Chromosome Res (2012) 20:47–55
DOI 10.1007/s10577-011-9258-8

Independent degeneration of W and Y sex chromosomes in frog *Rana rugosa*

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Published online: 6 December 2011

Abstract The frog *Rana rugosa* uniquely possesses two different sex-determining systems of XX/XY and ZZ/ZW, separately in the geographic populations. The sex chromosomes of both types share the same origin at chromosome 7, and the structural differences between X and Y or Z and W were evolved through two inversions. In order to ascertain the mechanisms of degeneration of W and Y chromosomes, we gynogenetically produced homozygous diploids WW and YY and examined their viability. Tadpoles from geographic group N (W^NW^N) containing three populations died of edema at an early developmental stage within 10 days after hatching, while tadpoles from the geographic group K (W^KW^K) that contained two populations died of underdeveloped growth at a much later stage, 40–50 days after fertilization. On the contrary, W^NW^K and W^KW^N hybrid embryos were viable, successfully passed the two lethal stages, and survived till the attainment of adulthood. The observed survival implies that the lethal genes of the W chromosomes are not shared by the two groups and thus demonstrates their independent degeneration histories between the local groups. In sharp contrast, a sex-linked gene of androgen receptor gene (*AR*) from the W chromosome was down-regulated in expression in both the groups, suggesting that inactivation of the *W-AR* allele preceded divergence of the two groups and appearance of the lethal genes. Besides, the YY embryos died of cardiac edema immediately after hatching. The symptom of lethality and the stage of developmental arrest differed from those for either of WW lethal embryos. We therefore conclude that the W and Y chromosomes involve no evolutionary common scenario for degeneration.

Keywords sex chromosome · lethal gene · gynogenetic diploid · androgen receptor gene · Amphibia

Abbreviations

AAT	ADP/ATP translocase
AR	Androgen receptor
cDNA	Complementary DNA
DG	Diploid gynogenesis
dpf	Day post fertilization
EF1α	Elongation factor 1 alpha
GD	Gynogenetic diploid
L^{WK}	Lethal gene on W chromosome of Kanazawa population

Responsible Editor: Tariq Ezaz and Jennifer Graves

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L^{WN}	Lethal gene on W chromosome of Niigata population
L^{Y}	Lethal gene on Y chromosome
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RT-PCR	Reverse transcription polymerase chain reaction

Introduction

Once a regional heterogeneity is established between a sex chromosome pair, the recombination is reduced and the Y and W chromosomes are forced to commence degeneration, even to the extent of causing eventual death of the whole chromosome and missing a master sex determining gene (Charlesworth and Charlesworth 2000; Charlesworth et al. 2005; Bergero and Charlesworth 2009; Graves 2006, 2008; Aitken and Graves 2002). The degeneration is accomplished by accumulation of deleterious mutations on the native genes, amplification of the repeated sequence and transposable element, along with structural rearrangements such as inversion, translocation and deletion (Bachtrog 2003; Steinemann and Steinemann 2005; Bergero and Charlesworth 2009). The sequential step of degeneration process from homomorphic state to highly degenerated is well seen through the phylogenetic stages in snakes and birds (Ohno 1967; Ray-Chaudhuri et al. 1971; Ray-Chaudhuri and Singh 1972; Takagi and Sasaki 1974; Nishida-Umehara et al. 2007). In eutherians, the degeneration process is independently processed since divergence from marsupials, by the addition of an autosomal part to both of the X and Y chromosomes (Waters et al. 2001). Even within eutherians, the members of degenerated genes on the Y chromosomes are different, indicating that the degeneration process of Y chromosome occurs independently in every species (Graves 2008; Waters et al. 2007). In contrast, quite a few such investigations on W chromosome have been carried out (Matsubara et al. 2006; Tsuda et al. 2007), and no investigations to compare the mechanism of degeneration between Y and W chromosomes were conducted. Generally, the latter issue could not be approached for investigation because their chromosomal origins are always different as seen in the avian W and mammalian Y chromosomes (Nanda et al. 1999).

Japanese frog *Rana rugosa* has two sex determining chromosome systems, existing separately, in the geographic populations (Fig. 1a) and provides us a unique opportunity to examine the difference in the process of degeneration of the Y and W chromosomes and of the W chromosomes from local populations within a species. The Northwestern Japan population, which is widely distributed along the Sea of Japan from north to south in Honshu, has a ZZ/ZW sex chromosome system, while the Central Japan population has an XX/XY sex chromosome system (Fig. 1b). These two systems share the evolutionary origin at hybridization between the two ancestral main populations (Ogata et al. 2003; Miura 2007): the Y and Z or X and W are morphologically homologous with each other, and they all share the same chromosomal origin (Fig. 1a). Between the Y and X or Z and W chromosomes, the structural differences are explained by two inversions: A chiasma at meiosis is observed at just one region of the pseudoautosomal region and almost no recombination occurs at the other part of the chromosomes (Miura et al. 1997, 2009). In this study, we produced gynogenetic diploids of WW and YY genotypes to detect degenerated, lethal genes on the sex chromosomes. In medaka fish, *Oryzias latipes* and common guppy, *Lebistes reticulatus*, the production of YY individuals is successful for detecting recessive, lethal genes on the Y chromosomes (Yamamoto 1964; Haskins et al. 1970). We found that the WW and YY embryos died during different developmental stages, and even WW embryos from two local groups died at different stages of development. We discuss the mechanisms of degeneration of the W and Y chromosomes and of sex chromosome differentiation.

Materials and methods

Diploid gynogenesis, artificial sex reversal, and sexing

Specimens of ZZ males and ZW females used for experiments were collected from five populations: locality 1, Hirosaki City in Aomori Prefecture; locality 2, Niigata City of Niigata Prefecture; locality 3, Toyama City of Toyama Prefecture; locality 4,

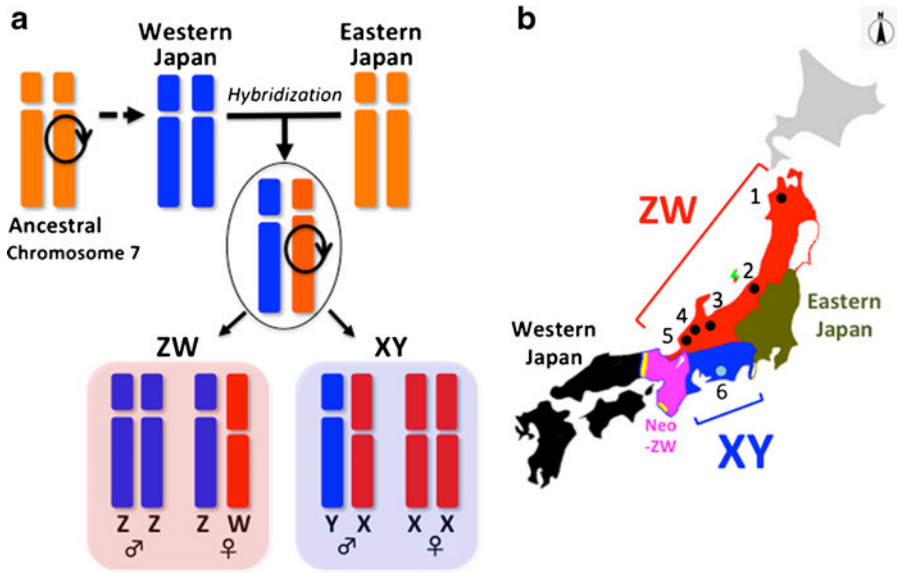

Fig. 1 The sex chromosomes of *Rana rugosa* and the geographic localities of specimens used. **a** The evolution of the XY and ZW sex chromosomes. Z and Y chromosomes, shown in *blue*, share the origin at chromosome 7 from the Western Japan population, while W and X chromosomes, shown in *red*, derived from chromosome 7 (in *orange*) of Eastern Japan population. Pericentromeric inversion, indicated by a *circle with an arrowhead*, occurred twice during the evolution of the chromosomes 7, once after emigration of an ancient population from Korea Peninsula to Western Japan, and then on the chromosome 7 from Eastern Japan population when hybridized with the Western Japan population. **b** The geographic localities of five ZW populations and one XY population used in this study: the cities of localities 1–6 are described in "Materials and methods"

Kanazawa City of Ishikawa Prefecture; and locality 5, Fukui City of Fukui Prefecture. XY males and XX females were collected from Hamamatsu City of Aichi Prefecture (locality 6).

Gynogenetic diploids were produced according to the method of Ohtani et al. (2003). The ovulation was induced by injection of a pituitary gland extraction of the frog, *Rana nigromaculata*, into the abdominal cavity of females. The eggs were fertilized with UV-irradiated sperm and were subjected to a cold shock at approximately 1°C for 1 h to suppress extrusion of the second polar body. The tadpoles were fed on boiled spinach in a glass container until metamorphosis at room temperature, and frogs after metamorphosis were fed on crickets and reared at plastic container at room temperature.

To artificially produce ZW males, the genetic ZW tadpoles at 30 days after fertilization were injected with 500 μg of testosterone propionate per tadpole (Enarmon dissolved in sesame oil, Teikokuzoki Inc.) into their abdominal cavities and were reared until maturation. Genotypic sex of frogs was identified using PCR-restriction fragment length polymorphism of a sex-linked gene ADP/ATP translocase (*AAT*) according to the method of Ohtani et al. (2003). Genotypes of hybrids WY, WX, ZX, and ZY were identified based on RFLP profiles of both *AAT* and *AR* genes. Since *W-AR* is inactivated, WY could be discriminated from ZX in a crossing of ZW female and XY and from XY in a crossing of WY female and XY male (Ogata et al. 2008).

RT-PCR and Northern blot hybridization

Total RNA of gynogenetic diploids WW, ZZ, XX, and YY were extracted from the embryos (ZZ and WW of Niigata population at 10 dpf; ZZ and WW of Kanazawa population at 40 dpf; XX and YY at hatching), and those of ZZ and ZW were from adult livers of Kanazawa population using a guanidinium thiocyanate-CsCl method (Miura et al. 1998). Complementary DNA (cDNA) was synthesized using 1 μg of the total RNA as the template in 20 μl of reaction solution containing 1 μl of reverse transcriptase

(SuperScript II, InVitrogen) and 25mer poly dT oligomer as the primer according to the manufacture's instruction. In addition, 0.5 μl of the cDNA solution was amplified in 50 μl solution containing 0.2 μl of Ex Taq polymerase (TaKaRa) and 1 μl each of 12.5 μM forward and reverse primers for one cycle at 94°C for 1 min and for 35 cycles for *AR* and 28 cycles for elongation factor 1 α (EF1α), at 94°C for 40 s, 62°C for 40 s, and 72°C for 1 min, followed by one cycle at 72°C for 2 min. The primer sequences of *AR* and *EF1α* are the same as those described by Ohtani et al. (2003). Northern blot hybridization was performed according to the method of Miura et al. (1998), using *AR* fragments of 2.5 kbp as the probe.

Results

WW embryos

The Northwestern Japan population has a female heterogametic sex determining system with heteromorphic sex chromosomes (Fig. 1). We chose five populations, locations of which range from north to south, and produced gynogenetic ZZ and WW embryos. The WW embryos from three populations (localities 1–3) died of edema within 10 days after hatching in contrast to the viable ZZ embryos (Fig. 2a and Table 1). In contrast, the WW embryos from the other two populations (locality numbers 4 and 5) passed the corresponding lethal stage with no symptom, but gradually fell into a state of undernourishment despite feeding and finally died within 50 days after fertilization (Fig. 2b and Table 1).

In order to verify whether the lethality of WW embryos is due to maternal allelic inactivation or degeneration (deleterious mutation) of the sex-linked genes responsible for development, we produced ZW sex-reversed males and crossed them with normal ZW females to obtain WW embryos, comprising both of the maternal and paternal W chromosomes. Testosterone propionate was injected into the abdomen of the tadpoles at 30 dpf, and finally 100% and 47.3% of the ZW female tadpoles from Kanazawa (K) and Niigata populations (N), respectively, were reversed to phenotypic fertile males. The sex-reversed ZW males (K and N) were crossed with normal ZW females and produced reciprocal WW embryos at a ratio of around 25% (Table 2). The WW embryos produced by crossing intra-population males and females showed the same lethality as that of the gynogenetic WW embryos. In contrast, the WW reciprocal hybrid embryos between groups N and K survived and normally matured. We conclude that the lethal genes on the W chromosomes underwent deleterious mutations, and they are not shared by the group N or K.

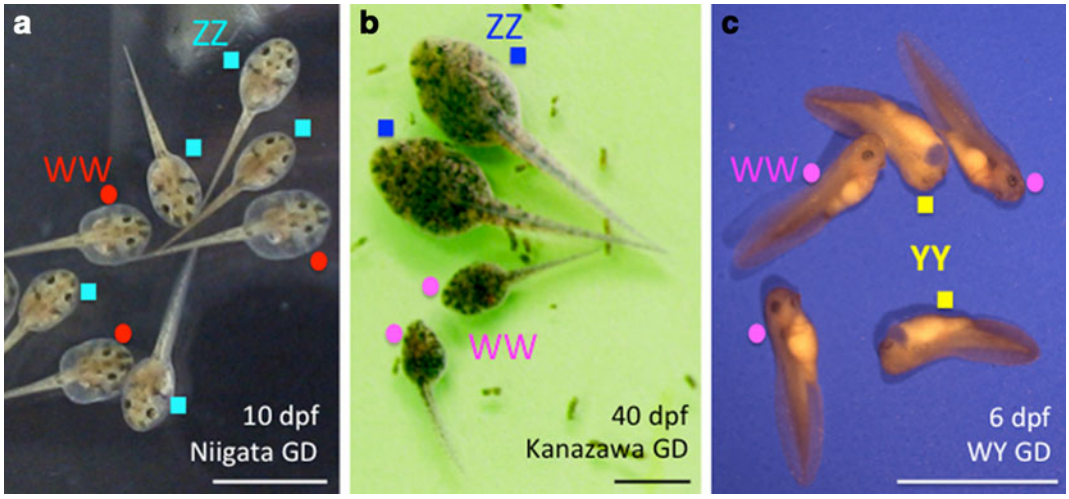

Fig. 2 Gynogenetic diploids WW and YY. Gynogenetic diploids ZZ and WW produced from a ZW female of Niigata population are shown in **a** and those from a ZW female of Kanazawa population are shown in **b**. The gynogenetic diploids YY and WW produced from a WY hybrid female between ZW female of Kanazawa and XY male of Hamamatsu populations are shown in **c**. Bar, 5 mm

Table 1 Survivorship of gynogenetic diploids *Rana rugosa* produced from ZW females of five local populations

Female	Population (locality no.)	Feeding tadpole (10 dpf[a])	Normal	Edema (WW)	Percent	Swimming tadpole (40 dpf)	Normal (ZZ)	Underdeveloped (WW)	Percent
H-ZW1	Hirosaki (1)	118	66	52	44.1	66	66	0	0
H-ZW2	Hirosaki (1)	51	26	25	49.0	22	22	0	0
N-ZW1	Niigata (2)	105	53	52	49.5	51	51	0	0
N-ZW2	Niigata (2)	121	63	58	47.9	57	57	0	0
T-ZW1	Toyama (3)	25	12	13	52.0	9	9	0	0
T-ZW2	Toyama (3)	31	16	15	48.4	14	14	0	0
K-ZW1	Kanazawa (4)	82	82	0	0.0	74	36	38	51.4
K-ZW2	Kanazawa (4)	103	103	0	0.0	98	47	51	52.0
F-ZW1	Fukui (5)	35	35	0	0.0	30	16	14	46.7
F-ZW2	Fukui (5)	33	33	0	0.0	29	16	13	46.4

[a] Days post-fertilization

Androgen receptor gene (AR) of W chromosome

AR located on the W chromosome is known to be extremely low in expression (Ohtani et al. 2003). We confirmed the *AR* expression in WW embryos from two different ZW populations using RT-PCR and Northern blot hybridization. The PCR product of *AR* was not detected in the WW embryos from Kanazawa or Niigata population, while ZZ, XX, and YY embryos showed a distinct *AR* product (Fig. 3a). The same results were obtained by Northern blot hybridization (Fig. 3b). Almost no signal was detected on RNA of the WW embryos from the Niigata or Kanazawa population. The hybridized signal of ZW females was weaker than that of ZZ males (Fig. 3b). Therefore, the regulatory mechanism of W-*AR* allele is probably inactivated both in the Niigata and Kanazawa populations of ZW type.

YY embryos

In order to obtain gynogenetic YY embryos, we first produced WY hybrid by crossing a ZW female from the Kanazawa population and an XY male from Hamamatsu population. The WY embryos normally grew to female adults. Then, we produced gynogenetic YY embryos from the WY hybrid mature females. The YY embryos, about half of the clutch,

Table 2 Crossing of a ZW female with a sex-reversed ZW male

Crossing	Female	Male	Feeding tadpole (10 dpf)	Normal (ZZ, ZW)	Edema (WW)	Swimming tadpole (40 dpf)	Normal (ZZ, ZW)	Underdeveloped (WW)
ZW × ZZ (control)	K-ZW1	K-ZZ1	69	69	0	69	69	0
	N-ZW1	N-ZZ1	63	63	0	63	63	0
ZW × ZW (intra-population)	K-ZW1	K-ZW1	98	98	0	98	73	25 (25.5%)
	K-ZW1	K-ZW2	66	66	0	66	47	19 (28.8%)
	N-ZW2	N-ZW1	50	39	11 (22%)	39	39	0
ZW × ZW (inter-population)	K-ZW2	N-ZW1	24	24	0	24	24	0
	K-ZW3	N-ZW1	55	55	0	55	55	0
	K-ZW4	N-ZW1	44	44	0	44	44	0
	N-ZW1	K-ZW1	78	78	0	78	78	0
	N-ZW1	K-ZW2	79	79	0	77	77	0

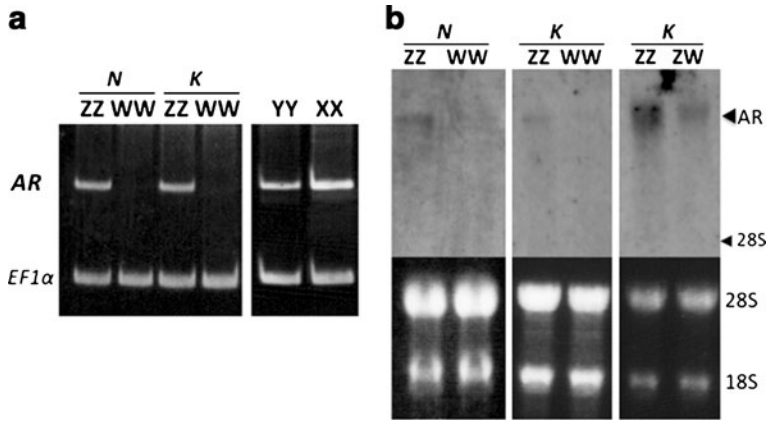

Fig. 3 RT-PCR and Northern hybridization of a sex-linked androgen receptor gene (*AR*). Amplified product of *AR* is not detected in WW embryos from Niigata (N) or Kanazawa (K) population, while ZZ, YY and XX ones showed a distinct *AR* band (**a**). *AR* hybridization signal is detected in ZZ RNA of both populations, but not in WW (**b**). Signal of ZW RNA is weaker than that of ZZ

died of cardiac edema at a very early developmental stage just after hatching (Fig. 2c and Table 3), while the WW embryos survived the lethal stage but finally died of under-nourishment at a much later stage, 40–50 dpf, despite feeding just like the WW embryos produced from a ZW female. Next, the WY females were crossed with normal XY males. The resultant YY embryos, about 25% of the offspring, died of the same cardiac edema at the same stage as those of gynogenetic YY embryos (Table 3). We conclude that the death of YY embryos was caused by a deleterious mutation, but not due to maternal inactivation, of one or more developmental gene(s) on the Y chromosome.

Discussion

Y and W chromosome degeneration

We discovered that the Y and W chromosomes of the frog *R. rugosa* have already undergone deleterious mutations of the genes responsible for the

Table 3 Production of YY embryos from WY hybrid females

Female	Male	Hatching tadpole (6 dpf)	Normal (WW, WX, WY or XY)	Cardiac edema (YY)	Percent	Average (%)of YY (WY1-8)
WY1	DG[a]	21	10	11	52.4	47.5
WY2	DG	19	12	7	36.8	
WY3	DG	115	55	60	52.2	
WY4	DG	51	31	20	39.2	
WY5	DG	17	8	9	52.9	
WY6	DG	88	51	37	42.0	
WY7	DG	84	38	46	54.8	
WY8	DG	32	16	16	50.0	
WY1	XY1	111	81	30	27.0	23.6
WY2	XY1	117	94	23	19.7	
WY3	XY2	143	119	24	16.8	
WY4	XY2	136	104	32	23.5	
WY5	XY2	103	83	20	19.4	
WY6	XY3	201	163	38	18.9	
WY7	XY3	140	106	34	24.3	
WY8	XY3	118	80	38	32.2	
WY6	XY4	196	157	39	19.9	
WY7	XY4	192	137	55	28.6	
WY8	XY4	110	78	32	29.1	

[a]Diploid gynogenesis

development, and the lethal genes are not shared with each other. Since the W and Y chromosomes are represented by the same chromosome 7 and share their recent evolutionary origin (Miura et al. 1998; Ogata et al. 2002), it is proved for the very first time that one partner of the sex chromosomes, Y for XY and W for ZW, has undergone degeneration independently, immediately after forming a heteromorphy regardless of the heterogametic sex (Fig. 4).

In the ZW system of this frog species, five populations examined here are divided into two groups N and K based on the lethality of WW embryos, and the two types of lethal genes are not shared by the two groups. This observation indicates that the W chromosomes independently underwent degeneration after divergence of the two groups originated from a common primary population. It is also implied that the W chromosome itself involves no evolutionary prior program for degeneration.

On the contrary, the sex-linked *AR* gene is not the case. Its W allele seems to be degenerated in the upstream regulatory region because the expression is extremely low. This observation was first reported by Ohtani et al. (2003) and later confirmed by Yokoyama et al. (2009) and now by us in the present study. The inactivated W allele is shared by the two groups N and K. This observation suggests that the degeneration of *W-AR* began just after or at the origin of the ZW system and before divergence of the two groups

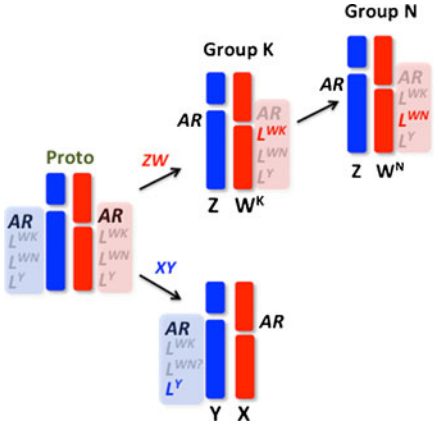

Fig. 4 Degeneration of the W and Y chromosomes. *AR* on the W chromosome is inactivated in both groups K and N, probably at very early stage in the primary ZW population. In contrast, lethal genes L^{WK} and L^{WN}, respectively, appear independently on the W chromosomes of the groups K and N. In the XY population, a different lethal gene L^{Y} specifically appears on the Y chromosome

and appearance of the lethal genes (Fig. 4). Precedence of W-*AR* inactivation to degeneration of the sex-linked developmental genes probably indicates its important role for ZW sex determining system, as proposed by Ohtani et al. (2003); Miura (2007) and Nakamura (2009), because the *W-AR* inactivation results in a sexually dimorphic expression: The *AR* expression level in ZW female gonads of tadpoles is almost half of ZZ ones. In fact, the treatment of ZZ male embryos with flutamide, an AR antagonist, induced upregulation of aromatase in gonads and then ovary differentiation at 30 dpf (Ohtani et al. 2003). This is a kind of "loss of function" analysis for sex determination by *AR*. Further critical functional analyses, such as transgenesis for overexpression of *AR* in ZW females and knockdown of *AR* in ZZ males, will be necessary to prove the sex determination role of *AR*.

Sex chromosome differentiation

The Z and W chromosomes of *R. rugosa* are similar in size and the W is still intact, with no abundant accumulation of constitutive heterochromatin at chromosomal level (Nishioka et al. 1993, 1994). Molecularly, W chromosome specific region is detected by comparative genomic hybridization around the pericentromeric region of the long arm, where a cluster of telomeric sequence of the ancestral chromosome before inversion continues to remain and transposase-like elements are also distributed (Miura et al. 2009). The molecular processing of sex chromosome differentiation and degeneration is now on going on the W chromosome, and this may have been triggered by creation of the non-recombining region with its partner Z chromosome through two inversions.

Besides, in amphibians, such a species with heteromorphic sex chromosomes is quite rare, occupying around 4% of the species examined, and most of the amphibian species still possess a homomorphic sex chromosome (Schmid et al. 1991; Eggert 2004). Basically, in highly evolved frog species, meiotic bivalents of homomorphic sex chromosomes and autosomes pair with their partners in a ring-shaped configuration, showing no chiasma except for the terminal regions of both arms (Molescalchi 1973; Okumoto 1980; Miura 1994). In fact, the recombination rate of genes is extremely lower in males than females in *Rana* species (Sumida and Nishioka 1994;

Nishioka and Sumida 1994b). If there were almost zero recombination in males, the Y chromosomes must experience degeneration because its life is eternally isolated from X chromosomes with no exchange of chromosomal materials, but this is not the actual case. Then, Perrin (2009) proposed that the homomorphy of abundant amphibian species could be explained by the occurrence of a rare sex reversal of XY male to female followed by a recombination revival in the XY females. This theory could be a case in point. However, despite almost no actual recombination between Y-linked genes themselves in males, a recombination between the sex-linked loci (or Y chromosomal specific region) and a male-determining locus definitely occurs at a low rate, to around 10%, in *Rana* frog species (Wright et al. 1983; Nishioka and Sumida 1994a; Sumida and Nishioka 1994; Miura 1994). Such a recombination seems to be sufficient to release the Y-linked genes from isolation and inhibit degeneration of the Y chromosome. Therefore, we propose another candidate mechanism that inhibits evolution of amphibian sex chromosomes toward heteromorphy. It is a rare occurrence of chromosomal rearrangement, particularly an inversion, in amphibian chromosomes. In 980 anuran species whose karyotypes are examined, 83% have a diploid chromosome number of 22–26 (Kuramoto 1990). The $2n=26$ chromosomes, consisting of five large and eight small pairs, of nine *Rana* species examined are almost completely homologous with each other with no chromosomal mutations, and particularly, the large chromosome pairs are highly conserved over genera (Miura 1995). These observations demonstrate a very high conservation of karyotypes in amphibians. We proved in the present study that if inversion occurs in one of the sex chromosomes, the degeneration process soon sets in even in a frog regardless of the heterogametic sex. Therefore, no occurrence of inversion on the sex chromosomes (on the large sex chromosomes), particularly at the evolutionary branching point of a large taxon, seems to be a reason for the evolutionary continuing homomorphy of abundant amphibian sex chromosomes. Together, a male determining gene on the large chromosome (1–4) is frequently replaced with another one in every species and even in local populations of one species, as seen in the *Rana* species (reviewed by Miura 2007). This frequent turnover of sex determining gene may not allow the sex chromosomes enough time to differentiate and leave them in a homomorphic state for a long period of time.

In summary, the Y and W chromosomes of *R. rugosa* have already undergone deleterious mutations on the genes for embryonic development. The lethal genes are not shared by the Y or W chromosomes and even by the W chromosomes from local populations. Thus, the degeneration of the Y and W chromosomes is processed independently and involves no evolutionary, prior common scenario. For the onset of degeneration of sex chromosomes, evolution of non-recombining region by inversions may be critically important. In amphibian species, the karyotypic evolution is highly conserved, and thus, the rare occurrence of inversion on the sex determining chromosomes could be one reason why they are kept in a homomorphic state. Surprisingly, S. Ohno had already predicted the critically important role of inversion for sex chromosome differentiation, as early as 1967 (Ohno 1967).

Acknowledgments This work was supported by a grant-in-aid for scientific research from Ministry of Education, Culture, Sports, Science and Technology of Japan to I.M (nos. 21570239 and 23132508).

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