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

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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 un-differentiated sex chromosomes (Soları 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 (Völff 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 (Völff 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; Souttler 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, Oludmr1lp (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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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 Solsoma, 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 fish 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 \(\mu\)g/ml methyl testosterone (Sigma Chemical, St. Louis) or 0.2 \(\mu\)g/ml 17\(\beta\)-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 \(\times\) XY male [Lz1; number of progeny \((n) = 190\), XX female \(\times\) XY male (Lz2; \(n = 95\)], and a sex-reversed XY female \(\times\) sex-reversed XX male (Lz3; \(n = 48\) ). Two BC1 progeny of O. latipes were produced: (Niitsu \(\times\) Hd-rR3) F1 \(\times\) Hd-rR3\(\#\) (ND1; \(n = 94\)) and (Niitsu \(\times\) Hd-rR3) F1 \(\times\) Hd-rR3\(\#\) (ND2; \(n = 94\)). Interspecific BC1 offspring were obtained from an (O. luzonensis \(\times\) O. curvinotus) F1 female crossed with an O. curvinotus male (CL1; \(n = 45\)).

**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.biolweb.ne.jp/~dl/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: 35 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C 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 (UKAT 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 Hdr-R strain of O. latipes (MATSUDA et al. 2001), was screened, and three clones—Md017311 (containing SL1), Md0172B19 (containing DMRT1), and Md0171M25 (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.
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. curvinitus* 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 DMRT1LG9. 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, MF018SA025F03, 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 heteromeric 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%; Evalue < e−6) 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
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
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. Sakazumi, 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...
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 Orzysz 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. Orzysz 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.

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LITERATURE CITED


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