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ORIGINAL ARTICLE

A sex-determining region on the Y chromosome controls the sex-reversal ratio in interspecific hybrids between *Oryzias curvinotus* females and *Oryzias latipes* males

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Oryzias latipes and *Oryzias curvinotus* are closely related medaka species that have the common sex-determining gene, *DMY*, on their homologous Y chromosomes. We previously reported that sex-reversed XY females were produced in hybrids between *O. curvinotus* females and *O. latipes* males (Hd-rR inbred strain). In this study we used HNI inbred strain males of *O. latipes* for mating with *O. curvinotus* females, and found that all the XY hybrids developed as males. To map the factor responsible for this strain-specific XY sex reversal, *O. curvinotus* females were mated with two Y-congenic strains (HNI.$Y^{Hd\text{-}rR}$ and Hd-rR.Y^{HNI}) and a recombinant congenic strain (Hd-rR.Y^{HNI}*rr*). HNI.$Y^{Hd\text{-}rR}$ produced sex-reversed females in the XY hybrids, whereas no sex-reversed females were obtained in the XY hybrids from Hd-rR.Y^{HNI} and Hd-rR.Y^{HNI}*rr*, demonstrating that a small region on the Y chromosome, which includes *DMY*, is responsible for the XY sex reversal. Sex-reversed hybrids were only produced in the presence of the Y-chromosomal region derived from the Hd-rR strain, suggesting that missense or regulatory mutations specific to the Hd-rR Y-chromosomal region induce the sex reversal.
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Introduction

Mutations or new combinations of genes that give rise to phenotypic differences provide novel insights into genes and their functions in the molecular pathways underlying development. Consequently, inconsistencies between the genetic sex (XX or XY) and the phenotypic sex (male or female) are useful for identifying the genes involved in the sex-determination pathway. In mammals, the sex-determining gene, *SRY/Sry,* located on the Y chromosome, induces the undifferentiated gonad to develop as a testis (Gubbay *et al*., 1990; Sinclair *et al*., 1990; Koopman *et al*., 1991; Capel *et al*., 1993). Some human XY sex reversals with gonadal dysgenesis have deletions or mutations in coding or regulatory regions of the *SRY* gene, providing compelling evidence of its sex-determining function (Berta *et al*., 1990; Jager *et al*., 1990; Page *et al*., 1990). An essential role of *Sox9* for testis differentiation was detected by analysis of XY sex-reversed patients with campomelic dysplasia (Foster *et al*., 1994). Furthermore, loss-of-function mutations in *R-spondin1* cause XX male sex reversal in humans (Parma *et al*., 2006). Many other autosomal and X-chromosomal genes have been identified as having roles in the sex-differentiation pathway based on the analyses of human sex-reversal cases and functional analyses in mice (reviewed in Koopman, 2001).

In the medaka, *Oryzias latipes*, which has an XX/XY sex-determining system (Aida, 1921), *DMY* was found to be the Y-specific sex-determining gene (Matsuda *et al*., 2002, 2007). In this fish species, many sex-reversal mutants (XX males and XY females) have been found in wild populations (Shinomiya *et al*., 2004; Otake *et al*., 2006, 2008). All the observed XY sex-reversed females had Y-linked gene mutations and were classified into two groups: a group with mutations in the amino acid coding sequence of *DMY* and a group with an intact coding region but reduced *DMY* expression during critical periods for sex determination (Matsuda *et al*., 2002; Otake *et al*., 2006). These findings show that *DMY* is required for male sex determination. In addition, a genetic analysis of an XX male suggested the presence of autosomal modifiers for sexual differentiation of the gonads (Shinomiya *et al*., 2004).

Oryzias curvinotus is a closely related species to *O. latipes*, and also has an XX/XY sex-determining system (Hamaguchi *et al*., 2004). The *DMY* gene was identified on the homologous Y chromosome, and is only expressed in XY embryos (Matsuda *et al*., 2003). The process of gonadal sex differentiation in *O. curvinotus* is similar to that in *O. latipes* (Shinomiya *et al*., 2006), suggesting that *O. curvinotus* and *O. latipes* have a common sex-determining pathway. Furthermore, interspecific hybrids between *O. curvinotus* and *O. latipes* are

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viable and develop normal secondary sexual characteristics, although males are sterile and females lay diploid eggs (Hamaguchi and Sakaizumi, 1992; Sakaizumi *et al*., 1992, 1993). In a previous study, we analyzed the genetic and phenotypic sexes of hybrids between *O. curvinotus* females and *O. latipes* males of the Hd-rR inbred strain, and found that this mating yielded not only XX females and XY males, but also XY females (Shinomiya *et al*., 2006). Male fish only appeared in the XY hybrids, indicating that *DMY* is required for male determination of the hybrids. However, 21% of the XY hybrids were females, demonstrating that *DMY* cannot always determine maleness in the hybrids.

In this study, we used *O. latipes* males of the HNI inbred strain for mating with *O. curvinotus* females. Unlike the results with the Hd-rR strain, all the XY hybrids developed as males, indicating that the Hd-rR and HNI inbred strains differ in their ability for male determination in the XY hybrids. We also found that the Y chromosome of *O. latipes* was involved in the sex reversal in the XY hybrids by using two Y-congenic strains, HNI.$Y^{Hd\text{-}rR}$ and Hd-rR.Y^{HNI}, and a recombinant congenic strain, Hd-rR.Y^{HNI}*rr*, derived from Hd-rR.Y^{HNI}. Sex-reversed hybrids were only produced in the presence of the Y-chromosomal region derived from the Hd-rR strain, irrespective of the autosomal background, suggesting that divergence in the Y-chromosomal region is associated with the different sex-reversal ratios in the XY hybrids.

Materials and methods

Fish

We used a laboratory stock of *O. curvinotus*, as well as two inbred strains (HNI and Hd-rR), two Y-congenic strains (HNI.$Y^{Hd\text{-}rR}$ and Hd-rR.Y^{HNI}) and a recombinant Y-congenic strain (Hd-rR.Y^{HNI}*rr*) of *O. latipes* (Figure 1). All the strains were supplied by a sub-center (Niigata University, Niigata, Japan) of the National Bioresource Project (Medaka) (http://www.shigen.nig.ac.jp/medaka/). The wild stock of *O. curvinotus* was originally collected in Hong Kong in 1986 (Takehana *et al*., 2005). HNI and Hd-rR were established from Northern and Southern Japanese Populations, respectively (Hyodo-Taguchi and Sakaizumi, 1993). HNI.$Y^{Hd\text{-}rR}$ is a Y-congenic strain established from HNI females and an Hd-rR male. An F_1 hybrid male was backcrossed to HNI females for 12 generations. This strain has the Y chromosome derived from Hd-rR on the HNI background. Similarly, Hd-rR.Y^{HNI} has the Y chromosome derived from HNI on the Hd-rR background (Matsuda *et al*., 1998). Hd-rR.Y^{HNI}*rr* is a recombinant strain derived from Hd-rR.Y^{HNI}, and has a small region of the Y chromosome of the HNI strain. This region is in an interval of 1.8 cM between two DNA markers, *SL1* and *51H7.F*, and includes *DMY* (Matsuda *et al*., 2002). The fish were maintained in aquaria under an artificial 14-h light:10-h dark photoperiod at 27 ± 2 °C.

Mating and sexing

O. curvinotus females and *O. latipes* males of each strain were crossed by pair mating, and the naturally spawned eggs were collected and incubated under the same conditions as the adult fish. The hatched hybrid fish were reared for 2–3 months, and examined to determine their phenotypic and genotypic sexes.

Phenotypic sex was judged on the basis of secondary sex characteristics, namely, the shapes of the dorsal and anal fins. Genotypic sex (XY or XX) was determined on the basis of the presence or absence of the *DMY* gene, evaluated using PCR amplification of caudal fin clip genomic DNA extracted from adult fish. PCR amplification of *DMY* and *DMRT1* was performed with the primers PG17.5 s (5′-CCGGGTGCCCAAGTGCTCCCG CTG-3′) and PG17.6U (5′-GATCGTCCCTCCACAGA GAAGAGA-3′) (Shinomiya *et al*., 2004) at an annealing temperature of 55 °C. The PCR products were analyzed by electrophoresis in a 1% agarose gel.

RNA extraction and reverse transcriptase PCR (RT-PCR)

Total RNA was extracted from fry at hatching using an RNeasy Mini Kit (Qiagen, Tokyo, Japan), 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 amplification conditions were: 30 min at 55 °C; 15 min at 95 °C; 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C; and 10 min at 72 °C. For *DMY*, the initial PCR products were diluted by 1:200 and re-amplified. The conditions for the second PCR amplification were: 5 min at 95 °C; 22 cycles of 20 s at 94 °C, 30 s at 65 °C and 30 s at 72 °C; and 3 min at 72 °C.

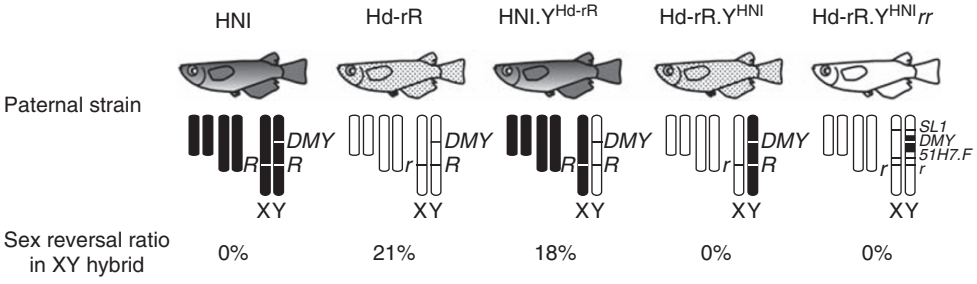

Figure 1 Genomic constitutions of XY individuals in five strains of *O. latipes* and sex-reversal ratios in their XY hybrids. The HNI inbred strain is derived from the Northern Japanese Population and the Hd-rR inbred strain is derived from the Southern Japanese Population (Hyodo-Taguchi and Sakaizumi, 1993). HNI.$Y^{Hd\text{-}rR}$ and Hd-rR.Y^{HNI} are Y-congenic strains. The former has the Y chromosome derived from Hd-rR on the HNI background, whereas the latter has the Y chromosome derived from HNI on the Hd-rR background. Hd-rR.Y^{HNI}*rr* is a recombinant strain of Hd-rR.Y^{HNI}, and carries a small region of the Y chromosome derived from HNI on the Hd-rR background. This region is in an interval of 1.8 cm between two DNA markers, *SL1* and *51H7.F*, and includes the *DMY* gene (Matsuda *et al*., 2002). The largest chromosomes represent the XY sex chromosomes, and the other chromosomes represent autosomes. Open, Hd-rR-derived chromosome; *r* and *R*, alleles of the *r* locus (a sex-linked pigment gene); Solid, HNI-derived chromosome.

The specific primers used were as follows: *DMY* first amplification: PG17.5s, 5′-CCGGGTGCCCAAGTGCTCC CGCTG-3′ and PG17.12U, 5′-GACCATCTCATTTTTTAT TCTTGATTTTT-3′; *DMY* second amplification: *DMY*spe, 5′-TGCCGGAACCACAGCTTGAAGACC-3′ and 48U, 5′-GGCTGGTAGAAGTTGTAGTAGGAGGTTT-3′; *β-actin*: actin3b, 5′-CMGTCAGGATCTTCATSAGG-3′ and actin4, 5′-CACACCTTCTACAATGAGCTGA-3′ (Otake *et al*., 2006).

Results

Strain differences in hybrid XY sex reversal

In a previous study, we showed that sex-reversed XY females were produced from interspecific hybridization between *O. curvinotus* females and Hd-rR strain males of *O. latipes* (Shinomiya *et al*., 2006), as shown in Table 1. In brief, all 138 XX hybrids were females, whereas the 140 XY hybrids consisted of 110 males and 30 females, indicating that 21% of XY individuals developed as females in the hybrids.

In this study, we used *O. latipes* males of another inbred strain, HNI, for mating with *O. curvinotus* females, and analyzed the phenotypic and genotypic sexes of the F_1 hybrids. The results showed that all 81 XX hybrids developed as females and all 70 XY hybrids developed as males, with no sex reversal (Table 1). The gonads of 68 XX females and 60 XY males were dissected out and observed using a stereomicroscope. All females had ovaries and all males had testes, indicating that HNI males did not produce sex-reversed XY females in the hybrids. These results suggest that the two inbred strains, HNI and Hd-rR, differ in their incidences of XY sex reversal in the interspecific hybrids.

Genetic mapping using Y-congenic strains

To test the possibility that the Y chromosome of *O. latipes* contributes to the strain-specific difference in the hybrid sex reversal, we mated males of two Y-congenic strains (HNI.$Y^{Hd\text{-}rR}$ and Hd-rR.Y^{HNI}) of *O. latipes* with *O. curvinotus* females (Table 1). HNI.$Y^{Hd\text{-}rR}$ males produced 42 XX hybrids that developed as females and 62 XY hybrids that consisted of 51 males (82%) and 11 females (18%). These findings indicated that HNI.$Y^{Hd\text{-}rR}$ males also produced sex-reversed XY females in the hybrids. In contrast, all 82 XX hybrids were females and all 75 XY hybrids were males in the mating with Hd-rR.Y^{HNI} males, indicating that Hd-rR.Y^{HNI} males did not produce sex-reversed XY females. These results clearly show that strain-specific differences in the Y chromosome of *O. latipes* contribute to the sex-reversal ratio in the XY hybrids.

To locate precisely the responsible region on the Y chromosome, we used Hd-rR.Y^{HNI}*rr*, a recombinant strain of Hd-rR.Y^{HNI}. This strain has a small region (including the sex-determining gene *DMY*) derived from the HNI strain on the Hd-rR genetic background (Matsuda *et al*., 2002). In this mating, all 110 XX hybrids developed as females and all 98 XY hybrids developed as males, demonstrating that the small region on the Y chromosome has a crucial role in the hybrid XY sex reversal.

Expression level of *DMY*

Reduced *DMY* expression levels have been observed in some XY sex-reversal mutants of *O. latipes* (Matsuda *et al*., 2002; Otake *et al*., 2006). To examine *DMY* expression in the hybrids during the sex-determining period, we performed RT-PCR analyses of fry at the hatching day. We analyzed the hybrids obtained from crosses between *O. curvinotus* and Hd-rR that produced sex reversal, and crosses between *O. curvinotus* and Hd-rR.Y^{HNI}*rr* and between *O. curvinotus* and HNI that produced no sex reversal. In addition, we analyzed the parental Hd-rR, Hd-rR.Y^{HNI}*rr* and HNI strains of *O. latipes*. Although *DMY* transcripts were detected in all XY fry, the expression levels were clearly lower in the XY hybrids between *O. curvinotus* and Hd-rR, compared with the other XY hybrids and the XY individuals of each strain (Figure 2). Similar reductions in the expression levels of *DMY* were also observed in the XY hybrids from crosses between *O. curvinotus* and Hd-rR at 10 and 15 days after hatching (data not shown).

Table 1 Genotypic and phenotypic sexes of the hybrids between *Oryzias curvinotus* females and *Oryzias latipes* males

Paternal strain	*XX*		*XY*		*Total*
	Female	*Male*	*Female*	*Male*	
Hd-rR[a]	138	0	30 (21%)	110	248
HNI	81	0	0	70	151
HNI.Y^{Hd-rR}	42	0	11 (18%)	51	93
Hd-rR.Y^{HNI}	82	0	0	75	157
Hd-rR.Y^{HNI}*rr*	110	0	0	98	208

[a]*Source*: Shinomiya *et al*. (2006).

Discussion

Our previous study showed that sex-reversed XY females were produced in the mating between *O. curvinotus* females and Hd-rR strain males of *O. latipes* (Shinomiya *et al*., 2006). However, this study has shown that no XY females were obtained in the hybrids between *O. curvinotus* females and HNI males. These findings indicate a strain difference in the ability to induce maleness in the XY hybrids. The two inbred strains, HNI and Hd-rR, were established from two regionally differentiated groups, namely Northern and Southern Japanese Populations, respectively (Sakaizumi *et al*., 1983; Takehana *et al*., 2003). They show high single-nucleotide polymorphism rates (Kasahara *et al*., 2007) and differ in terms of various quantitative traits, such as body shape, behavior and susceptibility to chemicals (Ishikawa, 2000). Furthermore, a recent analysis detected many differences in the craniofacial traits between the two inbred strains, and successfully identified chromosomal regions responsible for these traits using quantitative trait locus mapping (Kimura *et al*., 2007). Therefore, we consider that the hybrid XY sex reversal observed in this study is attributable to genetic differences between the HNI and Hd-rR strains, and that these differences make it possible to identify chromosomal regions associated with the sex reversal.

Using the two reciprocal Y-congenic strains (HNI.$Y^{Hd\text{-}rR}$ and Hd-rR.Y^{HNI}), we have clearly shown that the Y chromosome of *O. latipes* is responsible for the strain-specific difference in the hybrid sex reversal, as the former yielded XY sex reversal in the hybrids whereas the latter did not. Furthermore, a recombinant congenic

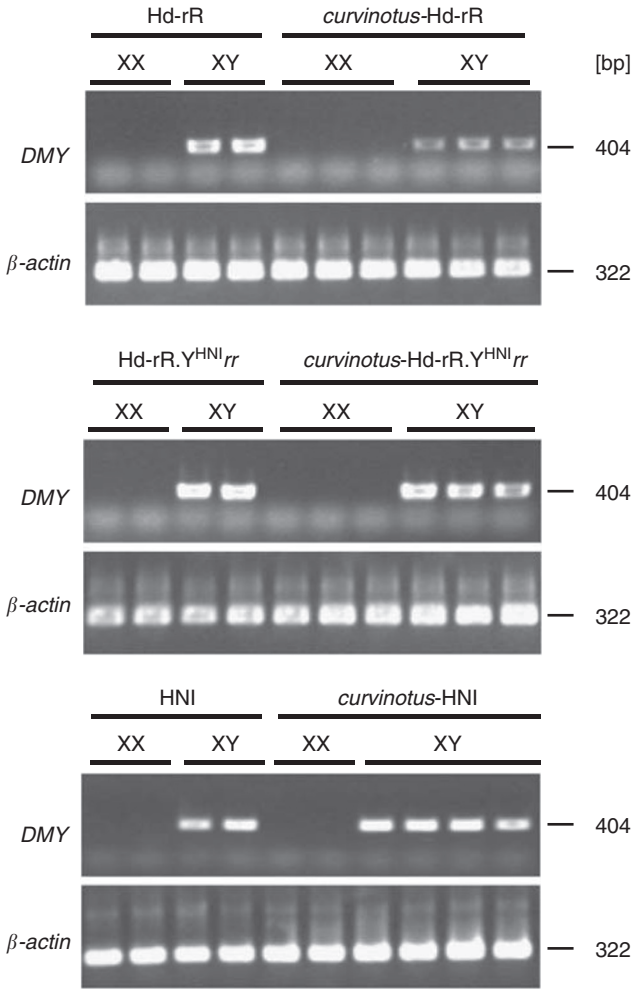

Figure 2 Expression of *DMY* in the XY hybrids. *DMY* mRNA expression at hatching was analyzed by reverse transcriptase PCR (RT-PCR). *β-actin* expression was determined for calibration. *DMY* transcripts are detected in all the XY embryos, but are clearly lower in the XY hybrids between *O. curvinotus* females and Hd-rR males.

strain (Hd-rR.Y^{HNI}*rr*) derived from the Hd-rR.Y^{HNI} strain produced no sex reversal in the hybrids, indicating that a small region of the Y chromosome contributes to the sex reversal. This critical region of the Y chromosome is in an interval of 1.8 cM between *SL1* and *51H7.F*, and contains the sex-determining gene, *DMY* (Matsuda *et al*., 2002). Taken together, these results suggest that the XY sex reversal in the hybrids results from incompatibility between the Hd-rR allele of the Y chromosomal locus and the *O. curvinotus* alleles of autosomal and/or X-chromosomal loci.

In *O. latipes*, mutations of *DMY* are associated with male-to-female sex reversal, and many mutations have been identified within the *DMY* open reading frame (Matsuda *et al*., 2002; Otake *et al*., 2006, 2008). Most of them were insertion/deletion mutations in the third exon of *DMY*, resulting in truncation of the DMY protein. As a consequence, all offspring that inherited these mutant alleles of *DMY* developed as XY females. On the other hand, some XY sex-reversal mutants with reduced expression of *DMY* produced both XY males and XY females in their progeny (Otake *et al*., 2006). The entire coding region of *DMY* was intact in these mutants, indicating that the XY sex reversal is associated with mutations that control *DMY* expression. Furthermore, the incidence of XY sex reversal was correlated with the *DMY* expression level, suggesting that a certain threshold level of *DMY* expression is required for male determination (Otake *et al*., 2006). These results suggest that the reduced expression of the *DMY* transcript can induce sex reversal in a subset of XY individuals having the same chromosome composition.

DMY expression first appears just before hatching, and morphological sex differentiation can be detected at hatching in both *O. latipes* and *O. curvinotus* (Matsuda *et al*., 2003; Kobayashi *et al*., 2004; Shinomiya *et al*., 2006). Our RT-PCR analyses indicated that the expression levels of *DMY* at hatching in the XY$^{Hd\text{-}rR}$ hybrids were severely reduced compared with those in the XY^{HNI} hybrids and in the XY individuals of the HNI and Hd-rR strains. This reduced expression suggests that the XY sex reversal in the hybrids could result from incompatibility between the *cis*-regulatory region of the *DMY*$^{Hd\text{-}rR}$ allele and autosomal or X-linked *trans*-acting loci of *O. curvinotus*. However, further expression analyses of *DMY* by quantitative PCR or *in situ* hybridization are necessary to determine whether the *DMY* expression levels are responsible for the XY sex reversal in the interspecific hybrids.

Similar conditions were found in mice, as XY sex reversal occurred when a certain variant of the *Mus musculus domesticus* type Y chromosome (Y^{DOM}) was transferred onto the C57BL/6J (abbreviated to B6) inbred strain background (Eicher *et al*., 1982). This B6-Y^{DOM} sex reversal was classified into three groups based on the gonadal phenotypes (Bullejos and Koopman, 2005). The first group, represented by Y^{POS} and Y^{TIR}, showed XY sex reversal with some ovaries or ovotestes on a B6 genetic background. The second group, represented by Y^{AKR} and Y$^{RF/J}$, had normal testes on a B6 genetic background, although the testis cord formation was delayed. The third group, represented by Y^{FVB} and Y^{SJL}, showed normal testis development on a B6 genetic background. It has been suggested that the B6-Y^{DOM} sex reversal is caused by abnormal interactions of B6-derived autosomal or X-linked loci with the *M. musculus domesticus* type Y chromosome. Furthermore, expression analyses showed reduced expression levels and delayed expression of *Sry*, which were correlated with the degree of sex reversal in each B6-Y^{DOM} strain, suggesting that regulatory mutations affecting the timing and/or levels of *Sry* expression are responsible for the sex reversal in B6-Y^{DOM} (Nagamine *et al*., 1999; Bullejos and Koopman, 2005).

As *O. latipes* and *O. curvinotus* are closely related species that are considered to have a common sex-determining pathway, sex reversal in their hybrids should arise from abnormal combinations of genes, rather than defective genes, in this pathway. Our results clearly showed that a Y-chromosomal region including *DMY* causes a strain-specific difference in the ability to induce maleness in the XY hybrids, suggesting that the hybrid sex reversal could result from incompatibility between this Y-chromosomal region of *O. latipes* and the *O. curvinotus* alleles of autosomal and/or X-chromosomal loci. Future analyses, including evaluation of the functional differences of *DMY* proteins among strains, analysis of the *DMY* expression patterns in the XY

hybrids, and identification of the *DMY* regulatory elements and the factors controlling *DMY* expression, will help to elucidate the molecular mechanism of the *DMY* action and sex reversal in the hybrids.

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