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The XX-XY Sex-determination System in *Oryzias luzonensis* and *O. mekongensis* Revealed by the Sex Ratio of the Progeny of Sex-reversed Fish

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ABSTRACT—The sex-determining gene in *Oryzias latipes* and *O. curvinotus* has been proved to be *DMY*. Although *O. curvinotus* has the DMY gene on the Y chromosome which is homologous to the Y chromosome of *O. latipes*, the sex-determining mechanism of other *Oryzias* fishes has not been identified. In order to uncover the sex-determining mechanism of *O. luzonensis* and *O. mekongensis*, which are most closely related species to *O. latipes* and *O. curvinotus*, we analyzed the sex ratio of the progeny of sex-reversed fish. We were able to obtain sex-reversed males by the administration of methyltestosterone, and found that these yielded all-female offspring in both species. These results indicate that *O. luzonensis* and *O. mekongensis* have the XX-XY sex-determination system.

Key words: *Oryzias*, sex-determination, sex chromosome, sex reversal

The genus *Oryzias* consists of 14 species endemic to Asia from India to Japan (Nelson 1994). From cytogenetical studies, Uwa (1986) divided these species into three groups: the mono-armed chromosome group, the bi-armed chromosome group, and the fused-chromosome group. This classification has been confirmed by molecular phylogenetic studies (Naruse *et al.,* 1992). The bi-armed group consists of four species, *O. latipes*, *O. curvinotus*, *O. luzonensis* and *O. mekongensis*, and the phylogenetical vicinity of these species has been evidenced by analyses of the nucleotide sequences of the mitochondrial *cytochrome b* gene as well as the 12S and 16S rRNA (Naruse 1996, Takehana *et al.,* in preparation).

Studies on the sex-determining mechanism of *O. latipes* began when Aida (1921) indicated that the *r* gene, which controls the presence or absence of yellow color pigment in xanthophores, was linked to a sex chromosome, and demonstrated that *O. latipes* had the XX-XY sex-determination system. Recently, we have identified molecular markers on the sex chromosomes (Matsuda *et al.,* 1997), and succeeded in visualizing the sex chromosomes (Matsuda *et al.,* 1998). In addition, through constructing a precise genetic map of the sex chromosomes (Sato *et al.,* 2001) and a BAC-library (Matsuda *et al.,* 2001), we cloned a candidate for the sex-determining gene of *O. latipes*, *DMY* (Matsuda *et al.,* 2002). The introduction of *DMY* into XX eggs induced the development of testes and caused functional sex-reversal from female to male (Matsuda *et al.,* in preparation), proving that *DMY* is the sex-determining gene of this fish.

In *O. curvinotus*, a Y-linked gene homologous to *DMY* has been identified (Matsuda *et al.,* 2003), and a syntenic relationship between the sex-chromosomes of *O. latipes* and *O. curvinotus* has been demonstrated (Kondo *et al.,* 2001, Sato *et al.,* unpublished data), indicating that these fishes have a common sex-determining mechanism. However, no *DMY* homologues were detected in other *Oryzias* fishes by a PCR survey of *Oryzias* fishes using several primer sets for the *DMY* gene of *O. latipes*. In addition, molecular phylogenetical studies have indicated that *DMY* originated from a duplication of *DMRT1* that occurred just prior to the speciation of *O. latipes* and *O. curvinotus* (Matsuda *et al.,* 2003). These facts suggest the possibility that fishes other than *O. latipes* and *O. curvinotus* adopt different sex-determining mechanisms. Therefore, from an evolutionary point of view, it is important to examine the sex-determining mechanism of *Oryzias* fishes other than *O. latipes* and *O. curvinotus*. In the present study, we investigated the sex-determination system of *O. luzonensis* and *O. mekongensis* to demonstrate whether they have the XX-XY type of sex-determination system.

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MATERIALS AND METHODS

Fish

The *Oryzias luzonensis* and *O. mekongensis* used in this study were from stocks in the Faculty of Science, Niigata University (*O. luzonensis*: collected by Formacion and Uwa 1982; *O. mekongensis*: collected by Magtoon and Uwa in 1984). They were kept indoors at 27±2°C, under a photoperiod of 14L-10D.

Sex steroid treatments for sex reversal

The sex-reversal experiment was carried out basically according to the method of Iwamatsu (1999). Briefly, hormones (methyltestosterone (MT) and estradiol-17β (E2); Sigma Chemical Co., St. Lous, MO) were dissolved in ethanol to make stock solutions and stored in a refrigerator. Just prior to use, stock solutions were diluted with aged tap water. Naturally spawned eggs were collected and incubated in the hormone-containing water at concentrations of 0.04 and 0.2 μg/ml of E2, and 0.001, 0.005 and 0.025 μg/ml of MT. Hatched fry were transferred to normal tap water and fed on a commercial pet-food until sexual maturation. We examined the secondary sex characters of the treated fish and judged the sex of the individuals. Treated fish were mated with normal males or females, and the sex of the offspring was examined histologically.

Histological method and judgment of the sex of the fry

At 20 days after hatching, fry were fixed *in toto* in Bouin's solution, sectioned at 6 μm in paraffin, and stained with hematoxylin and eosin for microscopic observation. We identified the sex of each fry from the histology of their developing gonads.

RESULTS

1) Sex ratios in the hormone-treated groups

The sex ratios of the E2-treated groups deviated toward females, suggesting that E2 induced sex reversal from male to female in both *O. mekongensis* and *O. luzonensis* (Table 1). In the groups reared under normal conditions, the sex ratios of *O. luzonensis* and *O. mekongensis* were almost 1:1 (data not shown). Therefore, the E2-treated groups are expected to contain sex-reversed females whose genetic sex is male (XY or ZZ).

The results for the MT-treated groups are also shown in Table 1. The sex ratios deviated from 1:1 toward males, indicating that MT could reverse the sex of the fish from female to male, that is, XX or ZW males are included in the treated fish.

Table 1. Sex ratios of the hormone-treated fishes of *O. luzonensis* and *O. mekongensis*.

Concentration	*O. luzonensis*		*O. mekongensis*	
(μg/ml)	Male	Female	Male	Female
Methyltestosterone				
0.001	–	–	11	2
0.005	35	5	7	4
0.025	30	2	17	4
Estradiol				
0.04	–	–	5	20
0.2	0	20	0	7

Table 2. Sex ratios of the offspring of mating between normal and hormone-treated fishes of *O. luzonensis.*

Offspring of fishes treated with estradiol				Offspring of fishes treated with methyltestosterone			
Mating	Male	Female	Total	Mating	Male	Female	Total
1	33	5	38	1	0	55	55
2	12	15	27	2	0	54	54
3	12	9	21	3	0	47	47
4	10	9	19	4	14	19	33
5	13	3	16	5	16	10	26
				6	0	15	15
				7	3	9	12

Table 3. Sex ratios of the offspring of mating between normal and hormone-treated fishes of *O. mekongensis.*

Offspring of fishes treated with estradiol				Offspring of fishes treated with methyltestosterone			
Mating	Male	Female	Total	Mating	Male	Female	Total
1	14	18	32	1	0	52	52
2	22	10	32	2	0	39	39
3	15	11	26	3	21	15	36
4	13	11	24	4	0	27	27
5	9	11	20	5	10	15	25
6	10	9	19	6	9	15	24
7	5	10	15	7	5	19	24
8	6	3	9	8	0	23	23
				9	11	8	19

2) Sex ratios of the offspring of the treated fish

For mating in *O. luzonensis* treated with sex hormones, we used 5 females obtained from the 0.2 μg/ml E2 treatment group and 7 males from the 0.025 μg/ml MT treatment group. For *O. mekongensis*, we used 8 females from the 0.04 μg/ml E2 treatment group and 9 males from the 0.001 μg/ml MT treatment group. The sex ratios of the offspring from each mating are shown in Table 2 (*O. luzonensis*) and Table 3 (*O. mekongensis*).

All females from E2 treatment group in both species produced both males and females, although the sex ratios were variable. On the other hand, 4 of 7 males from MT treatment group in *O. luzonensis* yielded all-female progeny. In *O. mekongensis*, all offspring from 4 of 9 males treated with MT were females. These results indicated that the MT-treatment groups contained XX sex-reversed males of *O. luzonensis* as well as *O. mekongensis*, that is, both species adopt an XX-XY sex-determination system.

DISCUSSION

The sex-determining mechanism in vertebrates is variable. Almost all mammalian species have the XX-XY sex-

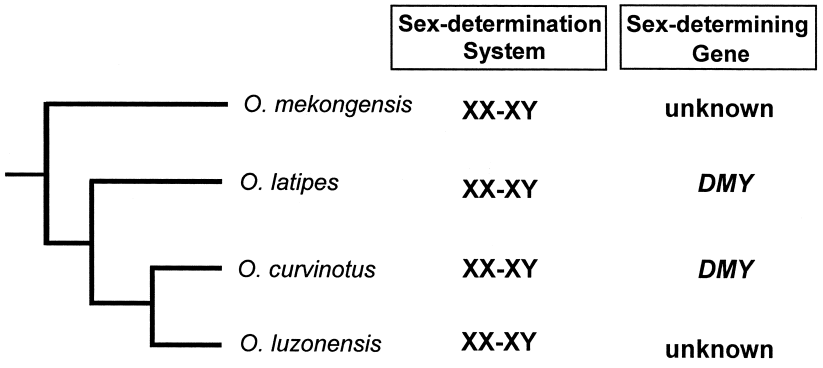

Fig. 1. The sex-determination systems and the sex-determining gene in bi-armed group of *Oryzias* fishes. The phylogentical relationship of the four *Oryzias* fishes is drawn. The present study indicated that *O. mekongensis* and *O. luzonensis* have XX-XY system as *O. latipes* and *O. curvinotus*. The *DMY* has proven to be originated in the common ancestor of *O. curvinotus* and *O. luzonensis*, and we failed to identify in *O. luzonensis* a homologous gene to DMY, therefore the sex determining genes of *O. mekongensis* and *O. luzonensis* may possibly be other genes than *DMY* homologues.

determination system, with *Sry* as the common male-determining gene, whereas birds have ZZ-ZW type sex chromosomes (reviewed by Solari 1994). In lower vertebrates, amphibians and fishes, both XX-XY and ZZ-ZW systems are known. Even within a genus or species, it has been shown that there are a variety of systems. In *Rana rugosa*, there is a geographical diversity in the sex chromosome types, that is, in west Japan they have XX-XY sex chromosomes, while the northern part of Japan populations have been reported to have ZZ-ZW sex chromosomes (Nishioka *et al.,* 1993, 1994). In fishes of the genus *Xiphophorus*, X, Y and Z sex chromosomes are known to exist (Volff and Schartl 2001).

With the exception of mammals, sex-determining genes were not identified until quite recently, and therefore it has been impossible to examine the diversity in the sex-determining mechanism at the molecular level. The recent identification of the sex-determining gene *DMY* in the medaka (Matsuda *et al.,* 2002) was a breakthrough in this regard. Now, we can utilize the variety of sex-determining mechanisms that occur in fish, especially in *Oryzias* fishes, to approach a common sex-determining mechanism in vertebrates.

In the present study, we investigated the sex-determination system as the first step to approaching the sex-determining genes in *O. mekongensis* and *O. luzonensis* which are the most closely related species to *O. latipes*. As summarized in Fig. 1, our results demonstrated that both fishes have male heterogametic system (XX-XY) as *O. latipes* and *O. curvinotus*.

In *O. latipes* and *O. curvinotus*, *DMY* has been proved to be the common male-determining gene on the Y chromosome (Matsuda *et al*., 2003), but it does not seem to be the common sex-determining gene in a variety of *Oryzias* fishes. *DMY* originates from *DMRT1*, which is believed to be a common important gene for male development in vertebrates. A duplicated copy of *DMRT1* became incorporated into a chromosome, and thereafter it acquired the male-determining function to become *DMY*. The molecular phylogeny of *DMY* and *DMRT1* shows that the duplication of *DMRT1* occurred just prior to the speciation of *O. latipes* and *O. curvinotus* (Matsuda *et al.,* 2003). This indicates that *O. mekongensis*, which first branched off from other *Oryzias* fishes of the bi-armed chromosome group, can hardly use *DMY* as the sex-determining gene. In fact, *DMY* has not been identified in *O. mekongensis* (Kondo *et al*., 2003). Considering these facts, it can safely be said that *O. mekongensis* has a male-determining gene other than *DMY,* in other words, the Y chromosome of this species may not be homologous to that of *O. latipes* and *O. curvinotus*.

O. luzonensis and *O. curvinotus* are a sister species pair to *O. latipes*, that is, they have a common ancestor, but we failed to demonstrate the presence of *DMY* in *O. luzonensis* (data not shown). It may also be probable that the Y chromosome of *O. luzonensis* is homologous to an autosome of *O. latipes*. Currently, we are searching for the sex chromosomes of *O. luzonensis* and *O. mekongensis*, using DNA markers established in *O. latipes*.

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