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ORIGINAL ARTICLE

An autosomal locus controls sex reversal in interspecific XY hybrids of the medaka fishes

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Although the two medaka species *Oryzias latipes* and *O. curvinotus* share the sex-determining gene *Dmy*, XY sex reversal occurs in interspecific hybridization between *O. latipes* females of the Hd-rR inbred strain and *O. curvinotus* males. In this Hd-rR-*curvinotus* mating, all XX and XY hybrids developed as females. In this study, we used another *O. latipes* inbred strain (HNI) for the mating, and found that 23% of XY hybrids developed as males, although all XX and the remaining XY hybrids developed as females. Linkage analysis using 236 XY hybrid males obtained from (Hd-rR × HNI) F_1 females showed that a single major locus, *Hybrid maleless* (*Hml*), on autosomal linkage group 17, contributed to the strain difference in the XY sex reversal. Furthermore, we found that crossing females of a different *O. latipes* inbred strain, HO4C, did not cause XY sex reversal in the interspecific hybrids, and that the XY hybrids from (Hd-rR × HO4C) F_1 females showed a 1:1 sex ratio. XY hybrid males had the HO4C allele at sequence-tagged site loci around the *Hml* locus whereas XY females had the Hd-rR allele, confirming the strong contribution of this locus to XY sex reversal. Reverse transcriptase PCR analysis showed a reduced expression of *Dmy*^{curvinotus} in XY fry of the Hd-rR-*curvinotus* hybrids at hatching. These results suggest that the Hd-rR allele at the *Hml* locus interfere with the function of *Dmy*^{curvinotus} on a hybrid background, thus resulting in XY sex reversal.
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Introduction

Gonadal sex determination is a fundamental process in vertebrates because the phenotypic sex depends on whether the gonad develops into a testis or an ovary. In most mammals, a single sex-determining gene on the Y chromosome, *SRY* is sufficient to induce the undifferentiated gonad to develop as a testis (Gubbay *et al*., 1990; Sinclair *et al*., 1990; Koopman *et al*., 1991; Capel *et al*., 1993). *SRY* encodes a high mobility group domain transcription factor, and its downstream target is the *Sox9* gene (Sekido and Lovell-Badge, 2008). Many other key downstream genes have been identified as having roles in the sex-differentiation pathway in human and mouse, although their molecular functions and the regulatory networks among these genes are unknown (Wilhelm *et al*., 2007).

A second vertebrate sex-determining gene was identified in the medaka fish, *Oryzias latipes*. This species also has an XX/XY sex-determination system (Aida, 1921), but initiation of the male-determination pathway begins with the expression of another Y chromosome-specific gene, *Dmy* (Matsuda *et al*., 2002). *Dmy* is the Y-specific paralog of the autosomal *Dmrt1* gene which appears to be involved in male sexual development in vertebrates (Nanda *et al*., 2002). Mutations with impaired *Dmy* function cause XY sex reversal (Shinomiya *et al*., 2004; Otake *et al*., 2006, 2008) and the presence of an exogenous *Dmy* gene induces XX sex reversal (Matsuda *et al*., 2007; Otake *et al*., 2009), suggesting that this gene is necessary and sufficient to induce testicular development. *Dmy* contains a DNA-binding motif (the DM domain), and is thus thought to encode a putative transcription factor that activates or represses downstream target genes to regulate sexual differentiation of the bipotential gonad. However, it remains unclear how *Dmy* controls the male-determination pathway and whether its downstream genes are conserved among vertebrates.

A straightforward approach for identifying genes involved in the sex-determination pathway is the genetic analyses of sex-reversal conditions. Our previous study demonstrated an intriguing sex reversal in the interspecific hybridization between *O. latipes* and *O. curvinotus* (Shinomiya *et al*., 2006), despite these two species having a common sex-determining pathway with the *Dmy* gene on the homologous Y chromosome (Matsuda *et al*., 2003). In XY^{latipes} hybrids between *O. curvinotus* females and *O. latipes* males, Hd-rR strain males produced XY females in the hybrids (110 males and 30 females), whereas HNI males produced no XY females (70 males and 0 females). This demonstrates that there is a strain difference in the incidence of XY^{latipes} females (Shinomiya *et al*., 2006; Kato *et al*., 2010). A congenic approach successfully demonstrated that a small region on the Y chromosome, which included *Dmy*, was

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responsible for the strain-specific difference in this $XY^{latipes}$ sex reversal (Kato *et al*., 2010).

In the opposite cross between *O. latipes* females (Hd-rR strain) and *O. curvinotus* males, all $XY^{curvinotus}$ hybrids developed as females. This suggests that *Dmy* derived from *O. curvinotus* cannot determine maleness in the hybrid background (Shinomiya *et al*., 2006). A previous study reported that *O. latipes* females from some wild strains produced both males and females in the interspecific hybrids (Sakaizumi *et al*., 1992), implying that there were genetic background differences in the incidence of the $XY^{curvinotus}$ sex reversal. However, these strain differences have not been confirmed because there were no sex chromosome-linked markers available in the hybrid fish until recently.

In the present study, we compared the ability to induce maleness in the $XY^{curvinotus}$ hybrids among three medaka inbred strains, Hd-rR, HNI and HO4C, using the *Dmy* gene as a Y chromosomal marker. Because these strains showed different incidences of XY sex reversal in the interspecific hybrids, we mapped the chromosomal region responsible for the sex reversal. Linkage analysis using the Hd-rR and HNI strains identified a single major locus on linkage group (LG) 17, which was associated with the strain differences in the XY sex reversal. This region also contributed to differences in the frequency of hybrid sex reversal between the Hd-rR and HO4C inbred strains, suggesting that the hybrid XY sex reversal may be caused by an incompatibility between a single autosomal locus derived from *O. latipes* and the *Dmy* allele of *O. curvinotus*. Further analysis will reveal the molecules that interact with *Dmy*, and allow us to understand the molecular mechanisms of sex determination that begin with *Dmy*.

Materials and methods

Fish

We used a laboratory stock of *O. curvinotus*, as well as three inbred strains (HNI, Hd-rR and HO4C) of *O. latipes*. All fish were supplied by a sub-center (Niigata University, 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 (see Takehana *et al*., 2005). HNI was established from the Northern Japanese Population, whereas Hd-rR and HO4C were from the Southern Japanese Population (Hyodo-Taguchi, 1996; Kinoshita *et al*., 2009). The fish were maintained in aquaria under an artificial 14-h light/10-h dark photoperiod at 27 ± 2 °C.

Mating and sexing

Hybrid fish were obtained from matings between *O. latipes* females and *O. curvinotus* males (XY or YY males) by pair mating. Naturally spawned eggs were collected and incubated under the same conditions as the adult fish. Hatched hybrid fish were reared until maturation for 2–3 months, and examined for their phenotypic and genotypic sexes.

Phenotypic sex was judged based on secondary sex characteristics, namely, the shapes of the dorsal and anal fins. Genotypic sex (XY or XX) was determined by the presence or absence of the *Dmy* gene of *O. curvinotus* evaluated by PCR amplification of caudal fin clip genomic DNA extracted from adult fish. PCR amplification was performed with the primers PG17.eS (5′-CGCCTTGAGGAGGCAGCAGG-3′) and PG17.20U (5′-GCATCTGCTGGTACTGCTGGTAGTTG-3′) with an annealing temperature of 65 °C (Shinomiya *et al*., 2006). Because the PCR primers amplify both *Dmy* and its autosomal paralog *Dmrt1*, individuals with only *Dmrt1* fragments were judged to have an XX genotype, and those with both *Dmy* and *Dmrt1* fragments were judged to have an XY genotype.

Production of YY males

Fertilized eggs of *O. curvinotus* were incubated in water containing estradiol-17β (Sigma, St Louis, MO, USA) at $0.2\,\mu g\,ml^{-1}$ until hatching. Hatched fry were transferred to normal tap water and fed on a commercial pet-food diet until sexual maturation. Sex-reversed XY females were identified by PCR genotyping of *Dmy* and subsequently mated with normal XY males. YY males were selected by PCR genotyping of *Dmy* and *Casp6* (Kondo *et al*., 2001; Matsuda *et al*., 2003).

Genetic mapping

Sixty expressed sequence tag (EST) markers covering twenty-four chromosomes were used to map the locus responsible for strain-specific differences in hybrid sex reversal. The primer sequences used in this study are listed in Supplementary Table S1, and the mapped positions of the EST markers are available in M Base (http://earth.lab.nig.ac.jp/~mbase/medaka_top.html). In addition, we designed sequence-tagged site (STS) primers based on genome sequence data (UTGB medaka genome browser: http://medaka.utgenome.org/ or Ensembl genome browser: http://www.ensembl.org), and used these STS markers for fine mapping. Primer sequences and chromosomal locations of these markers are listed in Supplementary Table S2. Individual PCR conditions were optimized for each primer pair, and products were digested with restriction enzymes if necessary. Separation of PCR products was performed by conventional polyacrylamide gel electrophoresis (Kimura *et al*., 2004).

RNA extraction and RT-PCR

Total RNA was extracted from fry at 8 days post fertilization (dpf) using an RNeasy Mini Kit (Qiagen, Tokyo, Japan), and subjected to reverse transcriptase PCR (RT-PCR) using a OneStep RT-PCR Kit (Qiagen). Aliquots (20 ng) of the total RNA were used as templates in 25 µl reaction volumes. The PCR amplification conditions were as follows: 30 min at 55 °C; 15 min at 95 °C; cycles of 30 s at 96 °C, 30 s at the annealing temperature and 60 s at 72 °C; and 5 min at 72 °C. The number of PCR cycles for *Dmy* and β-*actin* was adjusted to be 36 cycles and 22 cycles, and the annealing temperature at 63 °C and 55 °C, respectively. The primers for *Dmy* (cY331, 5′-AGG CTT CGT CCG GCC CTG AA-3′ and cY4-5U, 5′-GAG GCT CCT GGT GCA GAA CG-3′) amplified a 449-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 (Otake *et al*., 2006). Aliquots of 8 and 2 µl of the *Dmy* and *β-actin* RT–PCR products, respectively, were electrophoresed in a 2% agarose gel and stained with ethidium bromide.

Results

The HNI strain produces XY males in the hybrids

Our previous study indicated that interspecific hybridization between *O. latipes* females and *O. curvinotus* males caused complete XY sex reversal (Shinomiya *et al.*, 2006). We used Hd-rR inbred strain females for matings with *O. curvinotus* males (Hd-rR-*curvinotus*), and found that all 569 hybrids developed as females. Among 99 genotyped individuals, 55 individuals had the XX genotype and 44 had XY, demonstrating that all XY individuals were sex reversed (Table 1). In the present study, we used another inbred strain, HNI, for matings with *O. curvinotus* males (HNI-*curvinotus*). All 69 XX hybrids were female, whereas the 60 XY hybrids consisted of 46 females and 14 males (23%), indicating that the HNI strain could produce XY males in the hybrids. This means that the two inbred strains HNI and Hd-rR differ in their incidences of XY sex reversal in the interspecific hybrids. Next, we used (Hd-rR $\times$ HNI) F_1 females and ($F_1 \times$ Hd-rR) BC_1 females for matings with *O. curvinotus* males. The F_1 females produced 147 females and 38 males (21%) in the XY hybrids, and four out of five BC_1 females produced males in the XY hybrids wheras the one remaining female did not (Table 1).

A single major locus controls the sex reversal ratio in the XY hybrids

To map the chromosomal region(s) responsible for the strain differences in the XY sex reversal ratio, an initial linkage analysis was performed using EST markers established in *O. latipes*. In the cross between (Hd-rR $\times$ HNI) F_1 females and *O. curvinotus* males, the hybrids have either an Hd-rR- or an HNI-derived allele at each locus (Figure 1). Because XY males were found only in the HNI-*curvinotus* hybrids, we assumed that the chromosomal regions heterozygous for HNI and *O. curvinotus* were responsible for producing XY males. Among 38 hybrid males obtained from the cross, we selected 24 males and used them for genome-wide genotyping with 60 EST markers covering 24 chromosomes. Then we searched for loci that showed a significant deviation from the expected 1:1 ratio, using a classical chi-squared test. The tests revealed significantly high chi-squared values at four loci, *OLa21.11f*, *OLc30.09h*, *AU168385* and *MF01SSA074C05* ($P<0.01$), which are all located on LG 17 (Table 2). At these loci, HNI/*curvinotus* heterozygotes appeared at a significantly higher frequency than the 1:1 ratio, but other loci did not show a significant deviation from the expected 1:1 segregation. Furthermore, genotyping for the BC_1 females used in progeny tests (see Table 1) revealed that BC_1 females producing XY males had the HNI/Hd-rR genotype whereas those producing only XY females had the Hd-rR/Hd-rR genotype at the EST markers on LG 17 (data not shown). These results suggested that a single major locus, which we named *Hybrid maleless* (*Hml*), was associated with the strain differences in the hybrid sex reversal ratio.

Genetic mapping using all 38 XY hybrid males showed that the *Hml* locus was located on LG 17 flanked by the two molecular markers *AU171199* and *LG17-29* (Figure 2a). To map the *Hml* locus precisely, we obtained an additional 198 XY hybrid males from matings between (Hd-rR $\times$ HNI) F_1 females and *O. curvinotus* YY males, and genotyped new STS markers. This analysis narrowed the chromosomal location of *Hml* to a 1.2 cM interval between the two markers *gs20859* and *LG17–29*, with strong linkage to an additional four markers *LG17–54*, *sca3316*, *LG17–49* and *LG17–51* (0 recombination events in 236 meioses; Figure 2b). On the basis of the latest genome assembly (version 1.0), three scaffolds (354, 435 and 1423) were mapped to this region, although there remain large gaps (approximately

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

Parental females	XX	XY		Total
	Females	Females	Males (%)	
Hd-rR[a]	55	44	0 (0)	99
HNI	69	46	14 (23)	129
(Hd-rR × HNI)F_1	194	147	38 (21)	379
(F_1 × Hd-rR)BC_1#1	58	37	7 (16)	102
(F_1 × Hd-rR)BC_1#2	7	7	1 (13)	15
(F_1 × Hd-rR)BC_1#3	26	14	2 (13)	42
(F_1 × Hd-rR)BC_1#4	46	42	9 (18)	97
(F_1 × Hd-rR)BC_1#5	30	29	0 (0)	59

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

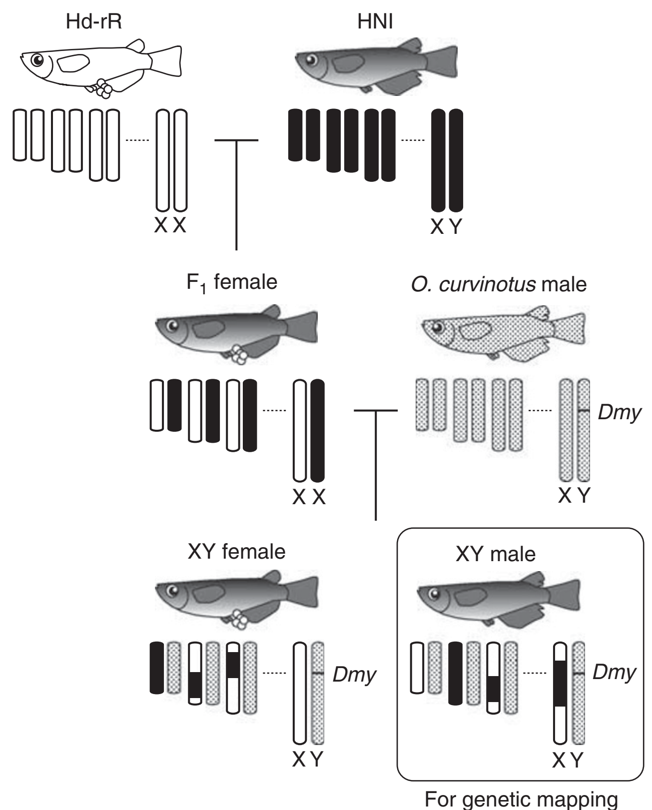

Figure 1 Mating scheme for linkage analysis. F_1 females between Hd-rR and HNI strains were crossed with *O. curvinotus* males, and the resulting XY males in the hybrids were used for genetic mapping. These XY males had an *O. curvinotus*-derived Y chromosome and various combinations of HNI chromosomal segments in the autosomes and the X chromosome. We searched for chromosomal regions heterozygous for HNI and *O. curvinotus* alleles, which could produce XY males. Solid, HNI-derived chromosome; open, Hd-rR-derived chromosome; dotted, *O. curvinotus*-derived chromosome. The position of the sex-determining gene *Dmy* is shown.

Table 2 EST markers associated with the strain difference in the XY sex reversal

LG	*EST marker*	*Position (Mbp)*	*D/C genotype*	*N/C genotype*	*Total*	χ^2 *value*	P
17	*OLa21.11f*	3.4	4	18	22	8.91	0.0028
17	*OLc30.09h*	4.9	3	20	23	12.6	0.00039
17	*AU168385*	13.4	1	22	23	19.2	0.000012
17	*MF01SSA074C05*	17.4	3	20	23	12.6	0.00039

Abbreviation: EST, expressed sequence tag.
Of 60 markers throughout the genome, only significant markers are listed in the table (threshold value, $P<0.01$, df = 1). D/C, heterozygous for Hd-rR and *O. curvinotus* alleles; N/C, heterozygous for HNI and *O. curvinotus* alleles. Positions of the markers were retrieved from the medaka ensemble database (Ensembl release 57; http://www.ensembl.org/).

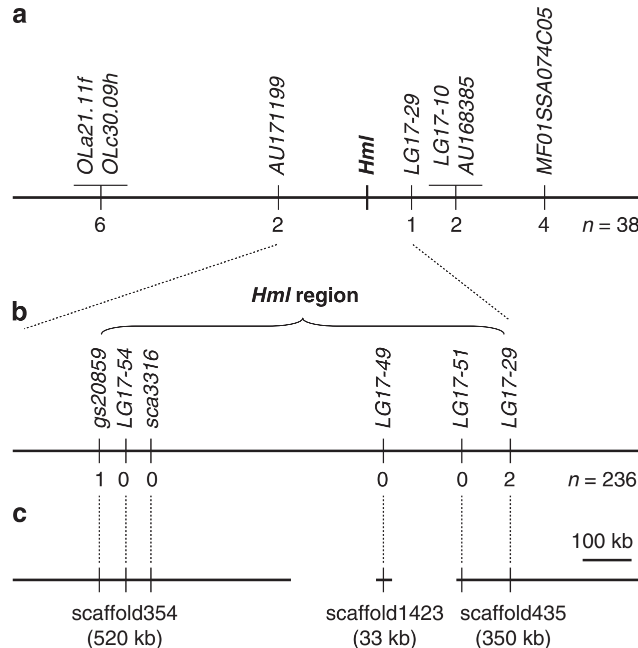

Figure 2 Genetic and physical map around the *Hml* locus. (**a**) A sparse genetic map around the *Hml* locus on linkage group 17. (**b**) A high-resolution recombination map around the *Hml* locus. The *Hml* gene was mapped to a 1.2 cM interval based on the genotyping of 236 XY hybrid males. The numbers under each map indicate the number of recombinants between *Hml* and the adjacent marker. (**c**) A physical map around the *Hml* locus based on the available genome sequence data (version 1.0; http://medaka.utgenome.org/). Three scaffolds (354, 435 and 1423) are mapped to this region but there remain gaps between the scaffolds.

120 and 140 kb) between the scaffolds (Figure 2c). To find candidate genes in this region, we surveyed mapped genes on these scaffolds using the UCSC genome browser (http://genome.ucsc.edu). Among 12 genes found in this region (chr17: 10.8–11.6 Mb), seven encoded zinc finger proteins, suggesting a clustered organization of these genes on the *Hml* region.

The HO4C strain did not cause XY sex reversal in the hybrids

For further fine mapping, we used another inbred strain, HO4C, for matings with YY males of *O. curvinotus* (HO4C-*curvinotus*). In this cross, all 34 XY hybrids developed as males with no sex reversal (Table 3), demonstrating that *Dmy*^{curvinotus} sufficiently induced maleness on this hybrid background. Then, we used

Table 3 Genotypic and phenotypic sexes of hybrids between *Oryzias latipes* females and *O. curvinotus* YY males

Parental females	*XY*		*Total*
	Females	*Males (%)*	
HO4C	0	34 (100)	34
(Hd-rR × HO4C)F_1	121	135 (53)	256

Table 4 Linkage analysis between phenotypic sex and STS markers on linkage group 17 in XY hybrids between (Hd-rR × HO4C)F_1 females and *O. curvinotus* males

STS marker	*D/C genotype*		*O/C genotype*		*Total*
	Females	*Males*	*Females*	*Males*	
gs20859	121	0	0	135	256
LG17–10	120	1	1	134	256

Abbreviation: STS, sequence-tagged site.
D/C, heterozygous for Hd-rR and *O. curvinotus* alleles; O/C, heterozygous for HO4c and *O. curvinotus* alleles.

(Hd-rR × HO4C) F_1 females for the matings, and found a 1:1 Mendelian ratio of males to females in the XY hybrids (135 and 121 progeny, respectively) (Table 3). Furthermore, two STS markers, *gs20859* and *LG17-10*, showed strong linkage to the phenotypic sex (Table 4). For these loci, XY females had the Hd-rR/*curvinotus* genotype whereas XY males had the HO4C/*curvinotus* genotype. These results strongly suggest that the *Hml* locus is involved in the different sex reversal ratios between Hd-rR-*curvinotus* and HO4C-*curvinotus* hybrids.

Reduced *Dmy* expression in Hd-rR-*curvinotus* XY hybrids

Reduced expression levels of the sex-determining gene *Dmy* have been observed in some XY sex-reversal mutants of *O. latipes* (Matsuda *et al*., 2002; Otake *et al*., 2006). To examine the relationship between the hybrid sex reversal and the *Dmy*^{curvinotus} expression level, we performed an RT-PCR analysis of fry at hatching (8 dpf) in the Hd-rR-*curvinotus* hybrids, the HO4C-*curvinotus* hybrids and the male parental strain of *O. curvinotus* (Figure 3). The *Dmy* mRNA level was slightly lower in XY fry of the HO4C-*curvinotus* hybrids than in that of *O. curvinotus*, although all the XY hybrids developed as males in adulthood. On the other hand, *Dmy* expression was not detected in XY fry of the Hd-rR-*curvinotus* hybrids, which developed as all females in adulthood,

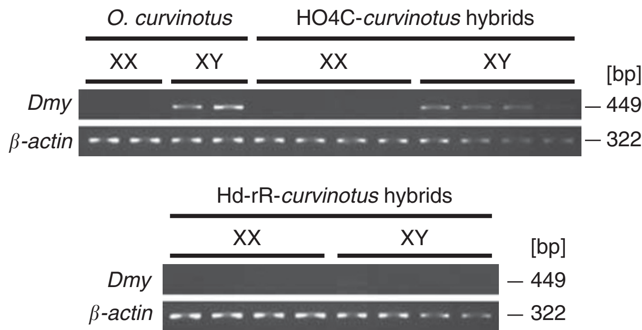

Figure 3 Expression of *Dmy* in the medaka hybrids. *Dmy*^{curvinotus} mRNA expression in fry at hatching (8 dpf) was analysed by RT-PCR. *β-actin* expression was determined for calibration. Depressed or eliminated expression of *Dmy* transcripts was observed in XY fry of the Hd-rR-*curvinotus* hybrids.

suggesting that reduced expression of *Dmy*^{curvinotus} was associated with the XY sex reversal in the Hd-rR-*curvinotus* hybrids.

Discussion

Our mating experiments clearly demonstrated that the incidence of XY females was different among inbred strains of *O. latipes*. All XY individuals (44/44) in Hd-rR-*curvinotus* hybrids were sex-reversed females, whereas 23% (14/60) of XY HNI-*curvinotus* hybrids developed as males. Furthermore, all XY (34/34) HO4C-*curvinotus* hybrids developed as males, indicating that there are strain differences in the ability to induce maleness in the XY hybrids. Among these hybrids, the Y chromosome and one set of autosomes are derived from *O. curvinotus* whereas another set of autosomes and the X chromosome are different, suggesting that the presence or absence of XY females in the hybrids can be attributed to the differences in the maternal genome. Notably, male fish appeared only in XY individuals, indicating that *Dmy*^{curvinotus} is necessary for the male determination of the hybrids. Taken together, these findings suggest that the XY sex reversal in the hybrids results from incompatibility between the *Dmy*^{curvinotus} allele and the *O. latipes* alleles of autosomal and/or X chromosomal loci.

The sex reversal condition observed in the medaka hybrids is similar to XY sex reversal in B6-Y^{POS} mice. This situation occurs when the Y chromosome derived from *Mus poschiavinus* (Y^{POS}) is transferred onto the C57BL/6J (B6) inbred strain that normally carries a *M. musculus* Y chromosome (Eicher *et al*., 1982). Genetic background differences have been characterized in the inbred mouse strains. The B6 background was particularly sensitive to XY sex reversal, whereas other strains, including DBA/2J and 129S1/SvImJ, were found to be completely resistant to Y^{POS}-associated sex reversal (Nikolova *et al*., 2008). Quantitative trait loci mapping using the B6-Y^{POS} and DBA/2J strains identified multiple loci that conferred some but not all of the observed sensitivity to XY sex reversal in B6 (Eicher *et al*., 1996). More recently, a congenic approach using the B6.129S1/SvImJ-Y^{POS} strain identified that a chromosome 11 region derived from the 129S1/SvImJ strain provided partial protection from sex reversal in XY^{POS} mice (Nikolova *et al*., 2008). These findings implicate the combined effects of many loci (rather than a single gene) in conferring the sensitivity to sex reversal in B6-Y^{POS}; this has been confirmed by a recent expression quantitative trait loci approach (Munger *et al*., 2009).

By contrast, a single locus is implicated in sex reversal in the medaka hybrids. (Hd-rR × HNI) F_1 females produced XY males in the interspecific hybrids, and four out of five (F_1 × Hd-rR) BC_1 females produced XY males, whereas the remaining BC_1 female did not, implying that a small number of genes contributed to the strain differences in the hybrid XY sex reversal. Our linkage analysis using XY hybrid males obtained from (Hd-rR × HNI) F_1 females successfully identified the single major locus *Hml* on LG 17 that confers sensitivity to the XY sex reversal in the hybrids. In addition, genotyping for XY hybrids obtained from (Hd-rR × HO4C) F_1 females revealed strong linkage to STS markers around the *Hml* locus, suggesting that the same *Hml* locus contributed to the sex reversal in the different XY hybrids. These results suggest that a single gene, rather than a disrupted global network, causes XY sex reversal in the medaka hybrids. Thus, it is likely that allelic differences at the single *Hml* locus affect *Dmy*^{curvinotus} function in the male-determining process in the hybrid fish.

Dmy transcripts first appear just before hatching exclusively in XY individuals of *O. latipes* and *O. curvinotus* (Matsuda *et al*., 2003; Kobayashi *et al*., 2004; Shinomiya *et al*., 2006). Reduced expression of the *Dmy* gene causes male-to-female sex reversal in *O. latipes*, suggesting that a threshold level of *Dmy* expression is required for male development (Matsuda *et al*., 2002; Otake *et al*., 2006, 2008). Our RT-PCR analysis of the medaka hybrids also revealed that *Dmy* expression levels in XY fry differed between Hd-rR-*curvinotus* and HO4C-*curvinotus* hybrids, in agreement with their sex reversal ratio. Hd-rR-*curvinotus* hybrids showed a loss of *Dmy*^{curvinotus} expression in the XY fry, which developed as all phenotypic females, suggesting that the *Hml* gene is an upstream regulator of *Dmy*. Thus, the XY sex reversal in the hybrids may result from an incompatibility between the *cis*-regulatory region of the *Dmy*^{curvinotus} allele and some *Hml* alleles of *O. latipes* as a *trans*-acting factor.

In this study, only 23% of XY individuals developed as males in the HNI-*curvinotus* hybrids. XY hybrids harboring identical genotypes developed as males or females, suggesting an incomplete penetrance for this phenotype. In the medaka mutants, a low level of *Dmy* expression below a threshold can induce sex reversal in a subset of XY individuals having the same chromosomal condition (Otake *et al*., 2006). Thus, a certain threshold level of *Dmy* expression may be required for male determination also in the hybrid backgrounds. Similar to the HNI-*curvinotus* hybrids, we obtained male XY hybrids from (Hd-rR × HNI) F_1 females. However, the incidence of XY males (21%) was higher than expected. In this cross, we estimated that approximately 11% of XY individuals would become males, because half of the XY hybrids would have the Hd-rR allele at the *Hml* locus, and develop as females, whereas the other half of the XY hybrids would have the HNI allele, and consist of males (23%) and females (77%). The observed high incidence of XY hybrid males is probably due to a

background effect when the HNI allele at the *Hml* locus is in the hybrid background containing a part of the Hd-rR genome. Therefore, the Hd-rR background in the hybrids seems to affect the *Dmy* expression level itself or the threshold for *Dmy* expression, and thus cause the higher frequency of the XY males. However, further expression analysis of *Dmy* is necessary to test these hypotheses.

In both *O. latipes* and *O. curvinotus*, the first appearance of morphological sex differentiation was a difference in the number of germ cells between XX and XY embryos, and a subset of germ cells in XX entered meiosis around hatching (Shinomiya *et al*., 2006). Later, morphological sex differences in somatic cells were observed. When *Dmy* function is impaired, germ cells in XY embryos start to proliferate and then enter meiosis just like XX embryos (Otake *et al*., 2006, 2008). These findings suggest that *Dmy* is involved in the regulation of germ cell proliferation at the early sex-determining stage and the formation of the testicular architecture. Previous histological observation of developing gonads in Hd-rR–*curvinotus* hybrids demonstrated that active proliferation of germ cells and oogenesis occurred in early gonadal development in the XY hybrids, identical to the early ovarian development in the XX hybrids (Shinomiya *et al*., 2006). Therefore, it is likely that the mismatching of the Hd-rR allele at the *Hml* locus with the regulatory region of $Dmy^{curvinotus}$ in the hybrids causes reduced expression of *Dmy* and disruption of the initial testis-determination steps including suppression of germ cell proliferation, thus resulting in XY sex reversal.

Detailed understanding of the molecular mechanisms underlying the sex reversal in the XY hybrids requires isolation and molecular characterization of the *Hml* locus. Although we found 12 candidate genes including 7 zinc finger proteins within the *Hml* region, other genes should be potentially located on the gap regions. To find them, we performed genomic synteny analysis using the Genomicus genome browser (version 60.01; http://www.dyogen.ens.fr/genomicus/). However, we could not find other candidate genes by this approach, because the genes around the *Hml* region were mapped to several chromosomes and/or scaffolds in other fish species including stickleback and *Tetraodon*. Furthermore, the gene order was not conserved among these fish species. Therefore, we have taken a positional approach to isolate the *Hml* gene, and started a chromosome walking using a bacterial artificial chromosome clones. So far, we have isolated two bacterial artificial chromosome clones that fill the gaps, and estimated the 1.2 cM region of interest to be 670–720 kb (with the gap region as 200–250 kb in total) which is similar to that in the genome data. To identify the *Hml* gene, we will determine the complete genomic sequence of these bacterial artificial chromosome clones, identify the functional genes in this genomic region and perform functional analyses of the candidate genes. Isolation of the *Hml* gene will help to define not only the processes and mechanisms underlying the hybrid XY sex reversal but also the transcriptional regulatory mechanism of *Dmy* in normal development of *O. latipes* and *O. curvinotus*.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Heredity website (http://www.nature.com/hdy)