

# Sex Determination and Sex Chromosomes in Amphibia

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## Keywords

Evolution · Heteromorphy · Homomorphy · Turnover

## Abstract

Sex chromosomes in most amphibians are homomorphic (undifferentiated) in both sexes and are characterized by frequent turnover. This is in sharp contrast to sex chromosomes in 2 major vertebrate groups, the mammals and birds, where they are heteromorphic in one sex and are highly conserved. Sex-determining mechanisms in anuran amphibians, particularly in relation to the turnover of sex-determining genes and sex chromosomes, are summarized and their evolution is discussed.

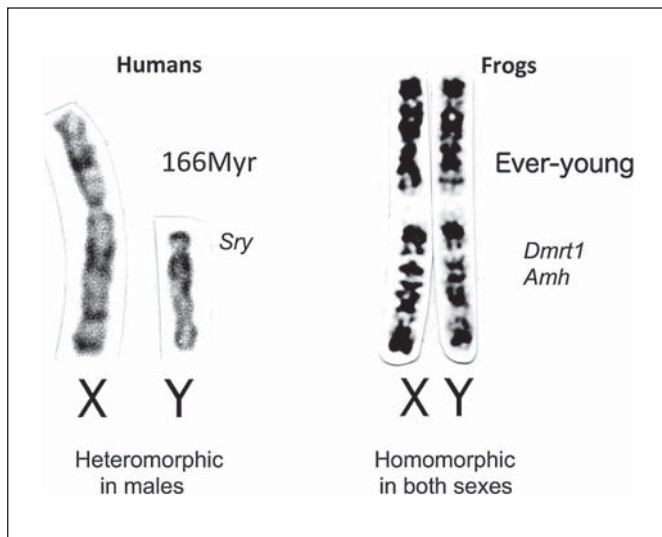
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## Turnover of Sex-Determining Genes and Sex Chromosomes

The heteromorphy or homomorphy (differentiated or undifferentiated) of sex chromosomes has a close correlation with the conservation of the sex-determining genes. If sex chromosomes are heteromorphic in one sex, the sex-determining gene is evolutionally conserved. For example, therian mammals share the heteromorphic sex chromosome pair, a large X chromosome and a tiny Y

chromosome, on which the male-determining gene *Sry* has been conserved for 166 million years (MY) [Graves, 2013] (Fig. 1). Similarly, in all birds, the ZW sex chromosomes are heteromorphic in females, and the male-determining gene *Dmrt1* on the Z chromosome has been conserved for 100 MY [Romanov et al., 2014]. On the other hand, if sex chromosomes are homomorphic in both sexes, for example as in 96% species of amphibians [Schmid et al., 1991; Eggert, 2004], the sex-determining gene on the sex chromosome is replaced easily and rapidly by another gene of a different chromosome (Fig. 1). This replacement is called “turnover of sex-determining genes and sex chromosomes” [Schartl, 2004] and is observed in abundant species of poikilothermic vertebrates. Turnover occurs between different taxa or even between geographic populations within a single species.

Turnover is well studied in the teleost fish medaka (*Oryzias*). Sex-linkage analysis in medaka indicated the existence of 7 independent linkage groups, which implies that 7 genes are expected to be candidate sex-determining genes: 2 genes for female and 5 for male sex determination, of which 3 male-determining genes have already been identified [Myosho et al., 2015]. In all vertebrates, sex-determining genes known to date are classified into 5 groups: *Sry* (*Sox3* in medaka), *Dmrt1* (*Dmy* in medaka), *Amh* (*Gsdf* in medaka belonging to the TGF- $\beta$  family), *Amhr*, and *Irf9* [O’Meally et al., 2012; Yano et al., 2012].



**Fig. 1.** Sex chromosomes of humans and frogs. The X and Y chromosomes are heteromorphic in human males, while the sex chromosome 1 is homomorphic in both sexes in frogs. Photographs of the human sex chromosomes are provided by Chizuko Nishida.

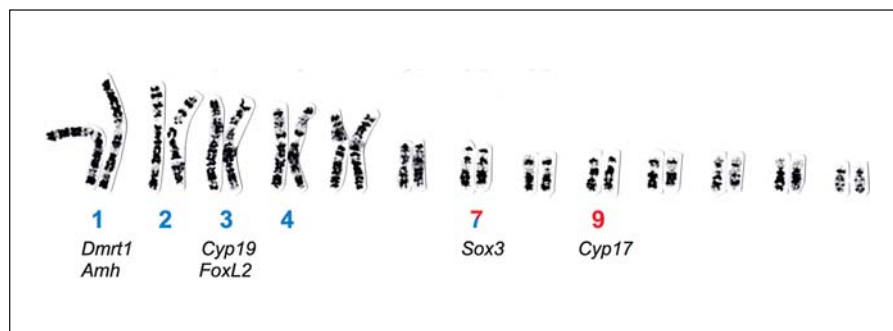
The list suggests that a sex-determining gene, which is the primary component of the sex-determining cascade, is selected from a limited number of candidates and not randomly chosen or recruited from all genes in the genome [Miura, 2007; Marshall Graves and Peichel, 2010].

On the basis of sex linkage and karyotypic data, 8 different genes on 6 different chromosomes are predicted to be the sex-determining candidates in frogs (Table 1). Two cases of turnover were reported in Japanese frog species. The pond frog *Pelophylax porosa brevipoda* is classically divided into 2 major geographic groups, Nagoya race in the east and Okayama race in the west. These 2 races differ in their external morphology, mating-call structure, and mitochondrial DNA. Both groups have a male heterogametic sex-determining mechanism, and among 13 chromosome complements, the allozyme loci linked with the male sex are located on chromosomes 3 and 4 in Nagoya and Okayama races, respectively [Nishioka and Sumida, 1994]. In the brown frog *Rana japonica*, the allozyme loci linked to the male sex are located on chromosomes 1 and 4 in the geographic populations of western and eastern Japan, respectively [Sumida and Nishioka, 1994]. Sex chromosome 4 is homomorphic in both sexes, and the X and Y chromosomes are indistinguishable by shape; however, a slight difference exists in the distribution pattern of the constitutive heterochromatin (C-band) in males [Miura, 1994]. A single block of hetero-

chromatin that is located in the region close to the centromere of the X chromosome is absent in the Y chromosome. The heteromorphy of constitutive heterochromatin distribution does not affect recombination between the X and Y chromosomes, because a genetic XY male with a YY (–/–) C-band morph was identified. This might have resulted from a cross between a recombinant XX female with a XY (+/–) C-band morph, which occurs naturally in the wild, and a normal XY male with a XY (+/–) C-band morph. In the northwestern Japanese populations, which originated by hybridization between 2 former populations of eastern and western Japan, linkage groups (LG) on chromosomes 1 and 4 (and 3) are not linked to the male sex, suggesting that the sex is determined by another gene in a different chromosome [Sumida and Nishioka, 1994]. An interesting case was reported in the European brown frog *R. temporaria*, which has homomorphic sex chromosomes in both sexes. LG2 on chromosome 1 is linked with the male sex in 7 Swedish populations from north to south. However, LG7 in addition to LG2 is also linked with the male sex in the northern boreal-population (Ammarunas), suggesting evolution of a neo-sex chromosome by chromosomal fusion or of a double sex chromosome system [Rodrigues et al., 2014, 2016]. Similarly, sex chromosome turnover was observed between geographic populations of water frogs, *P. esculentus* in Europe [Schempp and Schmid, 1981; Hotz et al., 1997] and *Lithobates pipiens* in the USA [Wright and Richard, 1983; Wright et al., 1983], between phylogenetically sister species of bullfrogs, *L. catesbeianus* and *L. clamitans* [Elinson 1981, 1983; Lambert et al., 2016], and among the water frog *L. pipiens* complex [Wright et al., 1983]. In all the populations and species described above, the sex chromosomes are homomorphic in both sexes, and banding karyotypes of  $2n = 26$  are perfectly conserved with essentially no chromosomal mutations at the banding level, indicating that their linkage groups are highly conserved [Miura, 1995]. In summary, homomorphic sex chromosomes in the above frogs repeat turnover among chromosomes 1, 2, 3, and 4 (Fig. 2; Table 1).

Sex chromosome 7 is heteromorphic in male stream brown frog *R. sakuraii* [Ryuzaki et al., 1999] and male or female wrinkled frog *Glandirana rugosa* [Nishioka et al., 1993, 1994], and sex chromosome 9 is heteromorphic in the female of the bell-ring frog *Buergeria buergeri* [Ohta, 1986]. In conclusion, homomorphic sex chromosomes under male heterogametic sex determination repeat turnover among the members of chromosomes 1, 2, 3, and 4, whereas chromosomes 7 and 9 are independently heteromorphic in the male and/or female in frogs [Miura, 2007]

**Fig. 2.** The potential sex chromosomes in frogs. The chromosome numbers of X and Y sex chromosomes among the complement of 13 haploid sets are indicated in blue, while those of Z and W sex chromosomes are shown in red. Chromosome 7 possesses a potential for both types of sex chromosomes. The sex-linked, sex-differentiation related genes are shown under the chromosome numbers.



(Fig. 2). Chromosome 7 of *Xenopus tropicalis*, which is homologous to chromosome 9 in the frogs described above, is a homomorphic sex chromosome of female heterogamety [Uno et al., 2008]. In addition, a *X. borealis* chromosome homologous to chromosome 7 in the above-described frogs is a homomorphic sex chromosome of female heterogamety [Furman et al., 2016]. Further, the heteromorphic sex chromosomes of female heterogamety in 4 species of the genus *Pseudis* (Hylinae) might be homologous to chromosome 7 or 9 in the frogs described above [Busin et al., 2008]. Thus, 6 male- (because 2 genes of *Amh* and *Dmrt1* on chromosome 1, described below) and 2 female-determining genes located on 6 different chromosomes in total are expected for turnover in anurans (Fig. 2; Table 1). In particular, chromosome 1 is a highly potential candidate sex chromosome in anurans as it is reported to be a sex chromosome in some species of other genera, such as *Hyla* and *Bufo* [Stöck et al., 2013; Brelsford et al., 2016]. To date, sex-determining genes on the 6 sex chromosomes remain unidentified, although 3 sex-determining genes identified in vertebrates are mapped in *G. rugosa*: *Dmrt1* and *Amh* on chromosome 1 and *SOX3* on chromosome 7 [Uno et al., 2008; Kodama et al., 2015]. *Cyp19/FoxL2* and *Cyp17*, mapped to chromosomes 3 and 9 [Oshima et al., 2006; Sakurai et al., 2008] and involved in syntheses of estradiol and testosterone, respectively, are attractive candidate genes for sex determination in frogs, because sex differentiation of gonads is under the control of sex steroid hormones (Table 1).

### Nature of a Female-Determining Gene in Female Heterogamety

In vertebrates, a female-determining gene of the ZZ-ZW system is identified in 3 species: *Dm-W* in a clawed frog, and lack of *Dmrt1* on the W chromosomes of chick-

en and flatfish [Yoshimoto et al., 2008; Smith et al., 2009, 2010; Chen et al., 2014]. Although the 3 genes are all *Dmrt1* or its derivative, it is primarily a male-determining gene. *Dm-W* is a truncated duplicate gene of *Dmrt1* lacking the transactivation domain and, therefore, might determine the female sex by a dominant-negative inhibition of the male-determining function of *Dmrt1* [Yoshimoto et al., 2010]. In chicken and flatfish, the *Dmrt1* locus is absent in the W chromosomes, and thus, half dosage of *Dmrt1* expression in ZW individuals compared to the double dosage in ZZ individuals determines the female sex. In all the ZZ-ZW systems described above, female-determining genes inhibit or reduce the male-determining function of *Dmrt1*, which is a negative regulation [Smith et al., 2010]. Furthermore, in the ZW system of the silkworm, *Bombyx mori*, the female-determining gene (female-enriched PIWI-interacting RNAs) on the W chromosome silences the male-determining function of the *Masculinizer* (*Masc*) gene located on the Z chromosome to determine the female sex [Kiuchi et al., 2014]. In addition, *SOX3* is a candidate female-determining gene on chromosome 7 in *G. rugosa* as *Sox3* of the W chromosome is highly expressed in the undifferentiated gonad of ZW tadpoles during the early stages of development [Miura et al., 2009; Miura et al., 2016], and a knockdown of *Sox3* results in ZW males [unpubl. data]. Further, *Cyp17* is a candidate female-determining gene on sex chromosome 9 of *B. buergeri* as it is located in the inverted region of the W chromosome, and the divergence of nucleotide and amino acid sequences is evident between *Cyp17* of Z and W chromosomes [Uno et al., 2015]. In the latter 2 species, the 2 candidate female-determining genes are primarily involved in testis differentiation and thus allow us to speculate that the genes on the W chromosomes would inhibit male-determining functions of homologous genes on the Z chromosomes to determine the female sex (Table 1).

**Table 1.** Sex chromosomes and sex-linked genes in frogs

Karyotype	Sex chromosome	Species (population)	2n	Sex determination	Morphology (heteromorphic)	Linked genes
Large pairs	Chromosome 1	<i>Rana japonica</i> (West)	26	XY	homo	<i>Dmrt1</i>
		<i>R. temporaria</i>	26	XY	homo	<i>Amh</i>
		<i>Lithobates clamitans</i>	26	XY	homo	
		<i>L. spinocephala</i>	26	XY	homo	
		<i>L. berlandieri</i>	26	XY	homo	
		<i>Hyla arborea</i>	24	XY	homo	
		<i>H. intermedia</i>	24	XY	homo	
		<i>H. molleri</i>	24	XY	homo	
		<i>Bufo siculus</i>	22	XY	homo	
		<i>B. shaartusiensis</i>	22	XY	homo	
		<i>B. turanensis</i>	22	XY	homo	
		<i>B. balearicus</i>	22	XY	homo	
	Chromosome 2	<i>L. blairi</i>	26	XY	homo	?
		<i>L. pipiens</i>	26	XY	homo	
	Chromosome 3	<i>Pelophylax porosa brevipoda</i> (Nagoya)	26	XY	homo	<i>Cyp19</i>
		<i>P. esculentes</i>	26	XY	homo (replication)	<i>FoxL2</i>
		<i>L. pipiens</i>	26	XY	homo	
	Chromosome 4	<i>R. japonica</i> (East)	26	XY	homo (C-band)	?
		<i>P. porosa brevipoda</i> (Okayama)	26	XY	homo	
		<i>P. nigromaculatus</i>	26	XY	homo	
		<i>L. catesbeianus</i>	26	XY	homo	
		<i>P. esculentus</i>	26	XY	homo	
Small pairs	Chromosome 7	<i>R. sakuraii</i>	26	XY	hetero	<i>Sox3</i>
		<i>Glandirana rugosa</i>	26	XY/ZW	hetero	<i>Sfl, Ar</i>
		<i>Xenopus borealis</i>	36	ZW	homo	
	Chromosome 9	<i>Buergeria buergeri</i>	26	ZW	hetero	<i>Cyp17</i>
		<i>X. tropicalis</i>	20	ZW	homo	
	Chromosome 7 <sup>a</sup>	<i>Pseudis p. paradoxia</i>	24	ZW	hetero	
		<i>P. p. platensis</i>	24	ZW	hetero	
		<i>P. bobodactya</i>	24	ZW	hetero	
		<i>P. fusca</i>	24	ZW	hetero	
	Chromosome 8 <sup>a</sup>	<i>Physalaemus ephippier</i>	24	ZW	hetero	

<sup>a</sup> The sex chromosome number does not yet correspond to the other frogs described here.

In sexual differentiation of the gonad and genital organs, it is well known that female is the base, while male is the derivative. This holds true for the morphological sex-differentiation of gonads in *X. laevis*. A mass-in-line-structure, which comprises several masses of somatic cells located along with the anterior-posterior axis, is commonly formed in the gonads of both sexes at early developmental stages [Mawaribuchi et al., 2014]. In ZZ males, the structure is soon broken and testis differentiation occurs with germ cells migrating into the cell masses, while in ZW female, the structure is maintained and ovary differentiation occurs, where germ cells are arranged around the outer of the masses and an ovarian cavity is formed in every cell mass. Therefore,

morphologically, female is the base in female heterogamety, whereas molecularly, male is the base and female sex determination is a negative regulation. An important question is whether a true principal gene that positively determines female sex without relying on anti-male determination exists in female heterogamety. Its absence suggests that the ancestral system of genetic sex determination (GSD) is a male heterogametic sex determination system from which a female heterogametic system, a derived form, has evolved. However, this hypothesis conflicts with the evolutionary theory of heterogametic sex determination in amphibians wherein female heterogamety is ancestral and changed to male heterogamety at several branching points of the phylog-

eny [Hillis and Green, 1990]. Hence, the identification of a female sex-determining gene in large numbers of species is essential.

### Ageing of Y and W Chromosomes

The evolutionary conservation of a sex-determining gene on heteromorphic sex chromosomes indicates the long lifetime and ageing of Y or W chromosomes. After heteromorphy of sex chromosomes is established by chromosomal mutations such as inversions, the recombination between the homologues is inhibited and Y or W chromosomes degenerate by accumulating transposons and repeated sequences and heterochromatin, thereby decaying the functional genes [Ohno, 1967; Marshall Graves, 2008]. The direction of morphological size change is towards dwarfism or enlargement. As mentioned earlier, the age of the Y chromosome of therians is 166 MY and that of the W chromosome of birds is 100 MY. The heteromorphy of sex chromosomes traps turnover and inhibits recombination, leading to lonely lives of sex chromosomes. In sharp contrast, in poikilothermic vertebrates sex chromosome differentiation occurs independently among different taxa, and thus they are homomorphic and intact in abundant species. Stöck and his group [2011] called it “Ever-young sex chromosomes.” Sex chromosomes do not degenerate as their homomorphy allows the recombination between homologues and turnover with another chromosome. In amphibians, sex chromosome heteromorphy did not occur at the branching points of large taxa; it always occurred within the lineage of a small group, species, or geographic populations. Thus, only 4% of species have differentiated heteromorphic sex chromosomes. Maintenance of homomorphy of sex chromosomes avoids their ageing, and sex-determining genes can escape the risk of disappearance from the genome through degeneration.

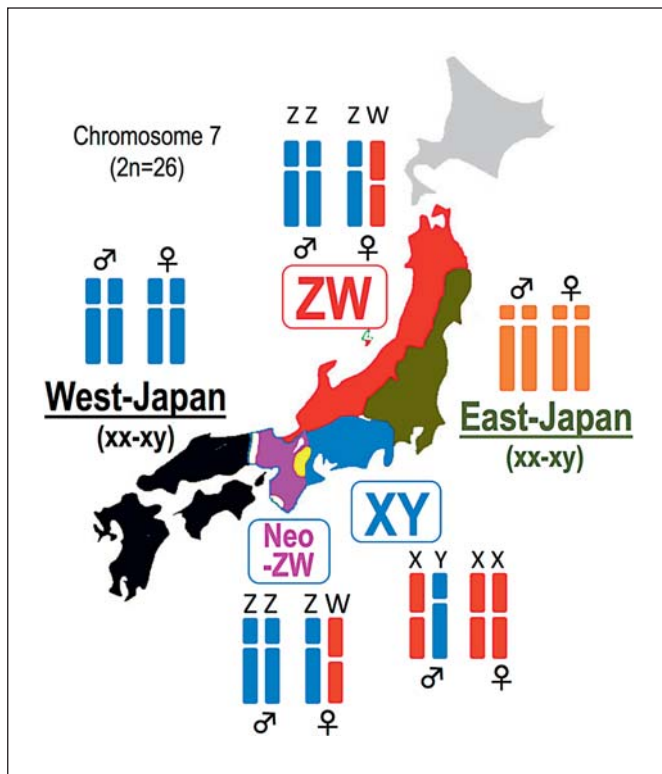
### Why Turnovers?

The establishment of heteromorphy of sex chromosomes traps turnover, while homomorphic sex chromosomes retain the opportunity to turnover. However, in the European tree frog group (*H. arborea*, *H. intermedia*, and *H. molleri*), it was observed that the sex chromosomes did not experience any turnover for a long time (5 MY), although they remain homomorphic in both sexes [Dufresnes et al., 2015]. In contrast, sex chromosomes turned over between geographic populations within a

single species of the Japanese, European, and North American frog species mentioned earlier. The question then is: what drives turnover? In the case of *P. porosa brevipoda* and *R. japonica*, the 2 groups with different sex chromosomes are geographically separated in the distribution, and the latter species is clearly suggestive. The geographic group in northwestern Japan, for which the sex chromosome is not yet identified, originated by hybridization between the western and eastern groups possessing sex chromosomes 1 and 4, respectively [Sumida and Nishioka, 1994]. Experimental results of artificial hybridization indicate that reciprocal crossings produced offspring with a highly skewed sex ratio favoring the male [Sumida, 1981]. Therefore, one hypothesis is that the original male-determining system does not function in hybrid populations with male bias, and thus, a new male- or female-determining gene was recruited from the other 7 candidate genes replacing the original system. A similar case was reported in *G. rugosa* wherein the skewed sex ratio, which is a result of the hybridization between the 2 ancestral groups, is hypothesized to promote the evolution of differentiated groups based on the new sex chromosome [Ogata et al., 2003]. In poikilothermic vertebrates, phenotypic sex is easily affected by environmental factors such as temperature and genetic disturbances such as hybridization through the invasion of genetically different populations [Walace et al., 1999; Eggert, 2004; Alho et al., 2010; Miura et al., 2016]. Therefore, sex bias could be a trigger for turnover of the sex-determining gene and sex chromosome to restore an even sex ratio.

### Sex Reversal

In amphibian species studied until now, the male or female sex is determined genetically. Temperature sex determination (TSD) or other environmental sex determination (ESD) has not been identified yet in any species. However, it is premature to assert that all amphibians follow GSD, because the information regarding sex chromosomes and sex determination in caecilians is relatively scant. Further research on this attractive and primitive lineage of amphibians is desirable. Nevertheless, all amphibians studied until now primarily follow GSD, even though the phenotypic sex is easily changeable in some species by temperature or exogenous administration of reagents such as sex steroid hormones and their inhibitors. In addition, the sensitivity varies among species, and it is unclear whether the sensitivity depends on the gametic sex or sex chromosome differentiation.



**Fig. 3.** Five major geographic groups of the Japanese frog *Glandirana rugosa*. All chromosome pairs shown are the 7th largest among the 13 haploid sets of complements. The intermingling populations located between XY and Neo-ZW groups in central Japan are shown in yellow.

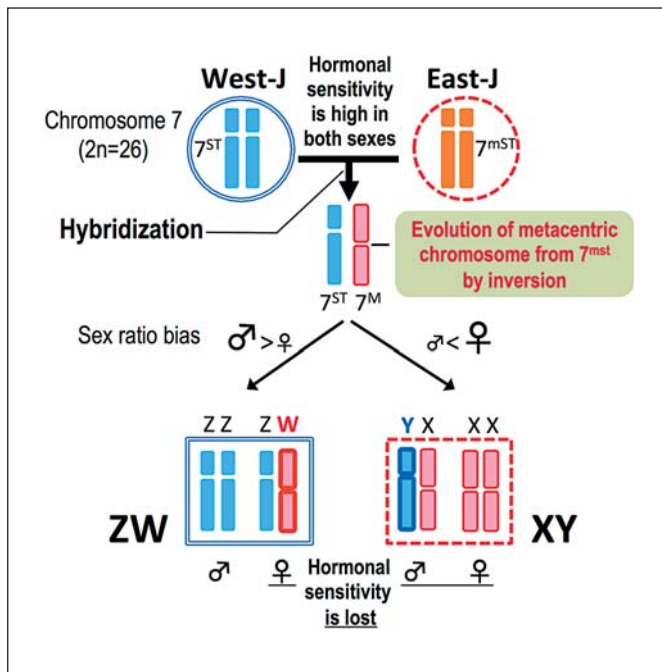
Studies of *G. rugosa* offer a solution, as this species is unique in sex chromosome differentiation and sex determination [Miura, 2007] and is constituted of 5 major geographic groups (Fig. 3): 2 groups have homomorphic sex chromosomes with male heterogametic sex determination (west and east Japan groups), whereas the other 3 groups have heteromorphic sex chromosomes with male (XY group in eastern central Japan) and female heterogametic sex determination (ZW and Neo-ZW groups in northwestern and western central Japan, respectively). Sex chromosomes of the latter 3 groups are the 7th largest in their haploid sets of 13 chromosomes and are homologous to each other, whereas those of the former 2 groups remain unidentified (chromosome 7 is not sex-linked in the west Japan population).

This species provides an opportunity to approach the following 2 questions regarding sex reversal induced by the administration of exogenous reagents. First, does sex reversal depend on sex chromosome differentiation, i.e.,

how is the sensitivity different between a population with homomorphic and heteromorphic sex chromosomes? Second, does the sensitivity to sex reversal depend on gametic sex, i.e., how does sensitivity differ between heterogametic XY and homogametic XX or between heterogametic ZW and homogametic ZZ populations? The results indicate that sex reversal is induced easily in the homomorphic sex chromosome groups in west and east Japan groups in both directions, from male to female and vice versa. In contrast, sex reversal is not induced in heterogametic ZW female in the ZW heteromorphic sex chromosome group or in homogametic XX female or heterogametic XY male in the XY heteromorphic sex chromosome group [Miura et al., 2016] (Fig. 4). Phylogenetically, the 2 previously mentioned homomorphic sex chromosome groups, west and east Japan, are ancestral, and the 2 heteromorphic sex chromosome groups, XY and ZW, evolved by their hybridization [Miura et al., 1998; Ogata et al., 2003; Miura and Ogata, 2013]. Cytogenetically, chromosome 7 of the west Japan group and the Y and Z chromosomes of both the XY and ZW groups are subtelocentric, and chromosome 7 of the east Japan group is more subtelocentric. These 2 chromosomes 7 are ancestral types. On the other hand, the metacentric X and W sex chromosomes are a derived type that differentiated by inversion [Miura et al., 1998] (Fig. 3). Therefore, it is evident that (a) mechanisms of gonadal sex differentiation evolved from being hormone sensitive to hormone resistant, which indicates that homomorphic sex chromosomes allow easy sex reversal, and (b) heterogametic sex, ZW and XY, and the homogametic XX bearing the metacentric chromosomes are resistant to sex reversal (Fig. 4). In conclusion, the sensitivity in hormonal gonadal sex reversal in *G. rugosa* depends on sex chromosome differentiation [Miura et al., 2016]. Based on previous studies, the metacentric X and W chromosomes were acquired through a sex ratio bias in hybridization accompanied by a turnover of sex chromosomes in the evolutionary process [Ogata et al., 2003; Miura and Ogata, 2013] (Fig. 4). Therefore, the metacentric chromosomes might hold the key to understanding the mechanisms of gonadal sex reversal under hormone sensitivity and elucidating the relationship between sex chromosome turnover and sex ratio control.

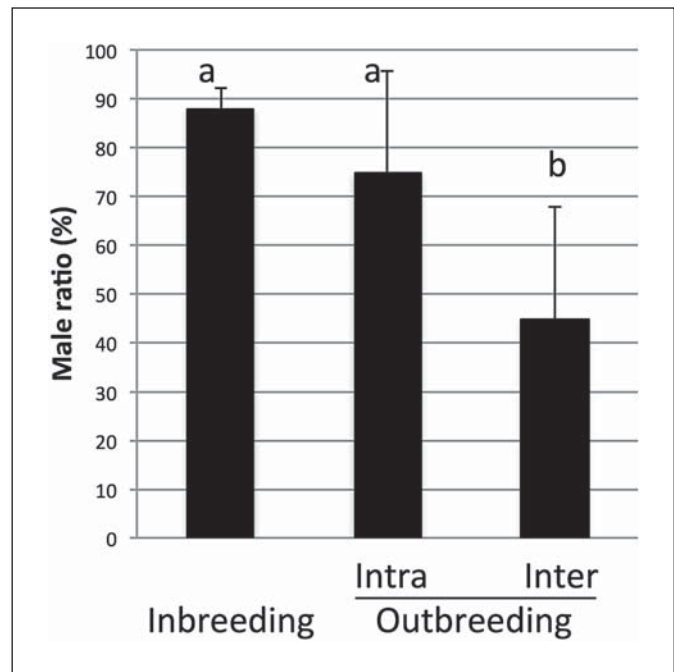
### Unusual Mechanism of Sex Determination

Finally, an unusual sex-determining system is observed in the Japanese rice frog *Fejervarya kawamurai* [Miura et al., 2015]. Normally, sex in animals is deter-



**Fig. 4.** Hybrid origin and evolution of heteromorphic sex chromosomes in ZW and XY populations of *Glandirana rugosa*. Subtelocentric chromosome 7 in west Japan, ZW, and XY populations are indicated in blue, the more subtelocentric chromosome 7, which has a smaller short arm, in the east Japan population is indicated in orange, and the metacentric chromosome 7 in ZW and XY populations is shown in red. Mitochondrial genotypes originating from the west Japan population are indicated using blue double-lined circles or squares, and those from the east Japan population are indicated using red dotted-lined circles or squares. The relative strength of the ovary or testis determination is indicated by the thickness of lines framing the outlines of chromosomes.

mined by 2 mechanisms, GSD and ESD, among which TSD is widely used in reptiles. For GSD, 2 major types of XX-XY and ZZ-ZW exist, and others are primarily variants or derivatives of these 2 types. In nonvertebrates, another mechanism of GSD, complementary sex determination (CSD), is widely used in Hymenoptera, such as ants and bees, where homo- or hemizygosity of the sex-determining locus induces the male sex, whereas heterozygosity induces the female sex. The results of sex ratio in the rice frog *F. kawamurai* are unusual, and it is difficult to explain the sex determination mechanism based on the established GSD and ESD systems. Briefly, inbreeding such as sibling-sibling crosses, backcrosses, and diploid gynogenesis result in a male-biased sex ratio ( $87.0 \pm 4.87\%$ ) with a high rate of developmental mortality. In contrast, outbreeding reduces mortality; particularly out-



**Fig. 5.** Male ratios in inbred and outbred crossings of the rice frog *Fejervarya kawamurai*. Bars represent the mean  $\pm$  SD for each breeding. Different letters indicate statistical differences ( $p < 0.05$ ) after ANOVA and a multiple comparison Tukey post hoc test [Miura et al., 2015].

breeding of females in a population with males from a geographically separate population restores the sex ratio (Fig. 5) [Miura et al., 2015]. The influence of temperature on gonadal sex differentiation does not account for the sex ratio bias, because the tadpoles and adults in the controls and experiments using the same females or males were always reared in the same room. The production of males in diploid gynogenesis suggests female heterogamy, and recovery of the female ratio in outcrosses between different separated populations suggests that heterozygosity of sex alleles determines females in CSD, because polymorphic sex alleles are not shared between geographically distinct populations. Thus, a kind of multi-factorial CSD is suggested to be the candidate mechanism. However, molecular analyses are necessary to prove the hypothesis and uncover the mechanism of the unusual sex determination in the rice frog.

In summary, sex chromosomes in most amphibian species are homomorphic (undifferentiated) in both sexes and repeat turnover, sometimes even between geographic populations in a single species. Thus, amphibians are excellent research materials on the turnover of sex

chromosomes and sex-determining genes. Based on sex linkage and karyological data in frogs, 6 different chromosomes harboring 6 and 2 candidate genes for male and female determination, respectively, are expected to turn over. The most significant is the large sex chromosome 1 bearing *Dmrt1* and *Amh*. An important question is whether a true principal gene that positively determines female sex without relying on anti-male determination exists in female heterogamety. Sex determination in Amphibia is primarily genetic, although temperature and steroid hormones can easily change the phenotypic sex. The evolution of new sex chromosomes associated with rearrangements in the case of *G. rugosa* might alter the mechanism of gonadal sex differentiation from a sex reversal-

sensitive to a resistant state, indicating the relationship between sex chromosome turnover and sex ratio control. Finally, the unexplainable mechanism of sex determination in the rice frog species was introduced. Amphibians bearing a novel sex determining mechanism are yet to be identified.

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