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Sex Chromosomes of *Rana rugosa* with Special Reference to Local Differences in Sex-determining Mechanism

By

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ABSTRACT

The karyotypes of the two populations, Kumano and Hirosaki, of *Rana rugosa* were compared with each other by the methods of conventional Giemsa staining, C-banding and LR(late replication)-banding. It was found that in the Kumano population, the 13 pairs of chromosomes are completely homozygous and there are no chromosome pairs which have any sexual differences, while chromosome pair No. 7 of the females in the Hirosaki population are heteromorphic and constructed of a subtelocentric Z and a metacentric W chromosome. The W chromosome also distinctly differs from the Z chromosome in both C-band and LR-band patterns. It is probable that the W chromosome was induced from the Z chromosome by two inversions on the basis of the LR-band pattern.

In the Kumano population, nearly all of the females are changed into males by injection of testosterone propionate at the tadpole stage. The offspring of the sex-reversed genetic females mated with normal females are almost females. This seems to show that the Kumano population of *Rana rugosa* is of the XX–XY type. In the Hirosaki population, it has been confirmed by the methods of conventional Giemsa staining, C-banding and LR-banding that this is of the ZW–ZZ type and there are no frogs whose sex is reversed by injection of testosterone propionate.

Sex ratios were examined in the offspring of reciprocal crosses between the Kumano and Hirosaki populations. It was found that the offspring (XZ) between the females (XX) of the Kumano population and the males (ZZ) of the Hirosaki population are almost all males, while the offspring (WX, WY, ZX, ZY) between the females (ZW) of the Hirosaki population and the males (XY) of the Kumano population include nearly the same number of females and males. It was quite evident that almost all the frogs having the W chromosomes were females, while all the frogs having the Z chromosomes were males, regardless of the existence of the X or Y chromosome.

INTRODUCTION

Rana rugosa SCHLEGEL is a small frog of dirty brownish color. This species is common in Japan, Korea and northeastern China. In Japan, it is abundantly found in rice fields and around ponds, lakes and mountain streams. The chromo-

some number of *Rana rugosa* was first reported by IRIKI (1928, 1932). It is 26 and 13 in diploid and haploid numbers, respectively. Later, SETO (1965) again studied the chromosomes of this species, but he could not detect any heteromorphic pair in males and females. KAWAMURA and NISHIOKA (1977) reported that *R. rugosa* distributed around Hiroshima City was of the male heterogametic type in sex-determining mechanism on the basis of an experiment by K. YANO (now K. KASHIWAGI) performed in our laboratory. This experiment showed that an overwhelming majority of the gynogenetic diploid frogs produced from the eggs of 10 females by refrigeration after insemination with irradiated conspecific sperm were females, while there were nearly an equal number of males and females in the control series.

TOBISHIMA and SAITOH (1989) analyzed the karyotypes of two populations collected from Hirosaki City, Aomori Prefecture, and Yuda-cho, Iwate Prefecture, by the conventional staining and C-banding methods. The results seemed to show that chromosome pair No. 7 of the male was homozygous, while that of the female was heterozygous.

The existence of two different types of sex-determining mechanisms in one and the same species seemed to be very interesting, as such a case has not yet been reported at least in amphibians, although the existence of two types of sex-determining mechanisms, XX–XY and ZW–ZZ, has been reported in a small Mexican freshwater fish, *Platypoecilus maculatus,* by GORDON (1947). Thus, the present authors performed a study to reconfirm the two types of sex-determining mechanisms in *R. rugosa* and to clarify the relationship of these two types. A preliminary report of the result of this study has been made by NISHIOKA, MIURA and SAITOH (1990).

MATERIALS AND METHODS

Rana rugosa SCHLEGEL were collected from two districts which are about 1120 km apart from each other in Japan. A group of 12 females, 15 males and four egg masses was collected from the districts around Hirosaki City, Aomori Prefecture, while a group of five females and six males was obtained from Kumano-cho, near Hiroshima City.

1. Preparation of chromosomes

Mitotic figures were obtained by the method of blood cell culture. The culture fluid was made by mixing 60% of RPMI 1640 (Gibco), 20% of calf serum, 20% of redistilled water and 3% of PHA-M (Phytohemagglutinin, Difco). Penicillin and streptomycin were added at a final concentration of 100 Iu/ml and 100 μg/ml, respectively. In 2 ml of this culture fluid, 0.1~0.2 ml venous blood was added and cultured for 3~5 days at 25°C.

Chromosome preparations were made by the conventional air-drying method. The hypotonic treatment was made in 0.075 M KCl solution and the cells were fixed in CARNOY's fluid (acetic acid : methanol = 1 : 3). The C-bands were mainly

detected by SUMNER's method (1972). The chromosome preparations which were air-dried for one day were treated for 40 minutes in 0.2 N HCl at room temperature. After rinsed in distilled water, the preparations were incubated in 5% $Ba(OH)_2$ solution at 35°C for 5~10 minutes. After rinsed in distilled water, they were incubated in 2×SSC fluid (0.3 M NaCl and 0.03 M sodium citrate) at 60°C for 60 minutes. After rinsed in distilled water, they were stained with 4% Giemsa solution in phosphate buffer (19.5 ml of 0.1 M NaH_2PO_4 and 30.5 ml of 0.1 M Na_2HPO_4; pH 7.0).

The late replication (LR) bands were mainly detected by the method of TAKAYAMA, TANIGUCHI and IWASHITA (1981). After cultivation of peripheral blood for 3~5 days, 5-bromodeoxyuridine (BrdU) was added to the cultures to make the final concentration 10^{-4} M 6 hours prior to the harvest. Colchicine was also added to make the final concentration 10 μg/ml 4 hours prior to the harvest. Chromosome preparations produced from the harvest were made by the conventional air-drying method. The BrdU-labelled chromosome preparations were allowed to age for 1~2 days at room temperature and then stained with 3% Giemsa solution at 40°C for 3~5 minutes. The Giemsa solution was made in 2% 4Na-EDTA aqueous solution (pH 11.5).

Comparison of karyotypes was done by the method of NISHIOKA, OKUMOTO and RYUZAKI (1987).

2. Sex reversal by hormone treatment

Tadpoles at stages III~IX (TAYLOR and KOLLROS, 1946) were injected with 250 μg of testosterone propionate per individual. The testosterone propionate used here is a commodity commercially available under the name 'Enarmon' from Teikoku-Zoki Pharmaceutical Company. This Enarmon contains 25 mg (1.250 units) of testosterone propionate in 1 ml of sesame oil.

Tadpoles were fed on boiled spinach, while frogs were fed on crickets, *Gryllus bimaculatus* DE GEER.

OBSERVATION

I. Sex chromosome of Rana rugosa

The chromosome preparations were made from the cultured venous blood of 11 frogs including six males and five females of the Kumano population and that of 27 frogs including 15 males and 12 females of the Hirosaki population. In the Kumano population, 172 mitotic figures including 70 from males and 102 from females were examined, while in the Hirosaki population, 310 mitotic figures including 179 from males and 131 from females were examined (Table 1).

1. Conventional staining

The karyotypes of the Kumano population were analyzed in 15 and 19 mitotic figures from four males and five females, respectively, while those of the Hirosaki

TABLE 1

Number of mitotic figures used for chromosome analyses and banding patterns of chromosome pairs Nos. 2 and 7 in the Kumano and Hirosaki populations of *Rana rugosa*

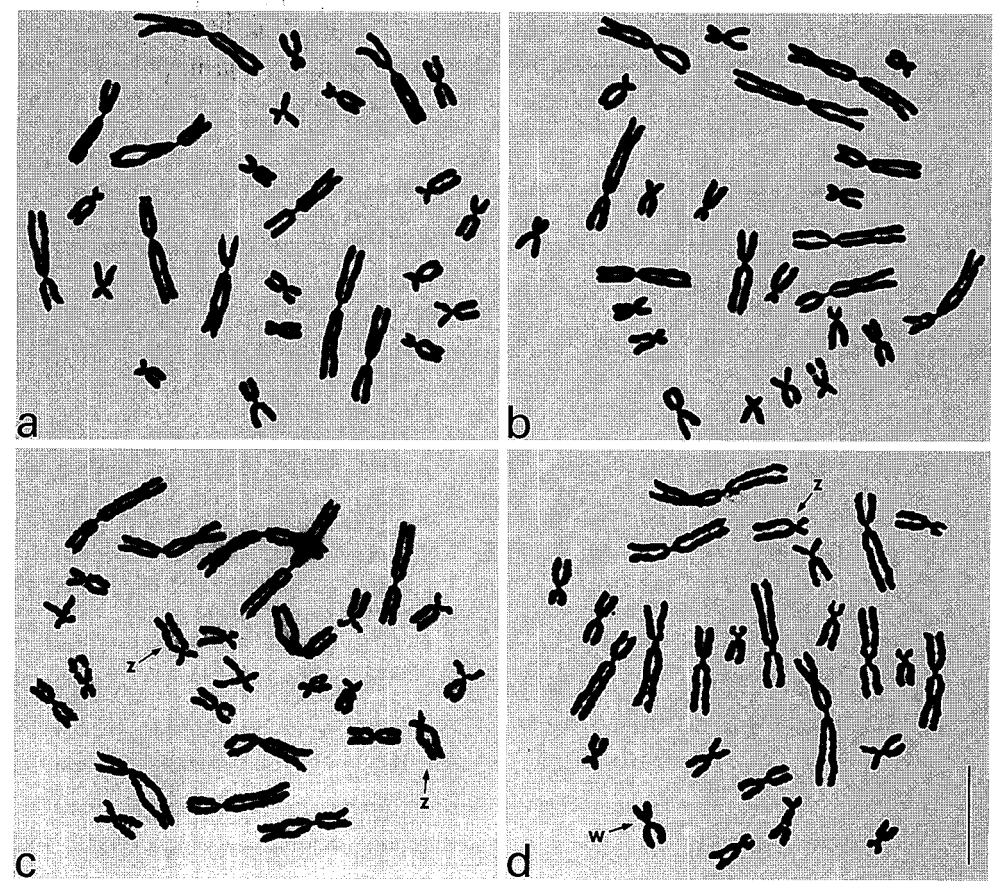
Population	Sex	No. of frogs	No. of mitotic figures observed by the methods of			Bivalent chr.	
			Giemsa staining	C-banding	LR-banding	No. 2	No. 7
Hirosaki	Male	4	13	36	41	OO	Z^AZ^A
		4	6	20	21	AO	Z^AZ^A
		1	—	1	—	AA	Z^AZ^A
		1	6	3	19	OO	Z^AZ^O
		4	4	3	5	AO	Z^AZ^O
		1	—	1	—	AA	Z^AZ^O
	Total	15	29	64	86		
	Female	6	10	18	50	OO	Z^AW
		6	18	22	13	AO	Z^AW
	Total	12	28	40	63		
Kumano	Male	4	15	24	19	BB	7^B7^B
		1	—	3	5	AA	7^B7^B
		1	—	4	—	AB	7^B7^B
	Total	6	15	31	24		
	Female	3	11	27	8	BB	7^B7^B
		2	8	20	28	AB	7^B7^B
	Total	5	19	47	36		

population were analyzed in 29 and 28 mitotic figures from 13 males and 12 females, respectively (Tables 2 and 3). It was found that all these figures were 2n=26, consisting of five pairs of large chromosomes and eight pairs of small chromosomes (Fig. 1). When these chromosomes were arranged in size and shape, as done in *Rana japonica* and *Rana nigromaculata* by NISHIOKA, OKUMOTO, UEDA and RYUZAKI (1987) and NISHIOKA, OKUMOTO and RYUZAKI (1987), each of the 13 pairs was completely homomorphic in the Kumano population. There were no sexual differences in the chromosomes. In contrast to the Kumano population, there were sexual differences in chromosomes No. 7 of the Hirosaki population. While chromosomes No. 7 of the males were homomorphic, those of the females were heteromorphic. They were constructed of a subtelocentric (Z) and a metacentric (W) chromosome. Thus, in the Hirosaki population, chromo-

Fig. 1. Metaphase plates and karyotypes of *Rana rugosa* from the Kumano and Hirosaki populations by conventional Giemsa staining.

a. Male of the Kumano population
b. Female of the Kumano population
c. Male of the Hirosaki population
d. Female of the Hirosaki population

No. 7 chromosomes are boxed. Bar represents 10 μm.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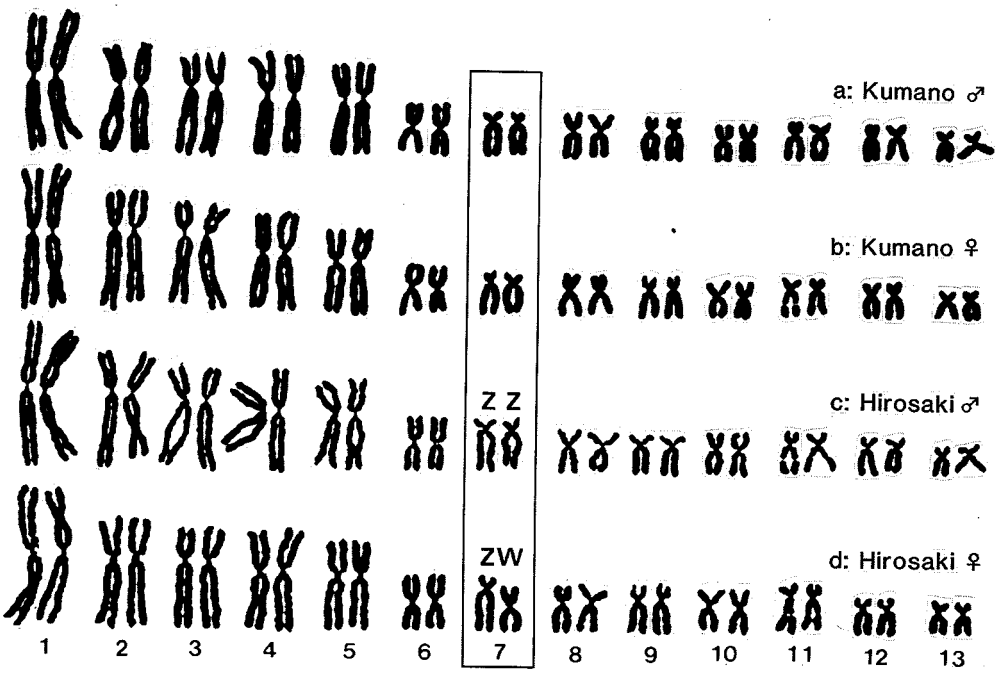
b
c
Z
Z
d
Z
W


a: Kumano ♂
b: Kumano ♀
Z Z
c: Hirosaki ♂
ZW
d: Hirosaki ♀
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some pair No. 7 of the males should be called ZZ and those of the females should be called ZW. In the Kumano population, chromosomes No. 7 of the males and females were similar to the Z chromosome of the Hirosaki population in size and

TABLE 2

Relative length, numerical values of centromere positions and types of metaphase chromosomes in the Kumano population of *Rana rugosa*

Male (XY)

Relative length (RL)				Numerical value of centromere position (NVC)				
Chromosome no.	Minimum	Maximum	Mean±	Chromosome no.	Minimum	Maximum	Mean±	Type
1	14.40	16.50	15.29±0.17	1	44.26	47.37	46.11±0.25	m
2	12.06	13.31	12.68±0.10	2	35.69	40.05	38.28±0.32	m
3	11.05	12.65	11.58±0.09	3	31.02	36.18	33.43±0.40	sm
4	10.48	11.58	11.17±0.08	4	40.29	44.66	42.42±0.36	m
5	10.04	10.81	10.38±0.07	5	37.84	43.14	40.12±0.36	m
6	5.45	6.54	5.83±0.08	6	45.96	50.86	48.42±0.36	m
7	4.97	6.02	5.27±0.07	7	23.68	29.50	26.82±0.37	sm
8	4.76	5.50	5.09±0.05	8	39.31	44.96	41.46±0.38	m
9	4.66	5.33	5.03±0.05	9	28.62	35.12	32.59±0.47	sm
10	4.28	5.03	4.60±0.06	10	41.03	47.84	44.14±0.45	m
11*	4.48	5.16	4.83±0.05	11*	29.68	36.40	32.73±0.49	sm
12	4.18	4.84	4.41±0.04	12	32.34	39.11	36.21±0.42	sm
13	3.47	4.28	3.84±0.05	13	34.30	41.60	37.26±0.49	sm

Female (XX)

Relative length (RL)				Numerical value of centromere position (NVC)				
Chromosome no.	Minimum	Maximum	Mean±	Chromosome no.	Minimum	Maximum	Mean±	Type
1	14.13	16.45	15.22±0.16	1	44.31	48.65	46.53±0.26	m
2	11.89	13.49	12.71±0.10	2	34.16	39.34	36.83±0.28	sm
3	10.93	12.90	11.81±0.10	3	28.67	33.46	31.22±0.27	sm
4	10.28	12.08	11.29±0.10	4	40.07	45.75	42.06±0.33	m
5	9.53	11.14	10.46±0.09	5	37.89	43.20	40.15±0.29	m
6	5.48	6.55	5.86±0.06	6	43.42	51.33	48.01±0.46	m
7	4.91	5.72	5.24±0.05	7	23.02	31.20	26.05±0.50	sm
8	4.45	5.60	4.96±0.06	8	37.99	42.74	40.91±0.36	m
9	4.53	5.59	5.03±0.05	9	26.33	33.56	30.75±0.50	sm
10	4.33	4.97	4.59±0.04	10	38.20	44.51	42.42±0.34	m
11*	4.31	5.16	4.68±0.05	11*	27.00	36.68	32.02±0.59	sm
12	3.99	4.80	4.49±0.05	12	33.37	42.00	36.42±0.48	sm
13	3.15	4.26	3.66±0.06	13	31.34	41.69	35.98±0.55	sm

$$RL=\frac{\text{Chromosome length}}{\text{Genome length}}\times 100$$

$$NVC=\frac{\text{Short-arm length}}{\text{Chromosome length}}\times 100$$

	NVC	Type
Chromosome type:	50.0~37.5	m
	37.4~25.0	sm
	24.9~12.5	st
	12.4~ 0	t

± Standard error of the mean

* Secondary constriction

TABLE 3

Relative length, numerical values of centromere positions and types of metaphase chromosomes in the Hirosaki population of *Rana rugosa*

Male (ZZ)

Relative length (RL)				Numerical value of centromere position (NVC)				
Chromosome no.	Minimum	Maximum	Mean±	Chromosome no.	Minimum	Maximum	Mean±	Type
1	13.68	15.78	15.00±0.11	1	44.44	48.96	46.64±0.23	m
2	11.42	12.77	12.14±0.07	2	33.67	41.43	37.51±0.28	m
3	10.76	12.72	11.47±0.08	3	30.50	37.01	33.75±0.31	sm
4	10.59	12.22	11.25±0.08	4	38.79	42.86	40.98±0.22	m
5	9.67	10.87	10.12±0.05	5	36.76	42.64	39.87±0.30	m
6	5.41	6.35	5.96±0.05	6	44.02	51.01	47.71±0.34	m
7(Z)	5.38	6.39	6.00±0.05	7(Z)	21.06	29.40	24.03±0.42	st
8	4.67	5.65	5.12±0.04	8	37.97	45.44	41.23±0.35	m
9	4.65	5.58	5.12±0.04	9	25.24	36.25	31.04±0.48	sm
10	4.34	5.37	4.93±0.05	10	38.67	45.75	41.75±0.31	m
11*	4.29	5.46	4.89±0.05	11*	25.53	34.94	30.17±0.43	sm
12	3.89	4.73	4.33±0.04	12	26.83	37.27	32.80±0.47	sm
13	3.20	4.29	3.65±0.04	13	32.98	43.59	36.83±0.44	sm

Female (ZW)

Relative length (RL)				Numerical value of centromere position (NVC)				
Chromosome no.	Minimum	Maximum	Mean±	Chromosome no.	Minimum	Maximum	Mean±	Type
1	14.11	15.86	14.84±0.09	1	44.99	49.53	46.91±0.21	m
2	11.38	13.25	12.16±0.08	2	33.69	43.03	37.04±0.40	sm
3	10.79	12.64	11.42±0.09	3	31.20	36.73	32.88±0.23	sm
4	10.72	12.06	11.38±0.06	4	36.92	44.35	41.84±0.31	m
5	9.35	10.80	10.06±0.06	5	38.40	43.04	40.87±0.23	m
6	5.64	6.33	5.98±0.03	6	45.41	52.07	48.90±0.25	m
7(Z)	5.58	7.07	6.10±0.07	7(Z)	17.56	27.46	23.72±0.41	st
7(W)	4.46	5.69	4.96±0.06	7(W)	37.50	47.33	42.98±0.43	m
8	4.74	5.51	5.14±0.04	8	37.46	45.11	41.31±0.35	m
9	4.80	5.40	5.12±0.03	9	27.00	36.90	31.31±0.33	sm
10	4.38	5.32	4.81±0.04	10	38.15	44.70	41.62±0.26	m
11*	4.41	5.55	4.93±0.05	11*	26.71	32.90	29.71±0.30	sm
12	3.93	4.82	4.30±0.04	12	27.81	36.21	32.94±0.36	sm
13	3.45	4.26	3.75±0.04	13	33.79	41.03	36.99±0.34	sm

The RL and NVC of the chromosomes of the females in the Hirosaki population were calculated by regarding the Z chromosome as chromosome No. 7. The RL of the W chromosome was calculated on the basis of the ratio of the Z and W chromosomes in length in each mitotic figure.

$$RL=\frac{\text{Chromosome length}}{\text{Genome length}}\times 100$$

$$NVC=\frac{\text{Short-arm length}}{\text{Chromosome length}}\times 100$$

	NVC	Type
Chromosome type:	50.0～37.5	m
	37.4～25.0	sm
	24.9～12.5	st
	12.4～ 0	t

± Standard error of the mean

* Secondary constriction

shape, although the Z chromosome of the Hirosaki population was somewhat larger than chromosome No. 7 of the Kumano population. Chromosomes No. 11 of both sexes in the Hirosaki and Kumano populations had a secondary constriction in the long arm.

The chromosomes of the males were compared with those of the females in relative length (RL) and numerical value of the centromere position (NVC) as reported by NISHIOKA, OKUMOTO and RYUZAKI (1987). RL and NVC of each chromosome in the Kumano population were examined in 15 and 19 mitotic figures from males and females, respectively. The results showed that there were no differences between the males and females. In the Hirosaki population, RL and NVC of each chromosome were examined in 29 and 28 mitotic figures from males and females, respectively. RL of chromosomes No. 7 in the females was shown by the values of the Z chromosomes, while RL of the W chromosome was calculated from the ratio of the W chromosome to the Z chromosome in each of the 28 mitotic figures. If the values of the W chromosomes are excluded, there were no differences between males and females, as shown in Table 3.

2. C-band pattern

The C-band patterns were observed in 31 and 47 mitotic figures from six males and five females, respectively, in the Kumano population and in 64 and 40 mitotic figures from 15 males and 12 females, respectively, in the Hirosaki population. The C-bands were observable at the centromeric regions of all the chromosomes and at the basal, intermediate and end portions of several chromosomes (Fig. 2).

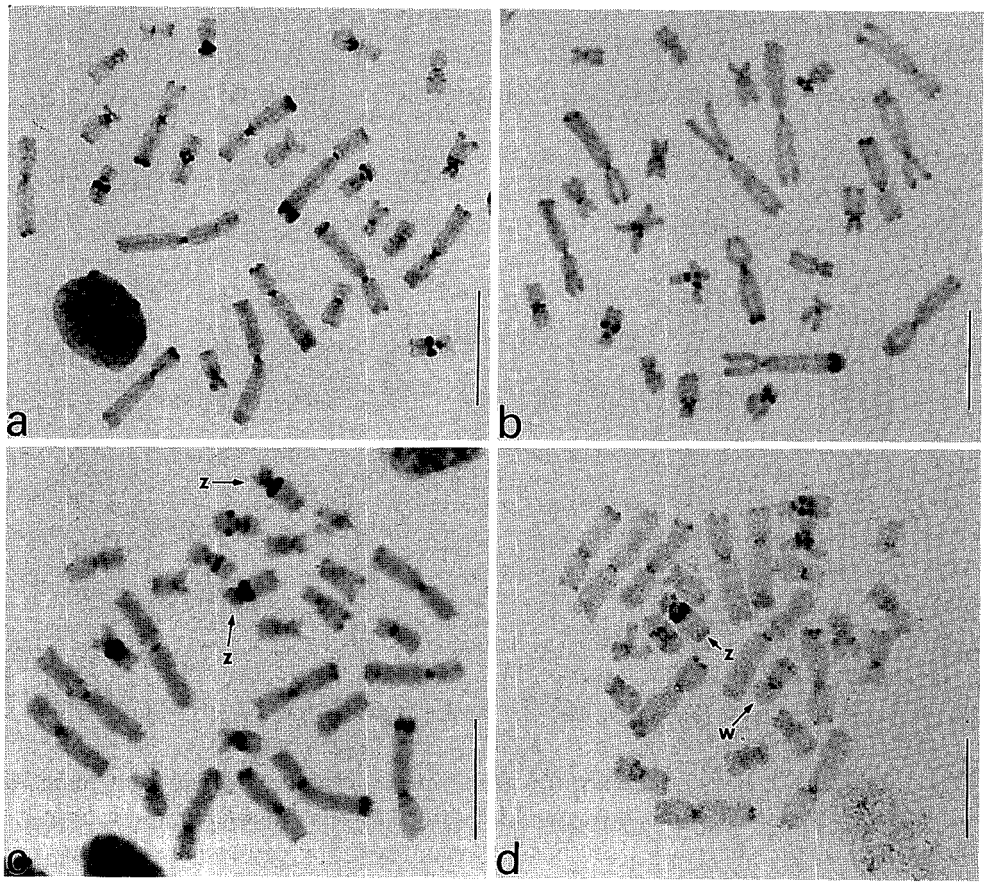
In chromosome pair No. 2, there were three kinds of chromosomes; one had a very intensely stained band (A), another had a somewhat weakly stained band (B) and the remainder had no band (O) at the end portion of the long arm. Each of these three kinds of chromosomes was found homozygously or heterozygously. Of the six males and five females of the Kumano population, one male was of the AA type, one male and two females were of the AB type and the remaining four males and three females were of the BB type. Of the 15 males and 12 females of the Hirosaki population, five males and six females were of the OO type, two males were of the AA type and the remaining eight males and six females were of the AO type (Table 1; Fig. 2). It was found that the presence of these bands on chromosomes No. 2 bore no relation to sex in both the Kumano and Hirosaki populations.

In chromosome pair No. 7 of the Hirosaki population, the subtelocentric (Z) chromosomes were divided into two kinds in the C-band pattern; one (Z^A) had a deeply stained band at the basal portion of the long arm and the other (Z^O) had no such band. Of the 15 males of the Hirosaki population, nine were of the Z^AZ^A type, and the other six were of the Z^AZ^O type. All the 12 females were of the

Fig. 2. C-banded metaphase plates and karyotypes of *Rana rugosa* from the Kumano and Hirosaki populations.

a. Male of the Kumano population
b. Female of the Kumano population
c. Male of the Hirosaki population
d. Female of the Hirosaki population

No. 7 chromosomes are boxed. Bars represent 10 μm.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b
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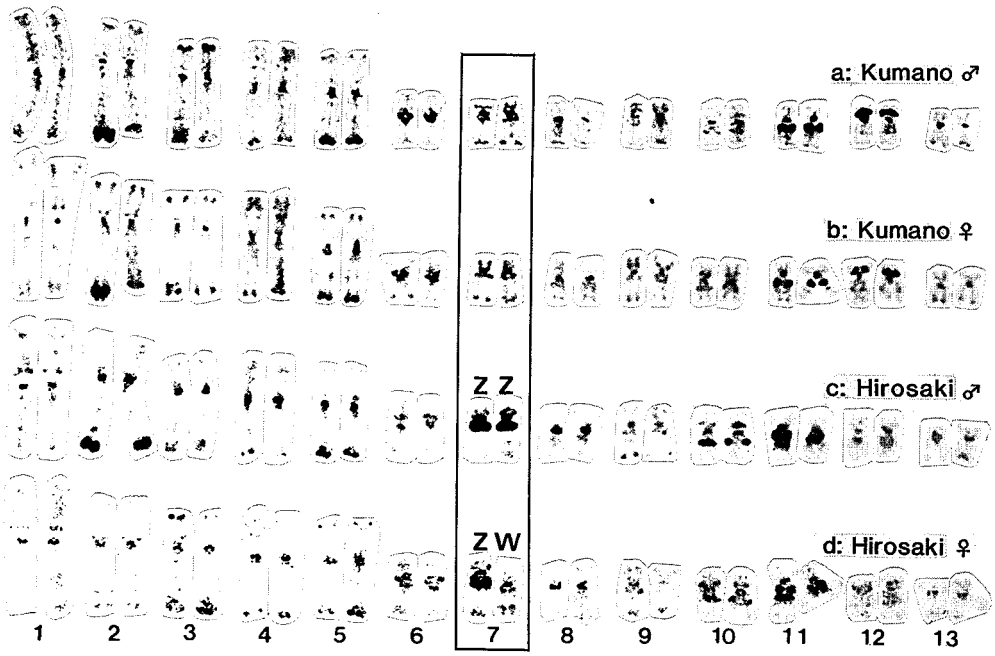
Z
c
Z
W
d


a: Kumano ♂
b: Kumano ♀
c: Hirosaki ♂
d: Hirosaki ♀
Z Z
Z W
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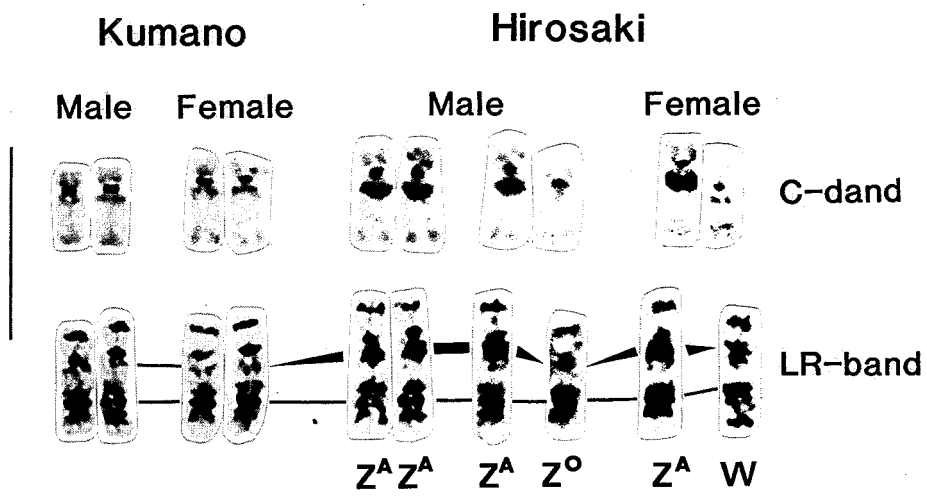


Fig. 3. Comparison of the C-band and LR-band patterns in chromosomes No. 7 between the Kumano and Hirosaki populations of *Rana rugosa*. Homologous LR-bands are joined by horizontal lines. Vertical bar represents 10 μm.

Z^AW type (Table 1; Fig. 3). Chromosomes No. 7 of the Kumano population had somewhat weakly stained bands (7^B7^B), although they were similar to the Z chromosome in shape, as well as in the position and number of C-bands (Figs. 2 and 3).

The metacentric (W) chromosome of chromosome pair No. 7 in the females of the Hirosaki population had three weakly stained bands situated at the intermediate portion of the short arm, the basal portion near the centromere and the end portion of the long arm.

3. LR(late replication)-band pattern

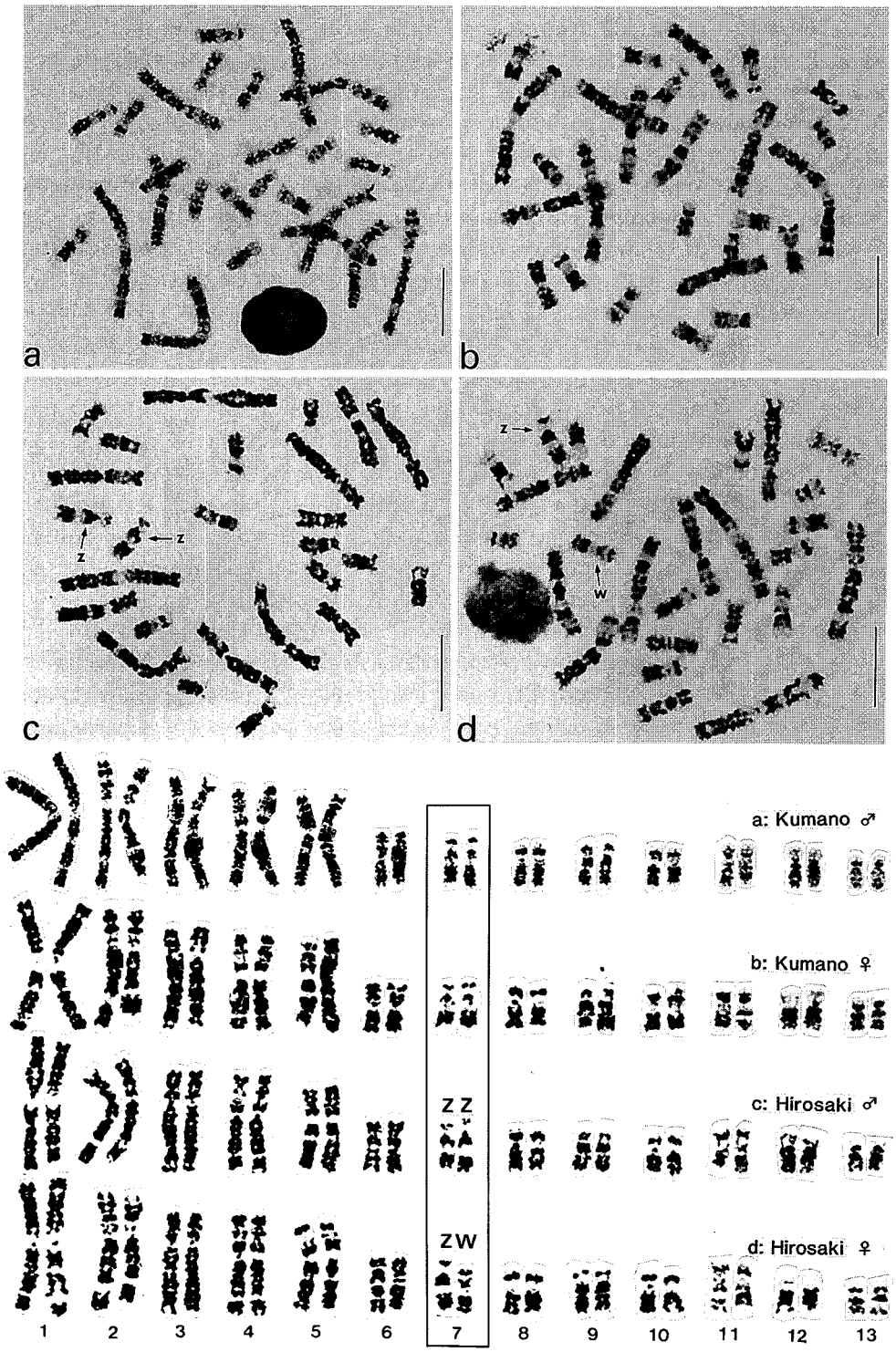
The LR-band patterns appearing during about 4 hours of the late S phase were observed in 24 mitotic figures from five males and 36 mitotic figures from five females of the Kumano population and in 86 mitotic figures from 13 males and 63 mitotic figures from 12 females of the Hirosaki population (Fig. 4). It was found that there was no sexual difference in the LR-band patterns of chromosomes No. 2, although there were some variations at the end portions of the long arms in both populations.

In the subtelocentric chromosome of chromosome pair No. 7 of the Hirosaki population, the LR-band corresponding to the C-band of the Z^A chromosome was deeply stained, while the LR-band corresponding to the C-band of the Z^O chromosome was very weakly stained. The W chromosome in the Hirosaki

Fig. 4. LR-banded metaphase plates and karyotypes of *Rana rugosa* from the Kumano and Hirosaki populations.

a. Male of the Kumano population
b. Female of the Kumano population
c. Male of the Hirosaki population
d. Female of the Hirosaki population

No. 7 chromosomes are boxed. Bars represent 10 μm.


a
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W
a: Kumano ♂
b: Kumano ♀
c: Hirosaki ♂
d: Hirosaki ♀
Z Z
Z W
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population distinctly differed from the Z chromosome in the LR-band pattern. From comparison of the LR-band pattern of the Z chromosome with that of the W chromosome, it was confirmed that the W chromosome was produced from the Z chromosome by two inversions (Fig. 5). On the other hand, there was no sexual difference in the LR-band patterns of chromosome pair No. 7 in the Kumano population. The LR-band patterns of chromosomes No. 7 of the males and females in the Kumano population were the same as those of the Z chromosomes of the Hirosaki population in the position and number of bands, while the LR-band situated at the position corresponding to the C-band at the basal portion of the long arm was intermediate in intensity between those of the Z^A and Z^O chromosomes (Figs. 3 and 4).

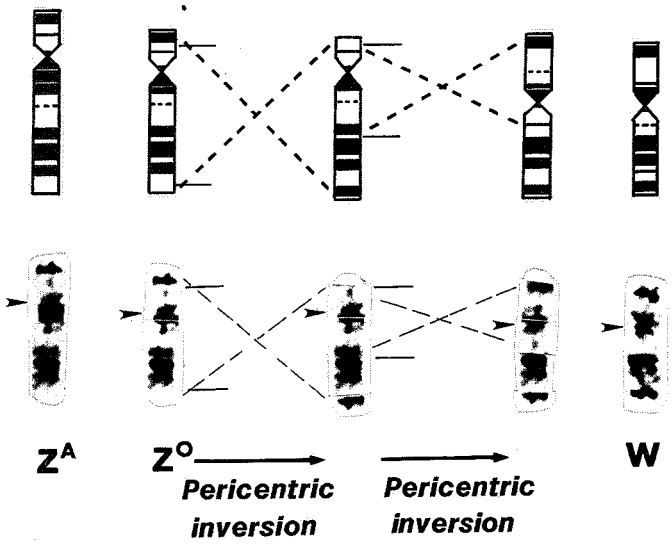

Fig. 5. Reconstruction of the W chromosome from the Z chromosome by two pericentric inversions. Horizontal bars indicate the positions of breakpoints in inversions. Dashed lines join the homologous regions between the chromosomes before and after inversions, respectively. Arrowheads denote the positions of centromeres.

II. Sex reversal by hormone

From the foregoing study on the morphology of chromosomes, it was evident that chromosomes No. 7 are the sex chromosomes in the Hirosaki population of *Rana rugosa*. Thus, the female is of the ZW type and the male is of the ZZ type. In contrast, the sex chromosomes have not yet been identified in the Kumano population. In order to clarify the difference in sex-determining mechanism between the Kumano and Hirosaki populations, an attempt was made to change the genetic females into males by injecting testosterone propionate into tadpoles of both populations. If ZW males (sex-reversed genetic females) were obtained in the Hirosaki population, the matings of normal females (ZW) with the sex-reversed genetic females (ZW) should produce three kinds of offspring, ZZ, ZW

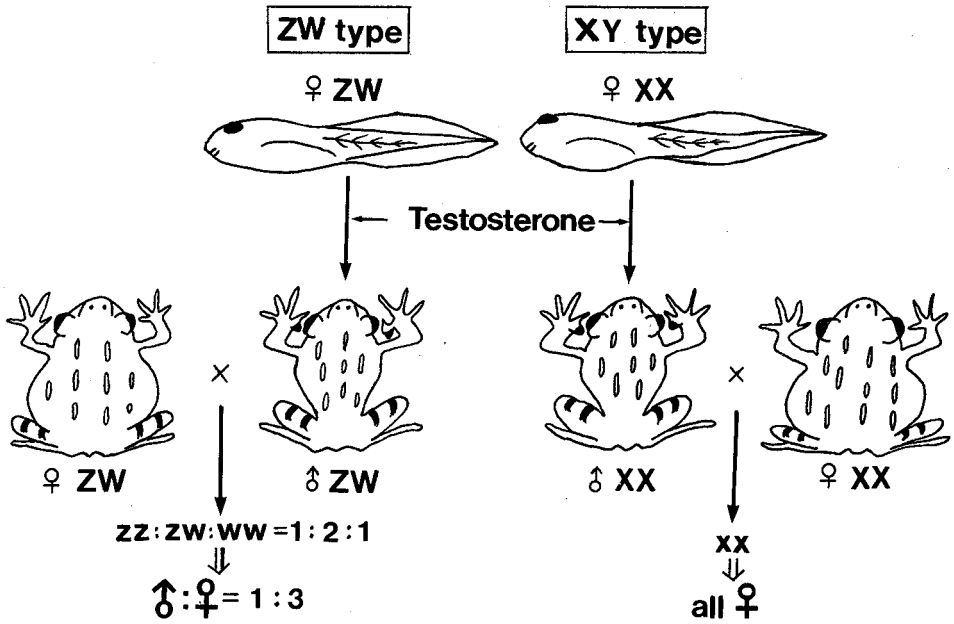

Fig. 6. Sex ratio and sex-chromosome constitution in the offspring of sex-reversed females having different sex-determining systems mated with the respective genetic females.

and WW in the ratio of 1:2:1, that is, males and females in the ratio of 1:3. If the population was of the XX–XY type, and XX males (sex-reversed genetic females) were produced by injection of the androgen, the matings of normal females (XX) with sex-reversed genetic females (XX) should produce only female offspring (Fig. 6).

1. Kumano population

a. Effect of testosterone propionate

In 1986, a total of 1088 normally feeding tadpoles, including 63, 103, 78, 78 and 766 tadpoles from five matings between three females (K♀, Nos. 1~3) and five males (K♂, Nos. 1~5), was obtained in the Kumano population. Of these tadpoles, 41, 93, 51, 72 and 658, 915 in total, became 22~38 mm, 31.1±0.3 mm on the average, at st. III~IX, 38~64 days after fertilization. Then, in about one-half of these tadpoles, 18, 43, 26, 37 and 327, 451 in total, were injected with 250 μg of testosterone propionate, while the other tadpoles were left as the controls. All the tadpoles of these experimental and control series were reared continuously beyond the time of metamorphosis. The results showed that 419 tadpoles of the experimental series and 408 tadpoles of the control series completed metamorphosis within 74~278 days after fertilization. There were no remarkable differences between the tadpoles of the experimental and control series in the number of days from fertilization to metamorphosis. When the sex of metamorphosed frogs was examined, it was found that four were females and 403 (99.0%) were males in the experimental series, while 155 were females and 224

TABLE 4

Sex ratio of the frogs raised from the testosterone-injected tadpoles in the Kumano (K) and Hirosaki populations of *Rana rugosa*

Series	Parents		No. of tadpoles	No. of metamorphosed frogs	No. of analyzed frogs		
	Female	Male			Total	Female	Male (%)
Cont.	K, No. 1	K, No. 1	23	18	18	8	10 (55.6)
	K, No. 1	K, No. 2	50	42	42	21	21 (50.0)
	K, No. 1	K, No. 3	25	25	24	12	12 (50.0)
	K, No. 2	K, No. 4	35	35	31	15	16 (51.6)
	K, No. 3	K, No. 5	331	288	264	99	165 (62.5)
	Total		464	408	379	155	224 (59.1)
Exp.	K, No. 1	K, No. 1	18	12	9	0	9 (100.0)
	K, No. 1	K, No. 2	43	39	36	0	36 (100.0)
	K, No. 1	K, No. 3	26	25	25	0	25 (100.0)
	K, No. 2	K, No. 4	37	37	33	0	33 (100.0)
	K, No. 3	K, No. 5	327	306	304	4	300 (98.7)
	Total		451	419	407	4	403 (99.0)
Cont.	Hirosaki, 86		108	108	95	38	57 (60.0)
Exp.	Hirosaki, 86		278	228	195	98	97 (49.7)

(59.1%) were males in the control series. Thus, about half of the males in the experimental series was considered to be genetic females whose sex was reversed by injection of testosterone propionate (Table 4).

b. Offspring of sex-reversed females

i) Control series

In 1987, eight control matings were made between eight females including five (Kw♀, Nos. 1~5) which were collected from the field in 1987 and three (Kc5♀, Nos. 6~8) which were produced from a mating, K♀, No. 3×K♂, No. 5, and four males including two (Kc3♂, Nos. 1 and 2) produced from a mating, K♀, No. 1×K♂, No. 3, and two (Kc5♂, Nos. 3 and 4) produced from a mating, K♀, No. 3×K♂, No. 5. The five females collected from the field were 47~55 mm, 51.0 mm on the average, in body length, while the other three females produced from a mating were 45~47 mm, 46.3 mm on the average, in body length. The four males were 37~44 mm, 40.5 mm on the average, in body length and had testes which were 3.2~3.8 mm, 3.44 on the average, in length and 2.5~2.8 mm, 2.65 mm on the average, in width.

It was found that 44.2~95.5% of the 43~306 eggs in the eight control series, 1209 (78.5%) of the 1541 eggs in total, cleaved normally. Of these eggs, 11.6~73.8%, 716 eggs (46.5%) in total, normally completed metamorphosis. When the metamorphosed frogs were continuously reared, 335 of the 694 frogs examined were females and the other 359 (51.7%) were males, that is, the sex ratio was about 1 : 1 (Table 5).

TABLE 5

Developmental capacity and sex ratio of the offspring of 17 males raised from the testosterone-injected tadpoles in the Kumano (K) population of *Rana rugosa*

Series	Parents		No. of eggs	No. of normal cleavages (%)	No. of normal tail-bud embryos (%)	No. of normally hatched tadpoles (%)	No. of metamorphosed frogs (%)	No. of analyzed frogs		
	Female	Male						Total	♀	♂ (%)
Cont.	Kw, No. 1	Kc3, No. 1	43	19 (44.2)	15 (34.9)	15 (34.9)	5 (11.6)	5	3	2 (40.0)
	Kw, No. 2	Kc3, No. 2	224	117 (52.2)	84 (37.5)	82 (36.6)	78 (34.8)	76	30	46 (60.5)
	Kw, No. 3	Kc3, No. 2	127	97 (76.4)	90 (70.9)	88 (69.3)	74 (58.3)	73	37	36 (49.3)
	Kw, No. 4	Kc5, No. 3	145	131 (90.3)	128 (88.3)	126 (86.9)	107 (73.8)	105	59	46 (43.8)
	Kw, No. 5	Kc5, No. 3	200	191 (95.5)	158 (79.0)	156 (78.0)	111 (55.5)	103	46	57 (55.3)
	Kc5, No. 6	Kc5, No. 4	306	258 (84.3)	189 (61.8)	165 (53.9)	111 (36.3)	111	54	57 (51.4)
	Kc5, No. 7	Kc5, No. 4	282	253 (89.7)	230 (81.6)	164 (58.2)	114 (40.4)	105	47	58 (55.2)
	Kc5, No. 8	Kc5, No. 3	214	143 (66.8)	137 (64.0)	132 (61.7)	116 (54.2)	116	59	57 (49.1)
	Total		1541	1209 (78.5)	1031 (66.9)	928 (60.2)	716 (46.5)	694	335	359 (51.7)
Exp.	Kw, No. 1	Ke3, No. 1	135	63 (46.7)	44 (32.6)	43 (31.9)	25 (18.5)	21	11	10 (47.6)
	Kw, No. 2	Ke3, No. 2	350	266 (76.0)	159 (45.4)	153 (43.7)	148 (42.3)	137	74	63 (46.0)
	Kw, No. 2	Ke3, No. 3*	334	300 (89.8)	209 (62.6)	203 (60.8)	174 (52.1)	**174**	**167**	**7 (4.0)**
	Kw, No. 2	Ke5, No. 4	214	187 (87.4)	136 (63.6)	133 (62.1)	112 (52.3)	111	55	56 (50.5)
	Kw, No. 3	Ke5, No. 5*	303	242 (79.9)	219 (72.3)	205 (67.7)	169 (55.8)	**168**	**163**	**5 (3.0)**
	Kw, No. 3	Ke5, No. 6*	290	241 (83.1)	208 (71.7)	202 (69.7)	154 (53.1)	**144**	**140**	**4 (2.8)**
	Kw, No. 4	Ke5, No. 7*	175	162 (92.6)	145 (82.9)	142 (81.1)	102 (58.3)	**99**	**99**	**0 (0.0)**
	Kw, No. 4	Ke5, No. 8*	203	191 (94.1)	160 (78.8)	153 (75.4)	115 (56.7)	**113**	**103**	**10 (8.8)**
	Kw, No. 4	Ke5, No. 9	223	211 (94.6)	176 (78.9)	165 (74.0)	133 (59.6)	133	60	73 (54.9)
	Kw, No. 5	Ke4, No. 10	224	214 (95.5)	207 (92.4)	205 (91.5)	182 (81.3)	178	89	89 (50.0)
	Kw, No. 5	Ke4, No. 11	216	213 (98.6)	202 (93.5)	202 (93.5)	178 (82.4)	175	72	103 (58.9)
	Kw, No. 5	Ke4, No. 12	233	225 (96.6)	204 (87.6)	198 (85.0)	162 (69.5)	136	61	75 (55.1)
	Kc5, No. 6	Ke2, No. 13	608	173 (28.5)	143 (23.5)	129 (21.2)	104 (17.1)	100	47	53 (53.0)
	Kc5, No. 7	Ke2, No. 14	245	211 (86.1)	201 (82.0)	201 (82.0)	160 (65.3)	149	72	77 (51.7)
	Kc5, No. 8	Ke5, No. 15*	173	147 (85.0)	139 (80.3)	98 (56.6)	67 (38.7)	**64**	**61**	**3 (4.7)**
	Kc5, No. 8	Ke5, No. 16*	203	179 (88.2)	172 (84.7)	133 (65.5)	67 (33.0)	**67**	**59**	**8 (11.9)**
	Kc5, No. 8	Ke5, No. 17*	231	172 (74.5)	129 (55.8)	129 (55.8)	99 (42.9)	**87**	**87**	**0 (0.0)**
	Total	8 males*	**1912**	**1634 (85.5)**	**1381 (72.2)**	**1265 (66.2)**	**947 (49.5)**	**916**	**879**	**37 (4.0)**
		9 males	2448	1763 (72.0)	1472 (60.1)	1429 (58.4)	1204 (49.2)	1140	541	599 (52.5)

w, field caught c, control series e, experimental series *, sex-reversed female

ii) Experimental series

In the five matings made in 1986 between three females and five males of the Kumano population, 403 (99.0%) became males by injection with testosterone propionate at the tadpole stage (Table 4). In 1987, 17 males including three (Ke3♂, Nos. 1~3) produced from a mating, (K♀, No. 1×K♂, No. 3), nine (Ke5♂, Nos. 4~9, Nos. 15~17) produced from a mating, (K♀, No. 3×K♂, No. 5), three (Ke4♂, Nos. 10~12) produced from a mating, (K♀, No. 2×K♂, No. 4), and two (Ke2♂, Nos. 13 and 14) produced from a mating, (K♀, No. 1×K♂, No. 2), were mated with eight females (Kw♀, Nos. 1~5, Kc5♀, Nos. 6~8), which were the same as those used in the control series. The 17 males were 35~44 mm,

38.9 mm on the average, in body length, and had testes which were 2.8~4.1 mm, 3.65 mm on the average, in length and 2.0~3.3 mm, 2.73 mm on the average, in width. The results showed that 28.5~98.6% of the 135~608 fertilized eggs in the 17 experimental series, 3397 (77.9%) of the 4360 eggs in total, cleaved normally, and 17.1~82.4% of respective numbers of eggs, 2151 eggs (49.3%) in total, normally completed metamorphosis (Table 5).

When the sex of the frogs was examined after reared for several months, it was found that the offspring of eight including one (KE3 ♂, No. 3) and seven (KE5 ♂, Nos. 5~8 and 15~17) of the 17 males were almost females. The male offspring produced from these males were 0~11.9% of the 64~174 frogs of the eight experimental series, 37 (4.0%) of the 916 frogs in total. The offspring of the other nine males were about 1:1 in sex ratio. Of the 21~178 frogs in these nine experimental series, 46.0~58.9%, 599 (52.5%) of the 1140 frogs in total, were males. Thus, eight of the 17 frogs were considered to be sex-reversed genetic females. The fact that the offspring of males which were considered to be sex-reversed genetic females became almost females, seemed to show that the Kumano population of *Rana rugosa* is of the XX–XY type in sex determination.

2. Hirosaki population

The feeding tadpoles at st. IV~VII raised from four egg masses collected from the field in 1986 were divided into two groups. One group contained 278 tadpoles which were 15~33 mm, 28.3±0.15 mm on the average, in total length. Each of these tadpoles was injected with 250 μg of the testosterone propionate. The other group contained 108 tadpoles which were continuously reared as the controls. As 228 and 108 tadpoles completed metamorphosis in the experimental and control series, respectively, they were reared for several months and their sex was examined. The results showed that 57 (60.0%) of the 95 frogs in the control series and 97 (49.7%) of the 195 frogs in the experimental series were males. There were no large differences between the numbers of females and males (Table 4).

In order to confirm the presence of sex-reversed individuals among these females and males, detailed examination of the chromosome preparations obtained from blood cell cultures was made in 14 males and two females of the control series and 54 males and 41 females of the experimental series by the methods of conventional Giemsa staining and C-banding.

a. Males raised from tadpoles injected with testosterone propionate

i) Control series

Chromosomes Nos. 2 and 7 were observed in detail in 377 mitotic figures at metaphase, 26.9 per male, obtained from 14 males of the control series. Two males had deeply stained C-bands situated at the end portions of the long arms of chromosomes No. 2 (AA), five males had no deeply stained bands (OO), and the remaining seven males had such bands heterozygously (AO). Chromosomes No. 7 of all the 14 males were of the ZZ type. Six of these males had chromosomes

No. 7 which were homozygous in having deeply stained bands on the basal portion of the long arm (Z^AZ^A), while the other eight males had heterozygous chromosomes No. 7 (Z^AZ^O), that is, one of the chromosomes had no such a band (Table 6).

ii) Experimental series

Chromosomes Nos. 2 and 7 were also observed in 1636 mitotic figures at metaphase, 30.3 per male, obtained from 54 males of the experimental series. It was found that chromosomes No. 2 were of the OO type in 11 males, of the AA type in 14 other males, and of the heterozygous AO type in the remaining 29 males. These males showed that the bands at the end portions of the long arms of chromosomes No. 2 had no relation to sex. On the other hand, chromosomes No. 7 of the 54 males were all of the ZZ type ; those of 37 of them were of the Z^AZ^A type, while those of the other 17 were of the Z^AZ^O type (Table 6).

It was found that all the frogs in which chromosomes No. 7 were of the ZZ type were males, regardless of the presence of the bands in chromosomes No. 2. There were no males produced by sex reversal.

TABLE 6

Banding patterns in chromosomes Nos. 2 and 7 of the frogs raised from the testosterone-injected tadpoles in the Hirosaki population of *Rana rugosa*

Sex	Series	No. of frogs	Type of banding patterns		No. of analyzed mitoses	
			Chromosome pair No. 2	Chromosome pair No. 7	Total	Per individual
Male	Cont.	5	OO	Z^AZ^A	156	31.2
		1	AO	Z^AZ^A	44	44.0
		6	AO	Z^AZ^O	157	26.2
		2	AA	Z^AZ^O	20	10.0
	Total	14			377	26.9
	Exp.	11	OO	Z^AZ^A	323	29.4
		15	AO	Z^AZ^A	552	36.8
		11	AA	Z^AZ^A	355	32.3
		14	AO	Z^AZ^O	357	25.5
		3	AA	Z^AZ^O	49	16.3
	Total	54			1636	30.3
Female	Cont.	2	AO	Z^AW	29	14.5
	Exp.	11	OO	Z^AW	220	20.0
		19(1)	AO	Z^AW	414	21.8
		5(1)	AA	Z^AW	93	18.6
		5	AO	Z^OW	90	18.0
		1	AA	Z^OW	6	6.0
	Total	41(2)			823	20.1

Parentheses show the frogs with ill-developed gonads.

b. Females raised from tadpoles injected with testosterone propionate

i) Control series

When chromosomes Nos. 2 and 7 were observed in 29 mitotic figures at metaphase, 14.5 per female, obtained from two females of the control series, it was found that chromosomes No. 2 were all of the AO type, and chromosomes No. 7 were all of the Z^AW type (Table 6).

ii) Experimental series

Chromosomes Nos. 2 and 7 were observed in 823 mitotic figures at metaphase, 20.1 per female, obtained from 41 females. In chromosomes No. 2, 11, 24 and six of these females were of the OO, AO and AA type, respectively, while in chromosomes No. 7, all the 41 females were of the ZW type. Of these females, 35 were of the Z^AW type, although two of them had poorly developed ovaries. The remaining six females were of the Z^OW type (Table 6). It was found that all the frogs, in which chromosome pair No. 7 were of the ZW type, were females and that there were no frogs whose sex was reversed (Table 6).

III. Matings between the Kumano and Hirosaki populations

1. Females of the Kumano (K) population and males of the Hirosaki (H) population

a. Development and viability

i) Control series, Kumano (K) ♀ × Kumano (K) ♂

In 1987, six matings were made between six females of the Kumano population including four (Kw♀, Nos. 2~5) collected from the field in 1987 and two (Kc5♀, Nos. 6 and 9) obtained from a mating (K♀, No. 3 × K♂, No. 5) in 1986, and four males of the Kumano population including one (Kc3♂, No. 2) produced from a mating (K♀, No. 1 × K♂, No. 3) and three (Kc5♂, Nos. 3~5) obtained from a mating (K♀, No. 3 × K♂, No. 5). The results showed that 52.2~95.5% of the 127~306 eggs in the six mating series, 1071 (82.8%) of the 1293 eggs in total, cleaved normally. Thereafter, 36.6~86.9% of the respective numbers of eggs, 839 eggs (64.9%) in total, became normally hatched tadpoles, and 34.8~73.8% of the respective numbers of eggs, 585 tadpoles (45.2%) in total, normally completed metamorphosis. This number corresponded to 54.6% of the normally cleaved eggs (Table 7).

ii) Experimental series, Kumano (K) ♀ × Hirosaki (H) ♂

In 1987, six matings were made between the same six females of the Kumano (K) population as those in the control series and four males (Hc♂, Nos. 1~4) of the Hirosaki (H) population which were raised from egg masses in 1986. It was found that 61.1~92.9% of 224~458 eggs in the six mating series, 1449 (76.6%) of the 1892 eggs in total, cleaved normally. Thereafter, 50.4~89.3% of the respective numbers of eggs, 1189 eggs (62.8%) in total, became normally hatched

tadpoles, and 37.2~53.0% of the respective numbers of eggs, 852 tadpoles (45.0%) in total, normally completed metamorphosis. This number corresponded to 58.8% of the normally cleaved eggs (Table 7).

b. Sex ratio of the offspring

i) Control series, Kumano (K) ♀ × Kumano (K) ♂

Of the 585 metamorphosed frogs raised from the six control series, 566 developed normally until their sex could be clearly identified. It was found that 277 of them were females and 289 were males. In the six series, the percentages of males were 43.8~60.5%, 51.1% on the average. There were no remarkable differences between the numbers of females and males (Table 7).

ii) Experimental series, Kumano (K) ♀ × Hirosaki (H) ♂

Of the 852 metamorphosed frogs raised from six mating series, 773 developed normally until their sex could be clearly identified. It was found that 39 were females and 734 (95.0%) were males. In each of the six series, 85.7~100% of the

TABLE 7

Sex ratio of the hybrids between the Kumano (K) and Hirosaki (H) populations in *Rana rugosa*

Series	Parents		No. of eggs	No. of normal cleavages (%)	No. of normal tail-bud embryos (%)	No. of normally hatched tadpoles (%)	No. of metamorphosed frogs (%)	No. of analyzed frogs		
	Female	Male						Total	♀	♂ (%)
Cont.	Kw, No. 2	Kc3, No. 2	224	117 (52.2)	84 (37.5)	82 (36.6)	78 (34.8)	76	30	46 (60.5)
	Kw, No. 3	Kc3, No. 2	127	97 (76.4)	90 (70.9)	88 (69.3)	74 (58.3)	73	37	36 (49.3)
	Kw, No. 4	Kc5, No. 3	145	131 (90.3)	128 (88.3)	126 (86.9)	107 (73.8)	105	59	46 (43.8)
	Kw, No. 5	Kc5, No. 3	200	191 (95.5)	158 (79.0)	156 (78.0)	111 (55.5)	103	46	57 (55.3)
	Kc5, No. 6	Kc5, No. 4	306	258 (84.3)	189 (61.8)	165 (53.9)	111 (36.3)	111	54	57 (51.4)
	Kc5, No. 9	Kc5, No. 5	291	277 (95.2)	272 (93.5)	222 (76.3)	104 (35.7)	98	51	47 (48.0)
	Total		1293	1071 (82.8)	921 (71.2)	839 (64.9)	585 (45.2)	566	277	289 (51.1)
Exp.	Kw, No. 2	Hc, No. 1	397	324 (81.6)	214 (53.9)	203 (51.1)	156 (39.3)	133	19	114 (85.7)
	Kw, No. 3	Hc, No. 1	289	255 (88.2)	208 (72.0)	207 (71.6)	149 (51.6)	116	0	116 (100.0)
	Kw, No. 4	Hc, No. 2	298	219 (74.5)	198 (66.4)	198 (66.4)	158 (53.0)	151	5	146 (96.7)
	Kw, No. 5	Hc, No. 2	226	138 (61.1)	115 (50.9)	114 (50.4)	84 (37.2)	78	4	74 (94.9)
	Kc5, No. 6	Hc, No. 3	458	305 (66.6)	290 (63.3)	267 (58.3)	213 (46.5)	204	11	193 (94.6)
	Kc5, No. 9	Hc, No. 4	224	208 (92.9)	200 (89.3)	200 (89.3)	92 (41.1)	91	0	91 (100.0)
	Total		1892	1449 (76.6)	1225 (64.7)	1189 (62.8)	852 (45.0)	773	39	734 (95.0)
Cont.	Hc, No. 1	Hc, No. 5	295	167 (56.6)	165 (55.9)	159 (53.9)	96 (32.5)	73	43	30 (41.1)
	Hc, No. 2	Hc, No. 6	440	304 (69.1)	161 (36.6)	142 (32.3)	105 (23.9)	81	42	39 (48.1)
	Total		735	471 (64.1)	326 (44.4)	301 (41.0)	201 (27.3)	154	85	69 (44.8)
Exp.	Hc, No. 1	Kc2, No. 6	581	437 (75.2)	330 (56.8)	270 (46.5)	223 (38.4)	186	100	86 (46.2)
	Hc, No. 2	Kc5, No. 7	711	358 (50.4)	198 (27.8)	191 (26.9)	134 (18.8)	121	52	69 (57.0)
	Hw, No. 3	Kw, No. 8	338	112 (33.1)	80 (23.7)	80 (23.7)	64 (18.9)	48	21	27 (56.3)
	Total		1630	907 (55.6)	608 (37.3)	541 (33.2)	421 (25.8)	355	173	182 (51.3)

w, field caught c, control series

respective numbers of frogs were males (Table 7). The offspring between the females (XX) of the Kumano (K) population and the males (ZZ) of the Hirosaki (H) population should have XZ chromosomes in chromosome pair No. 7. When the chromosomes of the offspring were examined, one chromosome of chromosome pair No. 7 was Z chromosome. Thus, almost all the frogs having a pair of XZ chromosomes were males (Table 7).

2. Females of the Hirosaki (H) population and males of the Kumano (K) population

a. Development and viability

i) Control series, Hirosaki (H) ♀ × Hirosaki (H) ♂

In 1987, control matings were made between two females (Hc ♀, Nos. 1 and 2) and two males (Hc ♂, Nos. 5 and 6) which were raised from four egg masses collected from the field in 1986. It was found that 471 (64.1%) of the 735 eggs cleaved normally, and 301 (41.0%) became normally hatched tadpoles. Eventually, 201 tadpoles (27.3%) normally completed metamorphosis. This number corresponded to 42.7% of the normally cleaved eggs (Table 7).

ii) Experimental series, Hirosaki (H) ♀ × Kumano (K) ♂

In 1987 and 1988, three matings were made between three females of the Hirosaki (H) population including the same two (Hc ♀, Nos. 1 and 2) as those used in the control matings in 1987 and one (Hw ♀, No. 3) collected from the field in 1988, and three males of the Kumano (K) population including one (Kc2 ♂, No. 6) obtained from a mating (K ♀, No. 1 × K ♂, No. 2) in 1986, one (Kc5 ♂, No. 7) obtained from a mating (K ♀, No. 3 × K ♂, No. 5) in 1986 and one (Kw ♂, No. 8) collected from the field in 1988. It was found that 907 (55.6%) of the 1630 eggs cleaved normally. When these eggs were reared, 421 eggs (25.8%) attained the completion of metamorphosis. This number corresponded to 46.4% of the normally cleaved eggs.

b. Sex ratio of the offspring

i) Control series, Hirosaki (H) ♀ × Hirosaki (H) ♂

Of the 201 metamorphosed frogs, 154 were reared until their sex could be clearly identified. It was found that 85 were females and 69 (44.8%) were males. Thus, there was no remarkable difference between the numbers of females and males. The control matings (ZW ♀ × ZZ ♂) produced offspring ZZ ♂ and ZW ♀ in the ratio of 1 : 1.

ii) Experimental series, Hirosaki (H) ♀ × Kumano (K) ♂

Of the 421 metamorphosed frogs, 355 were reared until their sex could be clearly identified. It was found that 173 were females and 182 (51.3%) were males. Thus, the sex ratio was about 1 : 1 (Table 7).

The matings, ZW ♀ × XY ♂, will produce four kinds of offspring which are ZX, ZY, WX and WY in sex-chromosome constitution. Of these offspring, WX and

ZY should become females and males, respectively, as these two kinds of offspring have no chromosomes which would lead them to the opposite sex. The ZX individuals should be males, as the mating XX♀×ZZ♂ produced almost males. The remaining WY individuals should be females, as the four kinds of offspring were 1 : 1 in sex ratio as a whole.

From each of the three matings between females of the Hirosaki (H) population and males of the Kumano (K) population (Hc♀, No. 1×Kc2♂, No. 6, Hc♀, No. 2×Kc5♂, No. 7 and Hw♀, No. 3×Kw♂, No. 8) 21, 19 and 12 females, 52 females in total, and 21, 20 and 20 males, 61 males in total, were obtained. The chromosomes of these females and males were analyzed by three kinds of methods, conventional Giemsa staining, C-banding and LR-banding, in chromosome preparations obtained from the blood cell cultures. In chromosome pair No. 7, the W chromosomes could be completely distinguished from the Z, X and Y chromosomes by the method of conventional Giemsa staining, as they were of the metacentric type in contrast to the Z, X and Y chromosomes which were of the subtelocentric or submetacentric type. Besides, the Z chromosomes could be distinguished from the X or Y chromosomes by the C-banding or LR-banding method, as they had deeply stained bands at the basal portion of the long arm. On the other hand, the X chromosome was not distinguished from the Y chromosome by any of the three kinds of staining methods. However, it was found that 60 of the 61 males had Z chromosomes, while the remainder had a W chromosome. Of the 52 females, 51 had W chromosomes, while the remainder was a triploid having two W chromosomes (Table 8). The observation of the chromosomes showed that each of the three female parents, Hc♀, No. 1, Hc♀, No. 2 and Hw♀, No. 3, had chromosome pair No. 7 of the Z^AW type, in which the Z chromosome had a deeply stained band at the basal portion of the long arm.

It was quite evident that in the offspring of reciprocal hybrids between the Kumano (K) and Hirosaki (H) populations of *Rana rugosa*, almost all the frogs

TABLE 8

Constitution of chromosome pair No. 7 of the hybrids between females of the Hirosaki population (H) and males of the Kumano population (K) in *Rana rugosa*

Parents		Sex	No. of analyzed frogs	Type of chromosome pair No. 7		
Female	Male			ZX or ZY	WX or WY	WWX or WWY
Hc, No. 1	Kc2, No. 6	Female	21	0	20	1
		Male	21	21	0	0
Hc, No. 2	Kc5, No. 7	Female	19	0	19	0
		Male	20	20	0	0
Hw, No. 3	Kw, No. 8	Female	12	0	12	0
		Male	20	19	1	0
Total		Female	52	0	51	1
		Male	61	60	1	0

c, control series w, field caught

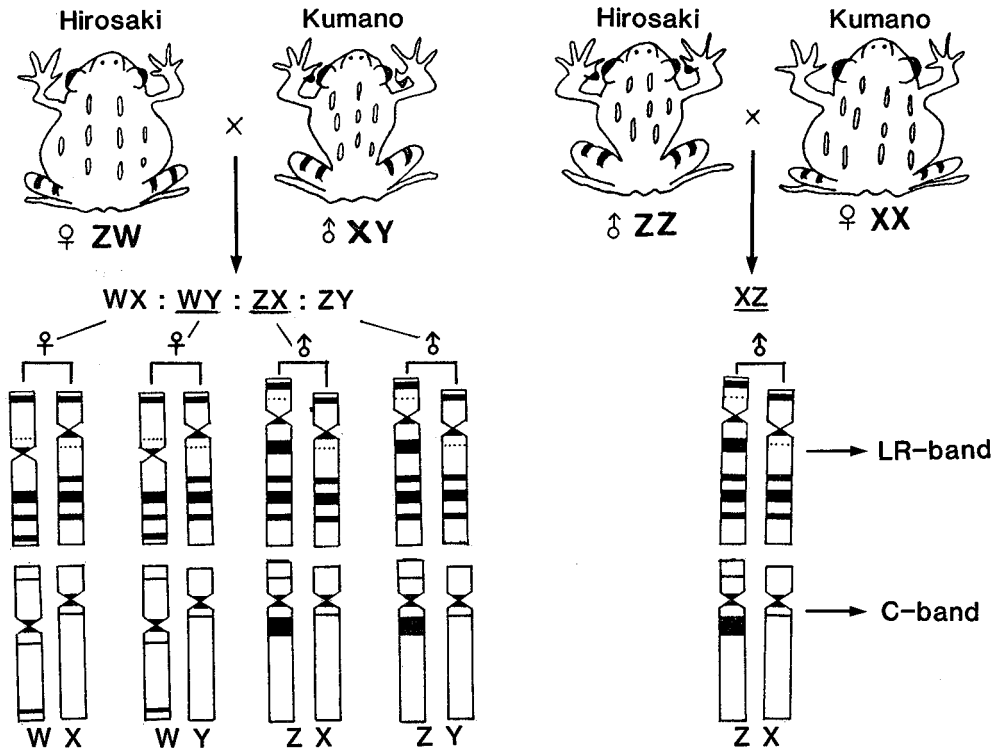

Fig. 7. Sex-chromosome constitution in the offspring from crosses between individuals with different sex-determining systems.

having W chromosomes were females, and all the frogs having Z chromosomes were males, regardless of the existence of the X or Y chromosome (Fig. 7).

DISCUSSION

As for the sex-determining mechanisms in anurans, X and Y chromosomes were first recognized in the germ cells of males by WITSCHI (1922, 1929) in *Rana temporaria*. GALLIEN (1953) and CHANG and WITSCHI (1955, 1956) have reported that *Xenopus laevis* is of the ZW type in sex-determining mechanism on the basis of the breeding of sex-reversed males. YOSIDA (1957) observed a pair of two unequal chromosomes in mitotic figures of male *Hyla japonica* and considered that they were X and Y chromosomes. This finding was confirmed by MATSUDA (1963) in cultured kidney cells. Heteromorphism of sex chromosomes was reported by WEILER and OHNO (1962) in *Xenopus laevis*. In this species, females were heterozygous (ZW) with the Z chromosome being acrocentric and the W chromosome being metacentric. KAWAMURA and NISHIOKA (1977) reported that six species of *Rana* including *R. rugosa, Hyla japonica* collected from Japan and *Bombina orientalis* from Korea were all of the XY type on the basis of the sex of gynogenetically produced diploid frogs.

SCHMID (1980) and SCHMID and BACHMANN (1981) observed the sex chromosomes of the ZW type in *Pyxicephalus adspersus*. In this species, chromosome pair No. 8 was the sex chromosomes and the W chromosome was considerably smaller than the Z chromosome. SCHEMPP and SCHMID (1981) ascertained that chromosome pair No. 4 of *Rana esculenta* was the sex chromosomes of the XY type, by observing that all males exhibited a very late replicating region in the Y chromosome. ITURRA and VELOSO (1981, 1989) reported that *Eupsophus migueli* and *E. roseus,* two species of small Chilean frogs, were of the XY type, and chromosome pair No. 14 was sex chromosomes. The X chromosome of *E. migueli* was telocentric, while that of *E. roseus* was metacentric, although the X and Y chromosomes were of the same size and the Y chromosomes of both species were metacentric. SCHMID (1983), SCHMID, HAAF, GEILE and SIMS (1983) and SCHMID, SIMS, HAAF and MACGREGOR (1986) confirmed in *Gastrotheca riobambae* that chromosome pair No. 4 was sex chromosomes of the XY type. In the karyotypes of males, the Y chromosome was evidently larger than the X chromosome. The sex chromosomes of the XY type were also observed in *Gastrotheca walkeri* and *G. ovifera* by SCHMID, STEINLEIN, FEICHTINGER, DE ALMEIDA and DUELLMAN (1988). In *G. walkeri*, chromosome pair No. 2 was sex chromosomes consisting of a large X chromosome and a small Y chromosome, while in *G. ovifera* chromosome pair No. 1 was sex chromosomes of the XY type. OHTA (1986) reported that chromosome pair No. 7 in the female of *Buergeria buergeri* was the sex chromosomes of the ZW type. While the somatic cells of female tadpoles had a single nucleolus, those of male tadpoles had two nucleoli. The nucleolus seemed to be derived from the nucleolar organizer of the Z chromosome. SCHMID, STEINLEIN and FEICHTINGER (1989) observed that the South American tree-frog, *Centrolenella antisthenesi,* was of the XY type in sex-determining mechanism. Chromosome pair No. 6 was constructed of the X and Y chromosomes in the male. These sex chromosomes seemed to be in the initial stage of differentiation.

In urodeles, HUMPHREY (1942, 1945, 1957) first reported that *Ambystoma mexicanum* and *A. tigrinum* were of the ZW type in sex-determining mechanism on the basis of the sex of the progeny of sex-reversed animals. GALLIEN (1951, 1954a, b) also demonstrated male homogamety in *Pleurodeles waltl* by obtaining offspring from sex-reversed genetic males produced by treatment with estradiol benzoate during the larval period. LACROIX (1968, 1970) also reported the ZW type of lampbrush chromosomes in oocyte nuclei of *Pleurodeles poireti*. In contrast, MANCINO (1965) and KEZER and MACGREGOR (1971) found chromosomes of the XY type in *Euproctus platycephalus* and *Plethodon cinereus*. In some salamandrid species, male heterogamety was also interpreted by cytological observations. However, MANCINO, RAGGHIANTI and BUCCI-INNOCENTI (1977) have stated that it is necessary to perform genetic tests for ascertaining the type of digamety as done in *Ambystoma mexicanum* by HUMPHREY (1945) and in *Pleurodeles waltl* by GALLIEN (1951).

SCHMID, OLERT and KLETT (1979) determined sex chromosomes in the karyotypes of *Triturus a. alpestris* (Chromosomes No. 4) and *T. v. vulgaris* (Chromo-

somes No. 5). The male of each species had one heteromorphic chromosome pair, of which one homologue had telomeric heterochromatin in the long arm. While chromosomes No. 5 of *T. h. helveticus* manifested identical morphology and C-band patterns in both sexes, there was a structural change in one of the two chromosomes No. 5, which led to an extreme reduction of the chiasma-frequency between the long arms in the male meiosis. Chromosomes of the XY type were also observed by SESSIONS (1980) in *Necturus maculosus*, and by SCHMID (1983) in *Triturus marmoratus* and *T. cristatus*.

In the present study, an interesting phenomenon on the sex-determining mechanism of *Rana rugosa* was observed. When the tadpoles of the Kumano population were injected with testosterone propionate, nearly all of the females were changed into males. When the sex-reversed females were mated with normal females, the offspring became almost all females. Such a result seemed to show that the Kumano population of *R. rugosa* is of the XY type in sex determination. In contrast, when the tadpoles of the Hirosaki population of *R. rugosa* were injected with testosterone propionate and reared for several months over metamorphosis, there were no large differences between the numbers of females and males. It was found that there were no frogs whose sex was reversed.

When the karyotypes of the Kumano population of *R. rugosa* were compared with those of the Hirosaki population of the same species by the methods of conventional Giemsa staining, C-banding and LR (late replication)-banding, it was found that the 13 chromosome pairs were completely homozygous and there were no chromosome pairs which had any sexual differences in the Kumano population, while chromosome pair No. 7 of the female in the Hirosaki population was heteromorphic and constructed of a subtelocentric (Z) and a metacentric (W) chromosome. Thus, it was confirmed that the Hirosaki population completely differed from the Kumano population in sex-determining mechanism.

When females (XX) of the Kumano population were mated with males (ZZ) of the Hirosaki population, almost all the offspring (XZ) were males, while the offspring (WX, WY, ZX, ZY) between females (ZW) of the Hirosaki population and males (XY) of the Kumano population consisted of nearly the same number of females and males. It was evident that almost all the frogs having W chromosomes and all the frogs having Z chromosomes were females and males, respectively, regardless of the existence of X or Y chromosome.

Hirosaki is situated at the northern end of Honshu of Japan, while Kumano is situated near the southwestern end. Kumano and Hirosaki are about 1120 km apart from each other. A rough survey performed by NISHIOKA, MIURA and HANADA (1991) showed that the populations of the XY type are mainly distributed in the southern part of Japan including the Kyushu, Chugoku and Tokai regions, while those of the ZW type are mainly distributed in the northern part of Japan including the Hokkaido, Tohoku and Hokuriku districts. While the Y chromosomes were not morphologically distinguishable from the X chromosomes in the populations of the Kyushu and Chugoku regions, they differed from the X chromosomes in shape and banding pattern in some populations of the Tokai

region and their vicinity.

A report on intraspecific differentiation of *R. rugosa* elucidated by electrophoretic analyses was published by NISHIOKA, KODAMA, SUMIDA and RYUZAKI (1993). The genetic distance between the Hirosaki and Kumano populations was 0.350, while those among the 40 populations distributed widely in Japan were 0.003~0.492. When a dendrogram was drawn from the genetic distances among these populations by the UPGMA method, it was assumed that the ancestor of *R. rugosa* was divided into the western group of the XY type and the eastern group which was divided again into three subgroups, the northern, intermediate and southern subgroups. Roughly speaking, the northern subgroup contained frogs of the ZW type, the intermediate subgroup contained frogs in which the sex-determining mechanism was obscure and sex chromosomes were not morphologically distinguishable, and the southern subgroup contained frogs of the XY type, in which the Y chromosome differed from the X chromosome in shape.

It now seems to be important to clarify the sex-determining mechanisms of the frogs belonging to the intermediate subgroup. All these frogs are distributed in and around the Kanto district, which is the central part of Japan. It may be probable that the sex-determining mechanism of the ZW type found in the northern part of Japan was derived from the XY type found in the southern part of Japan, passing through some intermediate stage of the two types.

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