on LG12 were examined for more sex-linked markers. Two genes, *b* and *eyeless* (Fukamachi *et al.* 2001; Loosli *et al.* 2001), two BAC end sequences (Fukamachi *et al.* 2001), and 22 ESTs were subjected to PCR direct sequencing. We identified four additional sex-linked SNPs (supplemental Table 4 at http://www.genetics.org/supplemental/). These seven markers were investigated for their sequence similarity and uniqueness by basic local alignment search tool (BLAST) searches against the medaka genome database (http://dolphin.lab.nig.ac.jp/medaka/). BLAST searches detected only one sequence with high similarity (sequence identity $> 89\%$; *E*-value $< e^{-45}$) for each (supplemental Table 4), indicating that all investigated sex-linked markers of *O. luzonensis* were orthologous to LG12 sequences of *O. latipes*.

FISH analysis (Figure 4) demonstrated that the sex chromosomes of *O. luzonensis* were submetacentric and

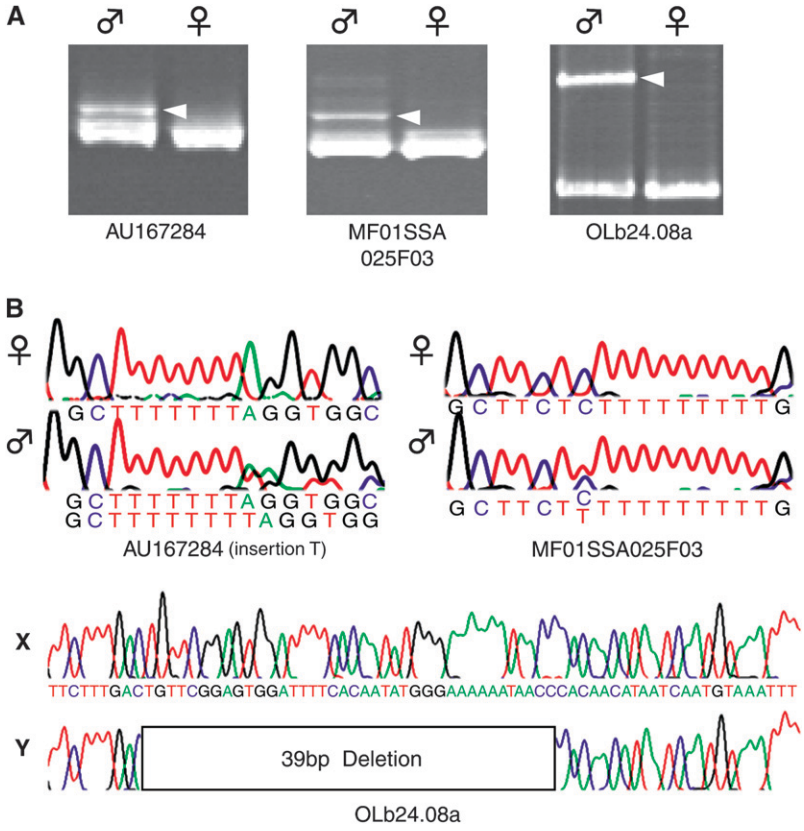

FIGURE 3.—Sex-linked polymorphisms of *O. luzonensis*. (A) Polyacrylamide gel electrophoresis (9%) of PCR products for AU167284, MF01SSA025F03, and OLb24.08a. Arrowheads mark male-specific bands. (B) Sequence analysis of polymorphic PCR products. For EST AU167284, the female PCR products show seven T repeats, whereas males display both seven and eight T repeats. In EST MF01SSA025F03, males show a (C/T) SNP. In OLb24.08a, the 39-bp deletion links to maleness.

not differentiated from one another cytogenetically. A BAC clone, Md0171M23, containing the tightly sex-linked gene *b*, hybridized on the long arms of the sex chromosomes, close to the centromere. Furthermore, it was confirmed that the sex chromosome of *O. luzonensis* was different from both the sex chromosome (LG1) and the *DMRT1*-bearing chromosome (LG9) of *O. latipes*.

The *O. luzonensis* sex chromosome appeared <5 MYA: *SRY/Sry* is the only known primary sex-determining gene in higher vertebrates and is believed to have arisen 130–170 MYA (MARSHALL-GRAVES 2002). By estimating the age of *DMY* as ~10 MY, KONDO *et al.* (2004) argued that the *O. latipes* sex chromosome is at an early stage of differentiation and concluded that the *O. latipes*

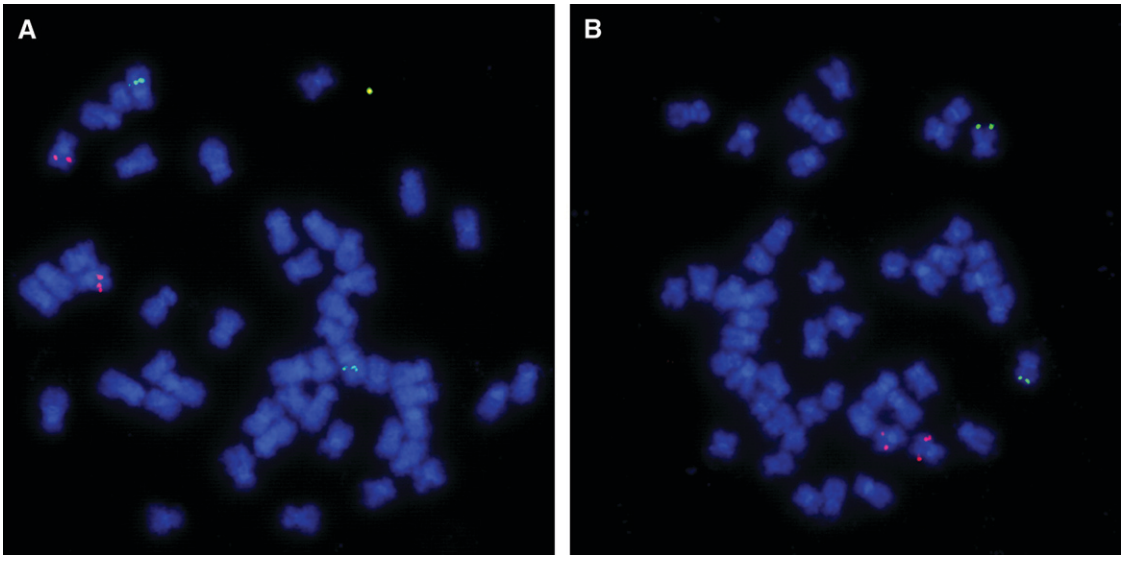

FIGURE 4.—FISH analysis of male metaphase chromosomes in *O. luzonensis* using *O. latipes* BAC clones. (A) Chromosomal location of the *O. luzonensis* sex-determining region (BAC Md0171M23, red) and of the *O. latipes* sex chromosomal marker *SL1* (BAC Md0173J11, green). (B) Chromosomal location of the *O. luzonensis* sex-determining region (BAC Md0171M23, red) and the *O. latipes DMRT1* gene (BAC Md0172B19, green).

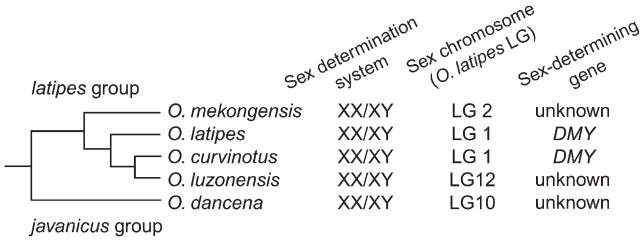

FIGURE 5.—Sex-determination mechanisms and sex linkage groups in Oryzias species. The phylogenetic information was taken from TAKEHANA *et al.* (2005).

Y chromosome is the youngest male-determining chromosome so far known in vertebrates.

O. latipes and *O. curvinotus* possess orthologous sex chromosomes (LG1) (MATSUDA *et al.* 2003; KONDO *et al.* 2004); in contrast, *O. luzonensis* displays a different sex chromosome (LG12), suggesting that the sex chromosome shifted from LG1 to LG12 after *O. luzonensis* diverged from *O. curvinotus*. The basal species, *O. mekongensis*, has an LG2 sex chromosome (A. KAWAGUCHI, A. SHINOMIYA, S. HAMAGUCHI and M. SAKAIZUMI, unpublished data), indicating that the new sex chromosome of *O. luzonensis* (LG12) is not the result of reversion to the old sex-determination system with *DMY* degeneration (Figure 5). Because the separation of *O. luzonensis* from *O. curvinotus* is estimated to be ~5 MYA on the basis of a molecular clock (Figure 1), the sex chromosome of *O. luzonensis* may be younger than 5 MY. *O. luzonensis* may have lost *DMY* and recruited a novel sex-determining gene on the new sex chromosome (LG12).

Comparison between the *O. luzonensis* sex chromosome and *O. latipes* LG12: We constructed comparative linkage maps between the sex-linkage group of *O. luzonensis* and the autosomal linkage group (LG12) of *O. latipes* (Figure 6). The order of markers was completely conserved between the two maps; *i.e.*, the sex chromosome of *O. luzonensis* is syntenic to *O. latipes* LG12. The *Sex* gene was tightly linked with the body-color gene *b* ($n = 141/141$) and located between *eyeless* and 171M23F. This region is equivalent to 859 kbp in the *O. latipes* genome, which includes 28 predicted genes (medaka genome sequencing project: KASAHARA *et al.* 2007; http://dolphin.lab.nig.ac.jp/medaka/). Thus, we expect that the primary sex-determining gene of *O. luzonensis* lies in this interval.

Although the only structural difference between the *O. latipes* X and Y is the Y-specific region (258-kb

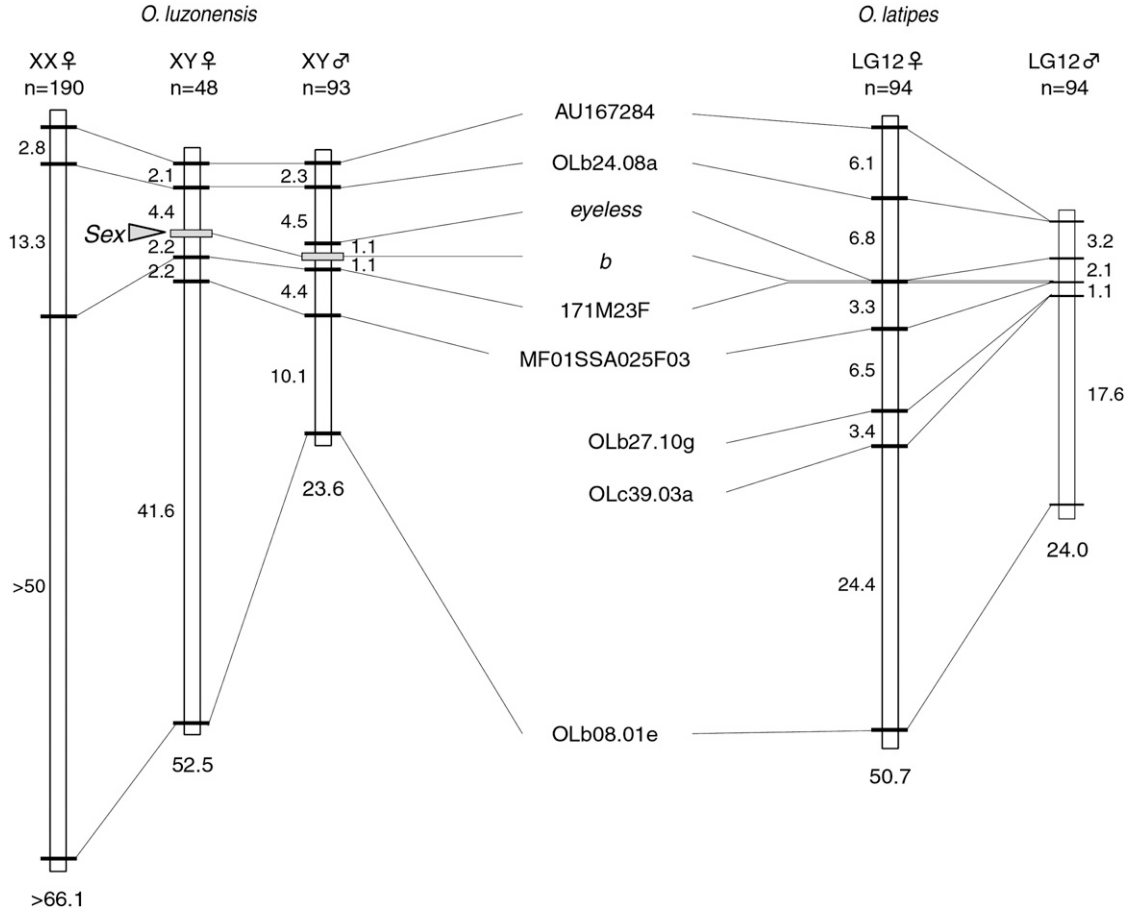

FIGURE 6.—Comparative recombination map between sex chromosomes of *O. luzonensis* and LG12 (autosome) of *O. latipes*. The sex-determining gene (*Sex*, arrowhead) of *O. luzonensis* is located adjacent to the *b* gene. Map distances between markers are shown in centimorgans and total map lengths are shown below each map.

insertion) (Kondo *et al.* 2006), the recombination rate in males is also strongly suppressed outside this region in a region that has a genetic map length of ~30 cM in females (Kondo *et al.* 2001). This restriction of recombination is observed in *O. latipes* sex-reversed XX males but not in XY females (Matsuda *et al.* 1999), indicating that the restriction is not caused by a structural difference between X–Y but by an unknown mechanism specific to phenotypic males. In contrast to the highly restricted sex chromosome of *O. latipes* (Figure 2 in Kondo *et al.* 2001), the sex chromosome of *O. luzonensis* (LG12) displays only a weak reduction of the recombination rate in males and recombines well around the sex-determining region (see AU167284–MF01SSA025F03 in Figure 6). A high recombination rate between the X and Y supports the argument that the sex chromosome of *O. luzonensis* (LG12) is younger than that of *O. latipes* (LG1).

Studies of such "young" sex chromosomes are important in understanding their early evolution (Charlesworth *et al.* 2005). As with Oryzias species, some salmonids and sticklebacks show different sex chromosomes among closely related species (Woram *et al.* 2003; Peichel *et al.* 2004). This suggests that frequent switching between different master sex-determining genes may have occurred in many species groups that possess undifferentiated sex chromosomes. Oryzias fishes may prove to be very informative systems for studying the evolutionary processes of the early stages of sex-chromosome differentiation and of the switching mechanisms of the master sex-determining gene.

We thank Masaru Matsuda of the National Institute for Basic Biology for his helpful technical advice. Medaka genome data were provided by the National Institute of Genetics and the University of Tokyo for use in this publication/correspondence only. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan to M.S. (16370094) and to K.N. (16310131).

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Communicating editor: D. Charlesworth