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Four Kinds of Sex Chromosomes in *Rana rugosa*

By

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ABSTRACT

The sex chromosomes of *Rana rugosa* distributed widely in Japan were analyzed by the methods of conventional staining, C-banding and late replication (LR)-banding on 196 frogs consisting of 105 females and 91 males belonging to 24 populations of one group and three subgroups. The chromosome numbers of these frogs were all of $2n=26$. The 12 pairs other than chromosome pair No. 7 had no sex differences in all the populations. In chromosome pair No. 7, sex-specific changes were found among some local populations.

Seven populations belonging to the northern subgroup of the eastern group, including the Asahikawa and Sapporo populations in Hokkaido region, the Hirosaki, Akita and Inawashiro populations in Tohoku region, the Murakami and Kanazawa populations in Hokuriku region and the Katata population in Kinki region of the southern subgroup, had chromosome pair No. 7 which was the sex chromosomes of the ZW type. The Z chromosome was subtelo- or submetacentric, while the W chromosome was metacentric. By the C-banding and LR-banding patterns, the Z chromosome was divided into five types, Z^A, Z^B, Z^C, Z^D and Z^O, while the W chromosome was divided into two types, W^1 and W^2.

Five populations of the southern subgroup of the eastern group, including the Toba population in Kinki region, and the Oigawa, Hamakita, Miyakoda and Yonezu populations in Chubu region, had chromosome pair No. 7 which was the sex chromosomes of the XY type. The X chromosome was metacentric and the Y chromosome was subtelo- or submetacentric. By the C-banding and LR-banding patterns, the Y chromosome was divided into two types, Y^A and Y^B, while the X chromosome was similar to the W^1 chromosome of the northern subgroup.

Seven populations of the intermediate subgroup of the eastern group, including the Daigo, Hitachiota, Ashikaga, Maebashi, Machida, Kamogawa and Isehara populations in Kanto region, had no sex differences in all the 13 chromosome pairs. All these populations were obscure in sex-determining mechanism. Chromosome pair No. 7 consisted of homologous subtelocentric chromosomes (7^C) in both males and females. Chromosome No. 7 was of 7^C type which was considered by the LR-banding pattern to have been produced from the 7^B type chromosome of the western group by a pericentric inversion. On the other hand, the X chromosome in the southern subgroup and the W^1 chromosome in the northern subgroup were considered to have been produced from 7^C type chromosome of this intermediate subgroup by a pericentric

inversion.

Four populations of the western group, including the Okayama, Kumano and Gotsu populations in Chugoku region and the Nagayo population in Kyushu region, had no sex differences in all the 13 chromosome pairs. However, this group was evidently of the XY type in sex-determining mechanism on the basis of the results of breeding experiments using males sex-reversed from genetic females. Chromosome pair No. 7 consisted of homozygous submeta- or subtelocentric chromosomes in both males and females. Chromosome No. 7 was divided into two types, 7^A and 7^B, by the C-banding and LR-banding patterns. The 7^A and 7^B type chromosomes were very similar to the Y^A and Y^B type chromosomes, respectively, in the southern subgroup.

INTRODUCTION

Rana rugosa SCHLEGEL is a comparatively small dark brown frog widely distributed in Japan, Korea and the northeastern part of China. IRIKI (1928, 1932) reported that the chromosome number of this species was 2n=26. Seto (1965) could not distinguish sex chromosomes from among 13 pairs. KAWAMURA and NISHIOKA (1977) and KASHIWAGI (1993) reported that *R. rugosa* distributed in the neighborhood of Hiroshima city is of the XX-XY type in sex-determining mechanism and of the male heterogamety, although the sex chromosomes were not yet distinguished. TOBISHIMA and SAITOH (1989) reported that *R. rugosa* collected from Aomori and Iwate Prefectures was of the female heterogamety, that is, the chromosome No. 7 of the female was heterozygous, when they examined the chromosomes by the conventional staining and C-banding methods. NISHIOKA, MIURA and SAITOH (1993) examined the differences between the population of *R. rugosa* collected from Hirosaki city, Aomori Prefecture, and that collected from Kumano-cho, Hiroshima Prefecture, by the method of hormone-induced sex-reversal and the analyses of the chromosomes of reciprocal hybrids between the two populations. In the Kumano population, the male was heterozygous and XX-XY type in sex-determining mechanism, although the sex chromosome was not distinguished, while in the Hirosaki population, the female was heterozygous and chromosome pair No. 7 was sex chromosomes. It was evident that the sex-determining mechanism of the Hirosaki population was ZW-ZZ type. NISHIOKA, KODAMA, SUMIDA and RYUZAKI (1993) calculated genetic distances among 25 loci controlling enzymes and blood proteins in 40 populations from gene frequencies by the method of NEI and then drew a phylogenetic tree by the UPGMA method. This phylogenetic tree clarified that this species was first differentiated into 14 populations of the western group and 26 populations of the eastern group. The eastern group was next differentiated into three subgroups, the northern, southern and intermediate subgroups.

In the present study, the authors will clarify the morphological differences in sex chromosomes of *R. rugosa* belonging to the western group and the northern, southern and intermediate subgroups of the eastern group. A preliminary report of this study was presented by NISHIOKA, MIURA and HANADA in 1991.

MATERIALS AND METHODS

Rana rugosa SCHLEGEL used as materials of this study belonged to 24 populations collected from Hokkaido, Tohoku, Hokuriku, Kinki, Chubu, Kanto, Chugoku and Kyushu regions, and contained 196 individuals consisting of 105 females and 91 males (Table 1). Of these materials, the Hirosaki population of the Tohoku region and the Kumano population of the Chugoku region have been previously reported by NISHIOKA, MIURA and SAITOH (1993).

TABLE 1
Collecting stations and the number of frogs examined in the present study

Group		Region	Prefecture	Station	Population	No. of frogs		
						Total	♀	♂
Eastern group	Northern subgroup	Hokkaido	Hokkaido	Asahikawa-city	Asahikawa	5	5	0
				Sapporo-city	Sapporo	3	3	0
		Tohoku	Aomori	Hirosaki-city	Hirosaki*	27	12	15
			Akita	Akita-city, Toyoiwashidazaka	Akita	4	2	2
			Fukushima	Yama-gun, Inawashiro-cho	Inawashiro	7	3	4
		Hokuriku	Niigata	Murakami-city, Hayakawa	Murakami	23	13	10
			Ishikawa	Kanazawa-city	Kanazawa	5	2	3
	Southern subgroup	Kinki	Shiga	Otsu-city, Katata-cho	Katata	3	3	0
			Mie	Toba-city	Toba	4	3	1
		Chubu	Shizuoka	Shita-gun, Oigawa-cho	Oigawa	8	5	3
				Hamakita-city, Kifune	Hamakita	26	12	14
				Hamamatsu-city, Miyakoda-cho	Miyakoda	5	2	3
				Hamamatsu-city, Yonezu	Yonezu	10	4	6
	Inter-mediate subgroup	Kanto	Ibaraki	Kuji-gun, Daigo-cho	Daigo	8	6	2
				Hitachiota-city	Hitachiota	6	5	1
			Tochigi	Ashikaga-city	Ashikaga	2	1	1
			Gunma	Maebashi-city, Kamikoide-cho	Maebashi	2	1	1
			Tokyo	Machida-city, Koyamadai	Machida	3	2	1
			Chiba	Kamogawa-city	Kamogawa	9	4	5
			Kanagawa	Isehara-city, Isehara	Isehara	8	4	4
Western group		Chugoku	Okayama	Okayama-city, Mitsu-cho	Okayama	4	2	2
			Hiroshima	Aki-gun, Kumano-cho	Kumano*	11	5	6
			Shimane	Gotsu-city, Arifuku-cho	Gotsu	7	3	4
		Kyushu	Nagasaki	Nishihigaki-gun, Nagayo-cho	Nagayo	6	3	3
Total		8	19		24	196	105	91

* These frogs were reported by NISHIOKA, MIURA and SAITOH (1993).

1. Conventional GIEMSA staining method

Mitotic figures were obtained by the method of blood cell culture. The culture fluid was prepared 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. Venous blood of 0.1~0.2 ml was collected with a glass pipette which contained 0.01~0.02 ml of heparin solution (10 mg/ml RPMI 1640). To 2 ml of the foregoing culture fluid, 0.1~0.2 ml venous blood was added and cultured for 3~5 days at 25°C. Chromosome preparations were produced 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).

2. C-banding method

The C-bands were obtained by SUMNER's method (1972) with slight modifications. The chromosome preparations which were air-dried for one day were treated for 40 minutes in 0.2 N HCl at room temperature. After rinsing in distilled water, the preparations were incubated in 5% $Ba(OH)_2$ solution at 35°C for 5~10 minutes. After rinsing again 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 rinsing 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).

3. Late replication (LR)-banding method

The late replication (LR) bands were obtained by the method of TAKAYAMA, TANIGUCHI and IWASHITA (1981) with slight modifications. 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 cell 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 made by the method of NISHIOKA (1972) and NISHIOKA, OKUMOTO and RYUZAKI (1987).

OBSERVATION

Chromosome preparations of 196 frogs consisting of 105 females and 91 males collected from 24 populations of eight regions, Hokkaido, Tohoku, Hokuriku, Kinki, Chubu, Kanto, Chugoku and Kyushu regions, were made and analyzed by the methods of conventional GIEMSA staining, C-banding and LR (late replication)-banding. The chromosome numbers of these frogs were all 2n=26, consisting of five pairs of large chromosomes and eight pairs of small chromosomes. Of these 13 pairs, each of the 12 pairs other than chromosome pair No. 7 consisted of

almost homologous chromosomes. In chromosome pair No. 7, there were four kinds of sex-specific changes among the local populations. The chromosome pair No. 7 in the females of the first kind of populations was heterozygous, while in the males it was homozygous. These populations were of the ZZ-ZW type in sex-determining mechanism. In the second kind of populations, the chromosome pair No. 7 in males was heterozygous and that in females was homozygous. Such populations were of the XX-XY type in which the X and Y chromosomes were differentiated from each other. In the third kind of populations, the sex chromosome was not distinguished and the sex-determining mechanism was quite obscure. There was the fourth kind of populations, which was XX-XY type in sex-determining mechanism, although the sex chromosomes could not be identified. Thus, there were four kinds of populations in sex chromosomes among the 24 local populations of *R. rugosa*.

I. First kind of populations (northern subgroup), which was ZZ-ZW type in sex-determining mechanism

1. Conventional GIEMSA staining

This kind consisted of eight populations, the Asahikawa and Sapporo populations of the Hokkaido region, the Hirosaki, Akita and Inawashiro populations of the Tohoku region and the Murakami and Kanazawa populations of the Hokuriku region belonging to the northern subgroup and the Katata population of the Kinki region belonging to the southern subgroup of *R. rugosa*. Observations of chromosomes were made on 286 mitotic figures obtained from 43 females and on 144 mitotic figures obtained from 34 males by conventional GIEMSA staining method. In each population, all the 13 chromosome pairs in males were homomorphic, while in females, chromosome pair No. 7 was heteromorphic and the other 12 pairs were all homomorphic (Fig. 1). Exceptionally, one of the three females in the Inawashiro population had all 13 homomorphic chromosome pairs.

The chromosome pair No. 7 in the female was a heteromorphic pair (ZW) composed of a subtelocentric (st) or submetacentric (sm) chromosome and a metacentric chromosome, while the chromosome pair No. 7 in the male was a homomorphic pair (ZZ) and was always composed of subtelocentric or submetacentric chromosomes. In the Murakami population, the numerical value of centromere position (NVC) of the Z chromosomes of males was 26.87~36.00, 31.26 on the average, while the NVC of the Z chromosomes of females was 27.09~36.89, 30.77 on the average. The W chromosome was divided into W^1 and W^2 chromosomes by a difference in the C-band pattern. The NVC of the W^1 chromosome was 39.13~48.65, 43.40 on the average, while the NVC of the W^2 chromosome was 33.33~44.33, 38.33 on the average (Table 2). The foregoing one female in the Inawashiro population, which had 13 homomorphic chromosome pairs, had a homozygous pair (ZZ) of submetacentric chromosomes.

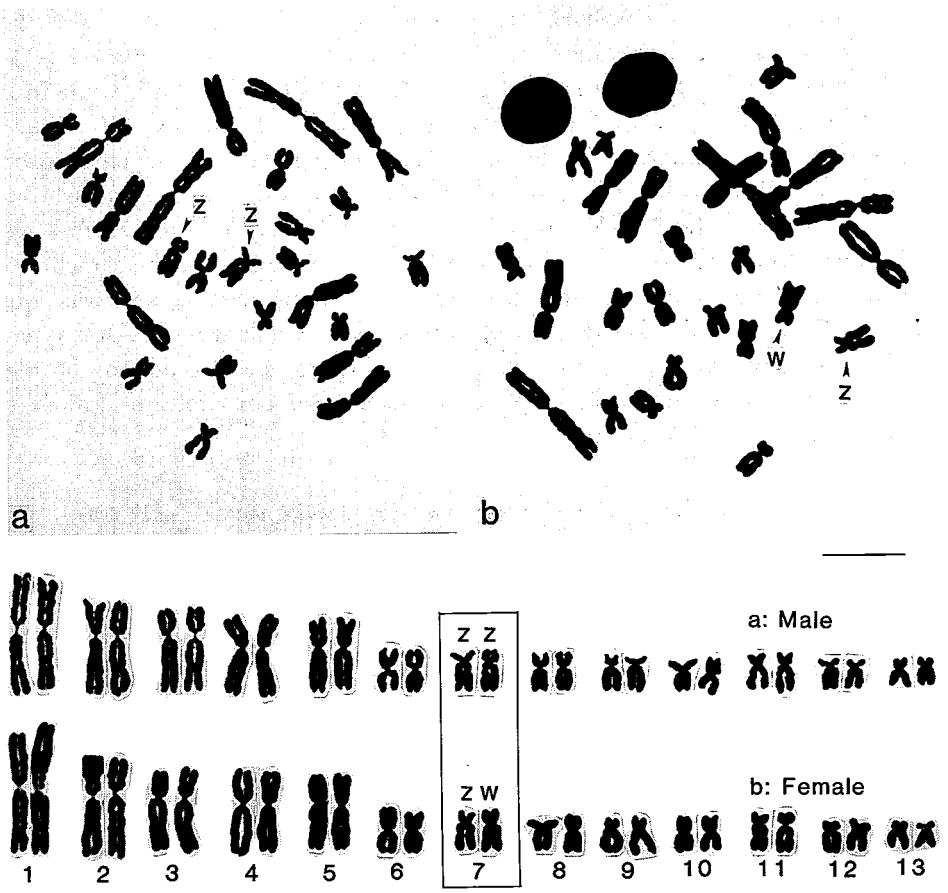

Fig. 1. Metaphase plates and karyotypes of *Rana rugosa* of the Murakami population, stained by the conventional GIEMSA staining method. Bar represents 10 μm.

2. C-band pattern

The C-band patterns were observed on 491 mitotic figures obtained from 43 females and on 295 mitotic figures obtained from 34 males of the foregoing eight populations. In all the populations, C-bands were observed at the centromere portions of the 13 pairs of chromosomes and at the basal, middle or distal portions of the long arms, or on the short arms of some chromosomes. In the 12 chromosome pairs other than chromosome pair No. 7, no sex difference was found in C-band pattern. In chromosome pair No. 7, there were five types of C-band patterns, Z^A, Z^B, Z^C, Z^D and Z^O, in the Z chromosome which was subtelo- or submetacentric. In the W chromosome which was metacentric, there were two types of C-band patterns, W^1 and W^2 (Figs. 2, 3).

Z^A type; this type had a wide deeply stained band at the basal portion of the long arm. In some chromosomes, there were weakly stained C-bands at the distal portion of the long arm and at the middle portion of the short arm. The Z chromosome of this type was subtelocentric.

TABLE 2

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

Male (ZZ)

Relative length (RL)				Numerical value of centromere position (NVC)				
Chromosome no.	Minimum	Maximum	Mean±SE	Chromosome no.	Minimum	Maximum	Mean±SE	Type
1	13.82	17.30	15.02±0.18	1	45.52	49.12	47.10±0.22	m
2	11.27	12.97	12.23±0.09	2	34.05	39.77	37.27±0.31	sm
3	10.57	11.95	11.34±0.08	3	30.85	35.84	33.64±0.31	sm
4	10.46	11.77	11.08±0.09	4	38.80	46.20	42.26±0.39	m
5	9.55	10.85	9.94±0.07	5	37.38	43.02	40.68±0.33	m
6	5.52	6.43	5.90±0.05	6	45.01	49.43	47.27±0.29	m
7	5.00	6.11	5.76±0.06	7	26.87	36.00	31.26±0.49	sm
8	4.71	5.55	5.15±0.05	8	34.92	45.03	40.56±0.55	m
9	4.59	5.64	5.14±0.06	9	25.77	33.77	30.02±0.53	sm
10	4.55	5.33	4.84±0.04	10	39.56	46.85	43.65±0.47	m
11*	4.66	5.62	5.16±0.06	11*	25.82	34.93	30.68±0.54	sm
12	3.75	4.96	4.41±0.07	12	25.57	35.77	30.62±0.55	sm
13	3.71	4.51	4.03±0.04	13	29.45	40.11	35.21±0.49	sm

Female (ZW)

Relative length (RL)				Numerical value of centromere position (NVC)				
Chromosome no.	Minimum	Maximum	Mean±SE	Chromosome no.	Minimum	Maximum	Mean±SE	Type
1	13.50	16.16	14.74±0.11	1	44.38	48.82	46.57±0.21	m
2	10.92	12.76	12.04±0.08	2	35.20	40.37	37.64±0.26	m
3	10.63	11.90	11.24±0.06	3	30.57	35.86	33.62±0.23	sm
4	10.49	11.96	11.18±0.07	4	39.86	46.38	42.57±0.31	m
5	9.34	11.05	10.26±0.07	5	36.40	45.59	40.19±0.33	m
6	5.59	6.53	5.98±0.04	6	43.49	50.28	47.80±0.27	m
7 (Z)	4.77	6.40	5.81±0.07	7 (Z)	27.09	36.89	30.77±0.46	sm
7 (W^1)	4.40	5.38	5.07±0.05	7 (W^1)	39.13	48.65	43.40±0.61	m
7 (W^2)	5.00	6.11	5.76±0.06	7 (W^2)	33.33	44.33	38.33±0.70	m
8	4.83	5.85	5.20±0.04	8	36.47	45.23	40.28±0.40	m
9	4.80	5.81	5.20±0.04	9	26.52	36.22	29.73±0.38	sm
10	4.39	5.25	4.90±0.04	10	38.82	48.53	44.13±0.41	m
11*	4.24	5.52	5.01±0.05	11*	26.37	35.34	31.17±0.45	sm
12	4.00	5.22	4.55±0.06	12	25.58	34.11	30.77±0.42	sm
13	3.52	4.39	3.89±0.04	13	31.53	40.47	36.04±0.40	sm

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

$$RL=\frac{\text{Chromosome length}}{\text{Genome size}}\times 100 \qquad NVC=\frac{\text{Short-arm length}}{\text{Chromosome length}}\times 100$$

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

SE: Standard error of the mean

*: secondary constriction

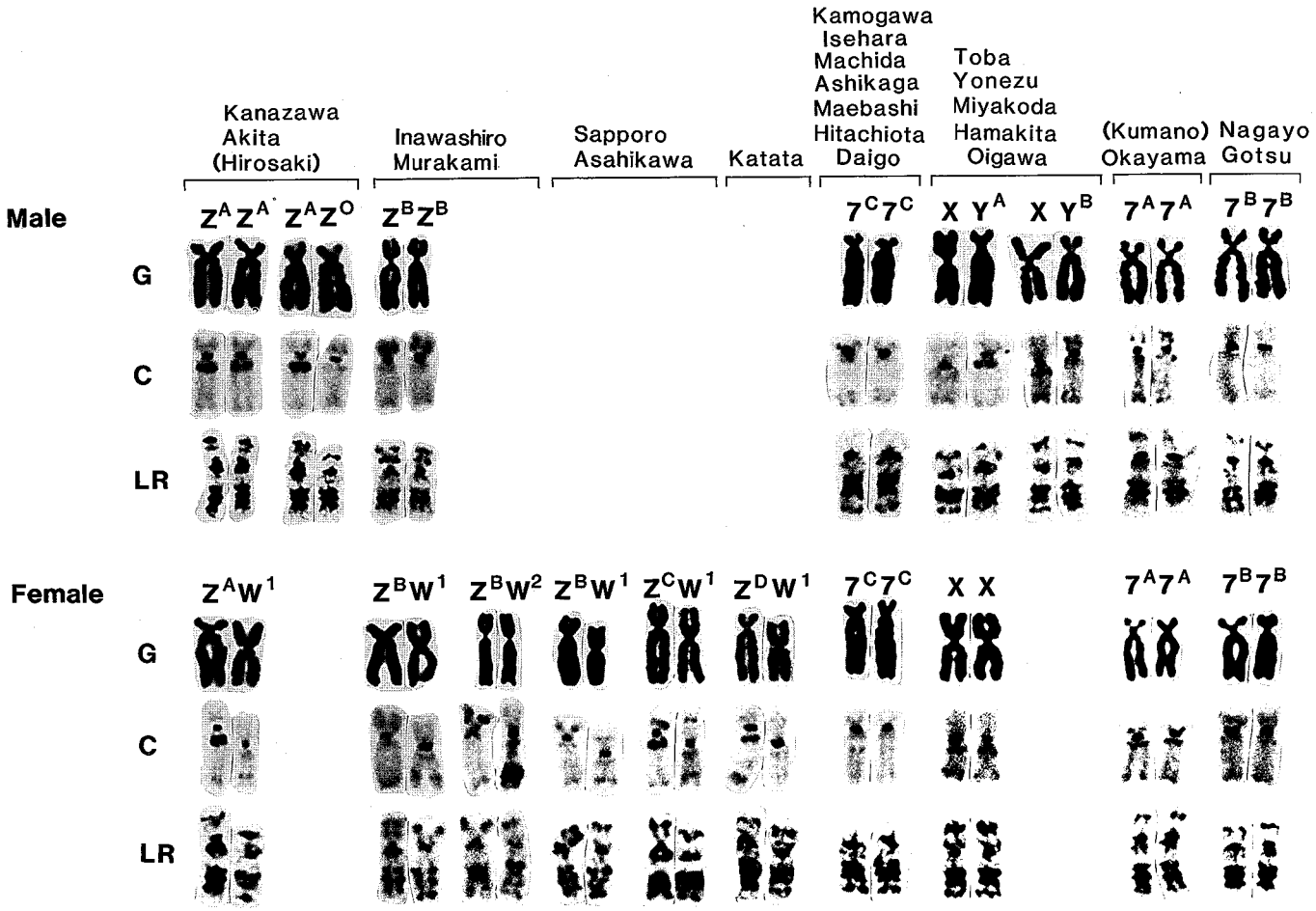

Fig. 2. Comparison of variations in chromosome pair No. 7 from 24 populations of *Rana rugosa*, stained by the conventional GIEMSA staining (G), C-banding (C) and late replication-banding (LR) methods.

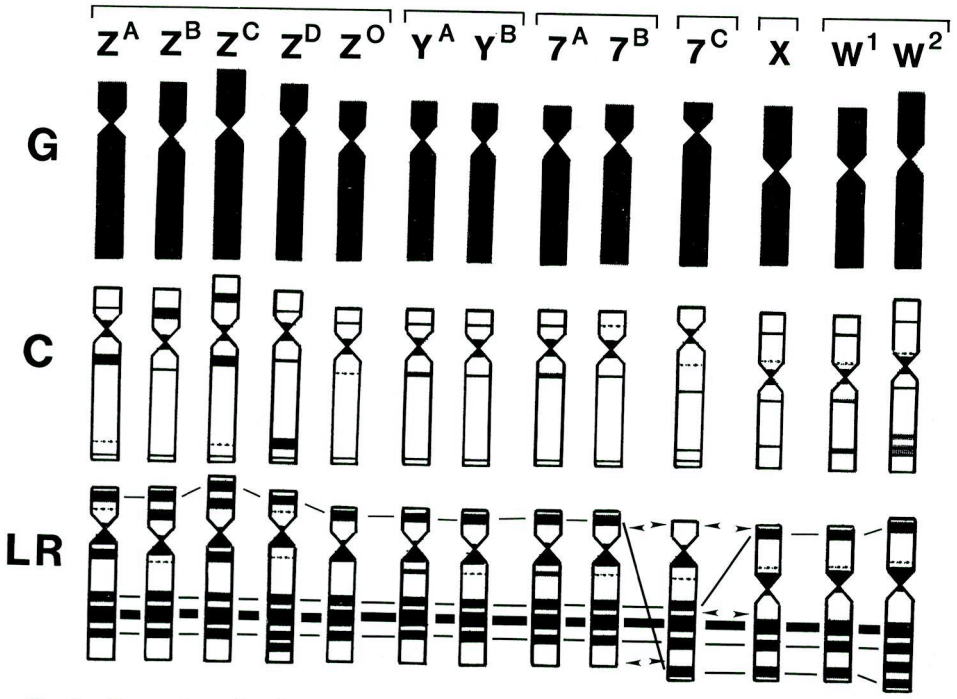

Fig. 3. Comparison of various types of chromosome No. 7. The W^1 and X type chromosomes show to have been produced from 7^C type chromosome by a pericentric inversion. The 7^C type chromosome shows to have been produced from the 7^B type chromosome by a pericentric inversion. Heavy or fine lines show homologous late replication (LR)-bands. Arrowheads indicate the presumed breaking point of inversion. G. Conventional GIEMSA staining. C. C-banding patterns. LR. Late replication-banding patterns.

Z^B type; this type had a wide deeply stained C-band at the middle portion of the short arm. In some chromosomes, there were weakly stained bands at the basal and distal portions of the long arm. The Z chromosome of this type was submetacentric.

Z^C type; this type had wide deeply stained C-bands at the basal portion of the long arm and at the middle portion of the short arm. The Z chromosome of this type was submetacentric and longer than the Z^A and Z^B type chromosomes.

Z^D type; at the basal and distal portions of the long arm, there were two deeply stained C-bands which were narrower than Z^A, Z^B and Z^C type chromosomes. The Z chromosome of this type was subtelocentric.

Z^O type; there were no deeply stained C-bands at other than the portion where the centromere was situated. The Z chromosome of this type was subtelocentric.

W^1 type; there was a weakly stained C-band at each of the basal and distal portions of the long arm and at the distal portion of the short arm. In the chromosome of this type, the ratio of the long and short arms was closer to 1 than that in the chromosome of W^2 type.

W^2 type; there was a wide deeply stained C-band at the distal portion of the

long arm, and there was a weakly stained C-band similar to that of the W^1 type chromosome at the basal portion of the long arm and at the distal portion of the short arm. In the W chromosome of this type, the long arm of the W^2 type was longer than that of the W^1 type chromosome by the length of the wide C-band at the distal portion of the long arm.

TABLE 3

Number of mitotic figures used for chromosome analyses and banding patterns of chromosome pair No. 7 in the southern and northern subgroups of *Rana rugosa*

Population	Sex	No. of frogs	No. of mitotic figures observed by the methods of			Bivalent chromosome No. 7
			Giemsa staining	C-banding	LR-banding	
Asahikawa	Female	1	9	15	5	Z^CW^1
	Female	4	56	59	19	Z^BW^1
Sapporo	Female	1	5	25	0	Z^CW^1
	Female	2	17	43	0	Z^BW^1
Hirosaki*	Female	12	28	40	63	Z^AW^1
	Male	9	19	57	62	Z^AZ^A
	Male	6	10	7	24	Z^AZ^0
Akita	Female	2	51	39	31	Z^AW^1
	Male	1	19	23	9	Z^AZ^A
	Male	1	3	11	0	Z^AZ^O
Inawashiro	Female	1	7	7	0	Z^BZ^B
	Female	2	11	17	13	Z^BW^1
	Male	4	5	30	0	Z^BZ^B
Murakami	Female	6	34	113	25	Z^BW^2
	Female	7	31	76	119	Z^BW^1
	Male	10	75	146	20	Z^BZ^B
Kanazawa	Female	2	0	11	7	Z^AW^1
	Male	3	13	21	3	Z^AZ^A
Katata	Female	3	37	46	33	Z^DW^1
Total	Female	43	286	491	315	
	Male	34	144	295	118	
Oigawa	Female	5	21	41	58	X X
	Male	2	11	18	0	X Y^A
	Male	1	6	14	4	X Y^B
Hamakita	Female	12	82	53	38	X X
	Male	11	63	46	40	X Y^A
	Male	3	27	16	0	X Y^B
Miyakoda	Female	2	7	19	34	X X
	Male	3	34	55	78	X Y^A
Yonezu	Female	4	19	38	55	X X
	Male	6	37	84	61	X Y^A
Toba	Female	3	8	56	13	X X
	Male	1	1	6	0	X Y^B
Total	Female	26	137	207	198	
	Male	27	179	239	183	

* These frogs were reported by Nishioka, Miura and Saitoh (1993).

In each of the Asahikawa and Sapporo populations, there was one female of the Z^CW^1 type and there were four and two females of the Z^BW^1 type in the two populations, respectively. In the Murakami population, six of the 13 females were of the Z^BW^2 type and the other seven were of the Z^BW^1 type. On the other hand, all of the 10 males were of the Z^BZ^B type (Fig. 4). In the Inawashiro population, two of the three females were of the Z^BW^1 type, while the remaining one female was of the Z^BZ^B type. Thus, the Z^BZ^B type female was considered to be a sex-reversed male. In the Hirosaki and Akita populations, there were 12 and two Z^AW^1 type females, respectively, while there were six and one Z^AZ^O type males and nine and one Z^AZ^A type males, respectively. In the Kanazawa population, there were two Z^AW^1 type females and three Z^AZ^A type males. In the Katata population, there were three Z^DW^1 type females (Table 3).

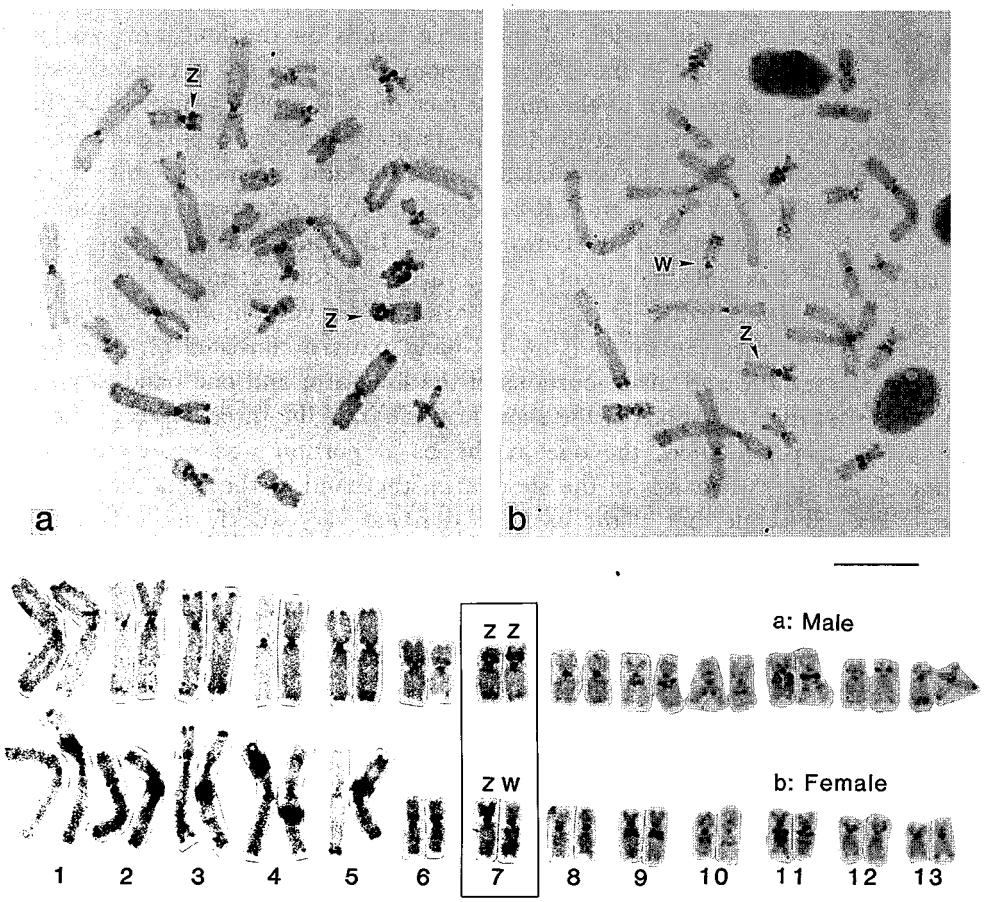

Fig. 4. Metaphase plates and karyotypes of *Rana rugosa* of the Murakami population, stained by the C-banding method. Bar represents 10 μm.

3. LR(late replication)-band pattern

The LR-band patterns appearing during about four hours of late S period were observed in 315 mitotic figures obtained from 39 females and 118 mitotic figures obtained from 29 males of the foregoing eight populations which had the sex chromosomes of the ZZ-ZW type. In all the populations, LR-bands were observed at the centromere portions of the 13 pairs of chromosomes. The LR-band patterns were found to be almost homozygous and there was no sex difference in the 13 chromosome pairs of the males and in the 12 chromosome pairs of the females other than the chromosome pair No. 7 (Figs. 2, 3).

In the Z chromosomes of four females of the Asahikawa population, in which the C-bands were of the Z^BW^1 type, the LR-band pattern consisted of one band at the basal portion, three bands between the middle and distal portions of the long arm, and one band at the middle portion and one band at the distal portion of the short arm. Of these six bands, the one band at the basal portion of the long arm was weakly stained, while the other five bands were deeply stained. Especially, the band situated in the middle of the three bands on the long arm was very deeply stained. Besides, in the Z^B chromosome, in which the C-band showed a deeply stained band pattern at the middle portion of the short arm, the LR-band corresponding to the C-band at the middle portion was very deeply stained. In contrast, in a female of the Asahikawa population and a female of the Sapporo population of the Z^CW^1 type, the LR-bands of the Z^C chromosome were similar to those of the Z^B chromosome. However, the LR-bands corresponding to the C-bands which was wide and deeply stained at the middle portion of the short arm and at the basal portion of the long arm. The W^1 chromosome of these populations was metacentric and the LR-band pattern consisted of three bands between the middle and distal portions of the long arm and one band at each of the basal and distal portions of the short arm. While the three bands of the long arm were deeply stained, the one at the basal portion was especially deeply stained. Of the two bands of the short arm, the band at the distal portion was deeply stained, while that at the basal portion was very weakly stained (Table 3; Figs. 2, 3).

In the Murakami population, ten males were all of the Z^BZ^B type as they were similar to those of the Asahikawa population in the LR-bands, band patterns, number and intensity of bands of the Z^B chromosome. In contrast, in the W chromosome of the females of the Murakami population, there were W^1 type and W^2 type as the C-band. The W^1 type was quite the same as the LR-bands of the W^1 type of the foregoing Asahikawa population, and there was no difference in the position and intensity of the bands. However, in the W^2 chromosome, which showed a wide and very deeply stained C-band at the distal portion of the long arm, the LR-band corresponding to the C-band was wide and very deeply stained (Table 3; Fig. 5).

In the Inawashiro population, the LR-band patterns of the Z and W chromosomes were quite the same as those of the Z^BW^1 type in the Asahikawa and Murakami populations. Four males were all of the Z^BZ^B type and two females

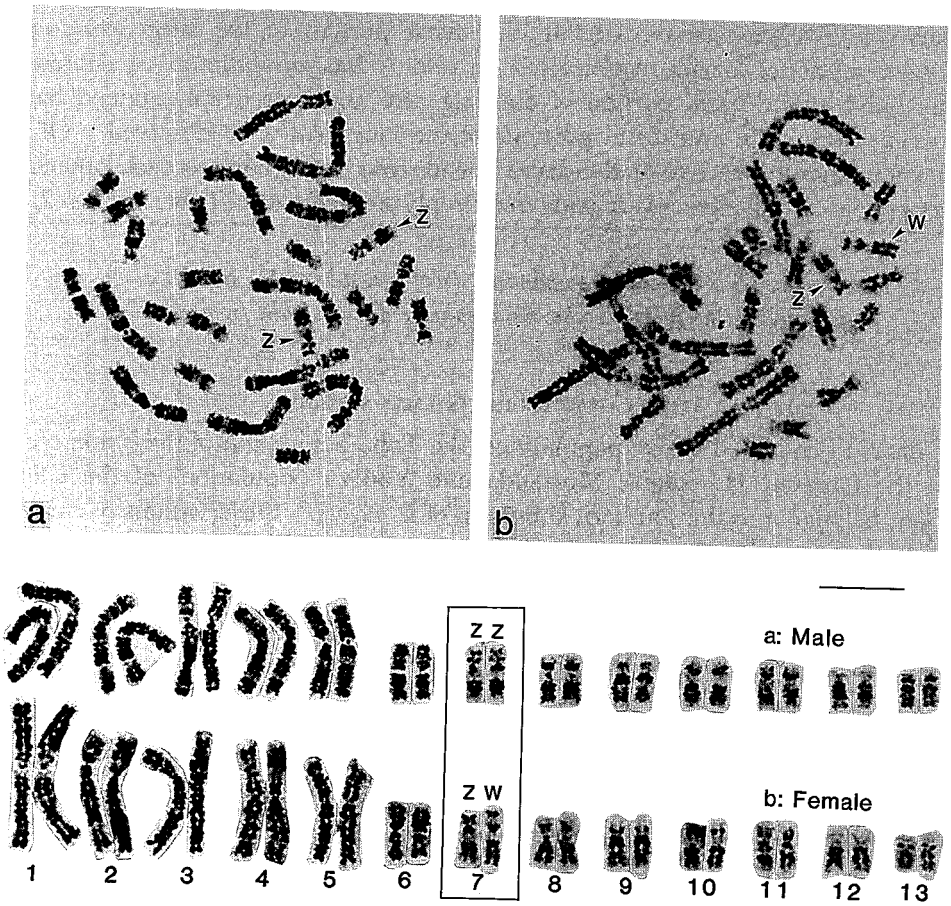

Fig. 5. Metaphase plates and karyotypes of *Rana rugosa* of the Murakami population, stained by the late replication (LR)- banding method. Bar represents 10 μm.

were of the Z^BW^1 type. However, the remaining one female was the same as the male which was of the Z^BZ^B type and homomorphic (Table 3).

In the Hirosaki and Akita populations, the W chromosome of the females had all the LR-band of the W^1 type. However, there were two types in the Z chromosomes. In the Z^A chromosome which showed a deeply stained band at the basal portion of the long arm, the LR-band corresponding to the C-band at the basal portion was deeply stained, while the LR-band corresponding to the C-band at the basal portion in the Z^O chromosome devoid of the C-band was very weakly stained.

In the Kanazawa population, the LR-band pattern was the same as that found in the Hirosaki population. All the Z chromosomes showed the band pattern of the Z^A type, while all the W chromosomes showed the band pattern of the W^1 type.

In the Katata population, the LR-band pattern of the W chromosome was all of the W^1 type, while the LR-band of the Z^D chromosome resembled a shape in

which one LR-band was added to the distal portion of the long arm to the LR-band of the Z^O type in the Hirosaki and Akita populations. A somewhat weak band at the basal portion of the long arm and four deeply stained bands between the middle and distal portions of the long arm were observed. Of the latter four bands, the two at the distal portion was contiguous to each other and often appeared to be like a very wide and deeply stained band (Table 3; Figs. 2, 3).

II. Second kind of populations (southern subgroup), XX-XY type in which the X and Y chromosomes were distinguished from each other

1. Conventional GIEMSA staining

This kind consisted of five populations, the Toba population of the Kinki region and the Oigawa, Hamakita, Miyakoda and Yonezu populations of the Chubu region, belonging to the southern subgroup of *R. rugosa*. Observations of chromo-

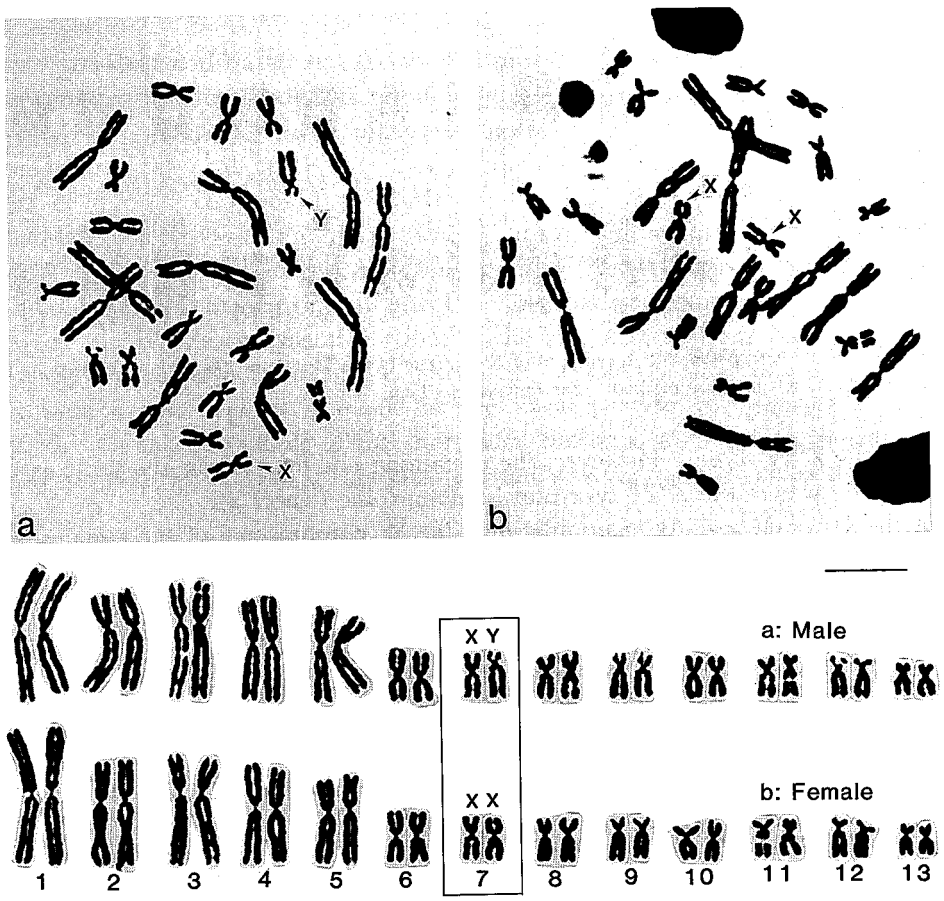

Fig. 6. Metaphase plates and karyotypes of *Rana rugosa* of the Hamakita population, stained by the conventional GIEMSA staining method. Bar represents 10 μm.

somes were made on 137 mitotic figures obtained from 26 females and on 179 mitotic figures obtained from 27 males by conventional GIEMSA staining method.

TABLE 4

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

Male (XY)

Relative length (RL)				Numerical value of centromere position (NVC)				
Chromosome no.	Minimum	Maximum	Mean±SE	Chromosome no.	Minimum	Maximum	Mean±SE	Type
1	13.50	16.00	14.67±0.07	1	44.78	50.08	46.73±0.13	m
2	11.00	13.37	12.18±0.06	2	32.84	40.16	37.50±0.18	m
3	10.76	13.40	12.13±0.07	3	30.14	38.37	34.21±0.21	sm
4	10.37	12.27	11.20±0.05	4	40.41	46.44	43.41±0.18	m
5	9.10	11.19	10.12±0.06	5	36.68	43.21	40.15±0.19	m
6	5.38	6.45	6.04±0.03	6	44.12	50.37	47.64±0.16	m
7 (X)	4.39	6.00	5.20±0.04	7 (X)	37.02	47.09	42.13±0.31	m
7 (Y)	4.82	6.04	5.41±0.03	7 (Y)	19.35	29.55	24.39±0.38	st
8	4.59	5.78	5.14±0.03	8	35.77	44.61	40.64±0.24	m
9	4.53	5.96	5.17±0.04	9	24.77	35.20	30.61±0.31	sm
10	4.36	5.52	4.91±0.03	10	32.75	46.80	41.83±0.31	m
11*	4.25	5.38	4.82±0.03	11*	23.78	36.89	31.38±0.34	sm
12	3.98	5.35	4.56±0.03	12	23.68	35.35	29.11±0.35	sm
13	3.33	4.67	3.87±0.04	13	30.22	41.79	36.49±0.36	sm

Female (XX)

Relative length (RL)				Numerical value of centromere position (NVC)				
Chromosome no.	Minimum	Maximum	Mean±SE	Chromosome no.	Minimum	Maximum	Mean±SE	Type
1	13.45	16.55	14.65±0.10	1	43.90	48.77	46.85±0.14	m
2	10.97	13.06	12.10±0.06	2	32.60	40.47	37.58±0.18	m
3	11.12	12.98	12.02±0.06	3	27.45	37.60	33.95±0.21	sm
4	9.76	12.23	11.19±0.06	4	40.14	46.10	43.30±0.19	m
5	9.21	10.96	10.05±0.06	5	36.81	43.75	40.02±0.19	m
6	5.48	6.44	5.97±0.03	6	45.06	49.99	47.75±0.17	m
7	4.73	5.92	5.30±0.03	7	38.07	47.22	42.98±0.28	m
8	4.77	5.84	5.22±0.03	8	30.21	45.35	40.44±0.34	m
9	4.68	5.98	5.25±0.04	9	26.90	43.47	31.75±0.34	sm
10	4.32	5.59	4.96±0.04	10	38.97	47.59	42.78±0.23	m
11*	4.21	5.62	4.84±0.04	11*	26.35	37.24	31.52±0.32	sm
12	4.11	5.32	4.56±0.04	12	24.19	36.39	30.27±0.34	sm
13	3.33	4.62	3.90±0.04	13	31.56	42.34	37.98±0.32	m

The RL and NVC of the chromosomes of the males in the Hamakita population were calculated by regarding the X-chromosomes as the chromosomes No. 7. The RL and NVC of the Y-chromosome were calculated on the basis of the ratio of the X- and Y-chromosomes in length in each mitotic figure.

$$RL=\frac{\text{Chromosome length}}{\text{Genome size}}\times 100 \qquad NVC=\frac{\text{Short-arm length}}{\text{Chromosome length}}\times 100$$

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

SE: Standard error of the mean

*: secondary constriction

In every population, all the 13 pairs of chromosomes in the females were homomorphic, while in the males, chromosome pair No. 7 was heteromorphic and all the other 12 pairs were homomorphic (Table 4; Fig. 6).

Chromosome pair No. 7 of the female in these populations was composed of homozygous metacentric chromosomes, while that of the male was heterozygous and composed of metacentric and subtelocentric chromosomes. In the Hamakita population, the numerical value of centromere position (NVC) in the X chromosomes of females was 38.07~47.22, 42.98 on the average, while the NVC in the X chromosomes of males was 37.02~47.09, 42.13 on the average. The NVC in the Y chromosomes was 19.35~29.55, 24.39 on the average (Table 4). Thus, it seems to be appropriate that chromosome pairs No. 7 of the female and male are called the XX- and XY-type, respectively, in the foregoing five populations of *R. rugosa*.

2. C-band pattern

The C-band patterns were observed on 207 mitotic figures obtained from the

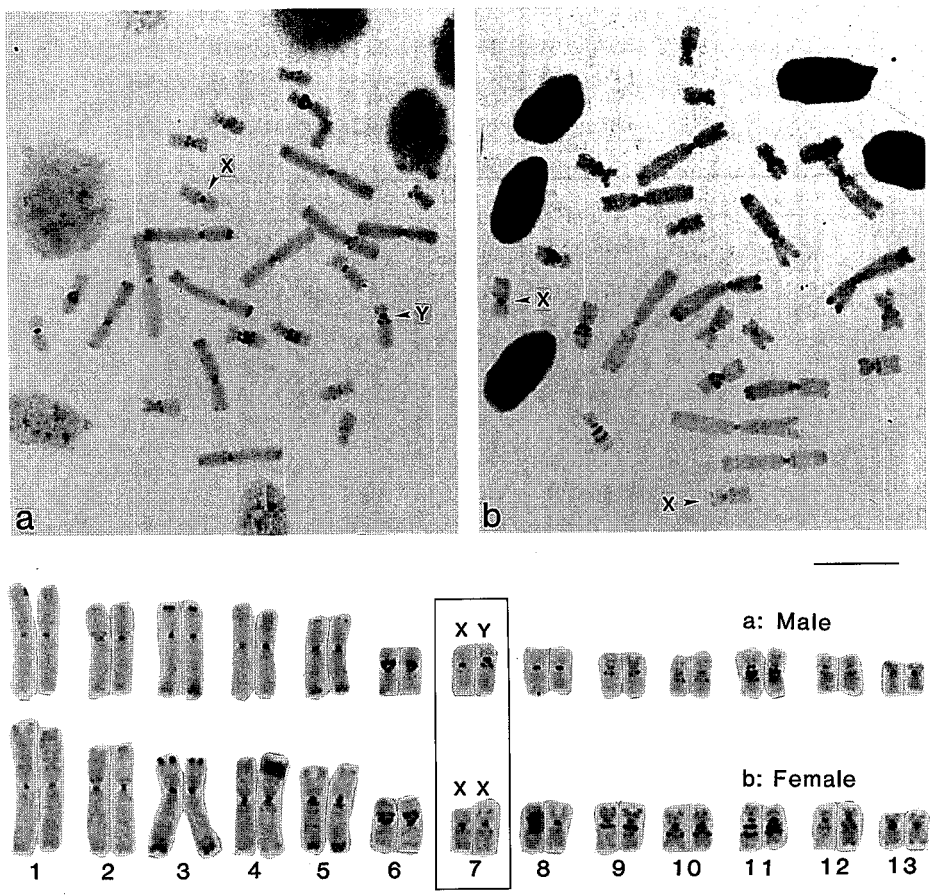

Fig. 7. Metaphase plates and karyotypes of *Rana rugosa* of the Hamakita population, stained by the C-banding method. Bar represents 10 μm.

foregoing 26 females and on 239 mitotic figures obtained from the foregoing 27 males of the five populations. In all the five populations, there were the C-bands in the centromere portions of all the 13 pairs of chromosomes, and at the basal, intermediate or distal portion of the long arms and the short arms of some chromosomes. In the 12 pairs of chromosomes other than chromosome pair No. 7, there was no sex difference in C-band patterns (Fig. 7).

The C-band pattern in the metacentric X chromosome was similar to the C-band pattern of the W^1 chromosome in the northern subgroup which had the sex chromosomes of the ZZ-ZW type. However, the C-band at the distal portion of the long arm in the X chromosome was distinguished from that of the W^1 chromosome by slight weakness in the staining. In the subtelocentric or submetacentric Y chromosome, there were two types in the C-band at the basal portion of the long arm. The Y^A type was deeply stained, while the Y^B type was very weakly stained. The Y^A band was weakly stained as compared with the Z^A band, while it was very similar to the C-band situated at the basal portion of the long arm of chromosome pair No. 7 in the Kumano and Okayama populations (Figs. 2, 3).

Of 14 males of the Hamakita population, 11 were of the XY^A type and three were of the XY^B type. Of three males of the Oigawa population, two were of the XY^A type and the remainder was of the XY^B type. Three and six males of the Miyakoda and Yonezu populations, respectively, were all of the XY^A type. The only one male of the Toba population was of the XY^B type (Table 3; Fig. 7).

3. LR-band pattern

The LR (late replication)-band patterns were observed on 198 mitotic figures obtained from the foregoing 26 females and on 183 mitotic figures obtained from the foregoing 21 males in the five populations of the southern subgroup. In all the five populations, there were the LR-bands in the centromere portions of all the 13 pairs of chromosomes. The LR-band patterns were homologous in almost all the 13 pairs of chromosomes in the female and in the 12 pairs other than chromosome pair No. 7 in the male (Fig. 8).

In chromosome pair No. 7 which was considered to be the sex chromosomes, the LR-band pattern of the X chromosome was the same as the LR-band pattern of the W^1 chromosome in the northern subgroup which had the sex chromosomes of the ZZ-ZW type. In the Y^A chromosome which showed a deeply stained band at the basal portion of the long arm by C-banding, the LR-band corresponding to the C-band at the basal portion was deeply stained, while in the Y^B chromosome, the LR-band corresponding to the C-band at the basal portion was very weakly stained. Thus, the LR-band patterns in the above five populations were the same as the differences by the C-band (Figs. 2, 3).

In the Hamakita and Oigawa populations, the female was of the XX type and the male consisted of two types, XY^A and XY^B. In the Miyakoda and Yonezu populations, the female was of the XX type and the male of the XY^A type. In the Toba population, the female was of the XX type and the male was of the XY^B

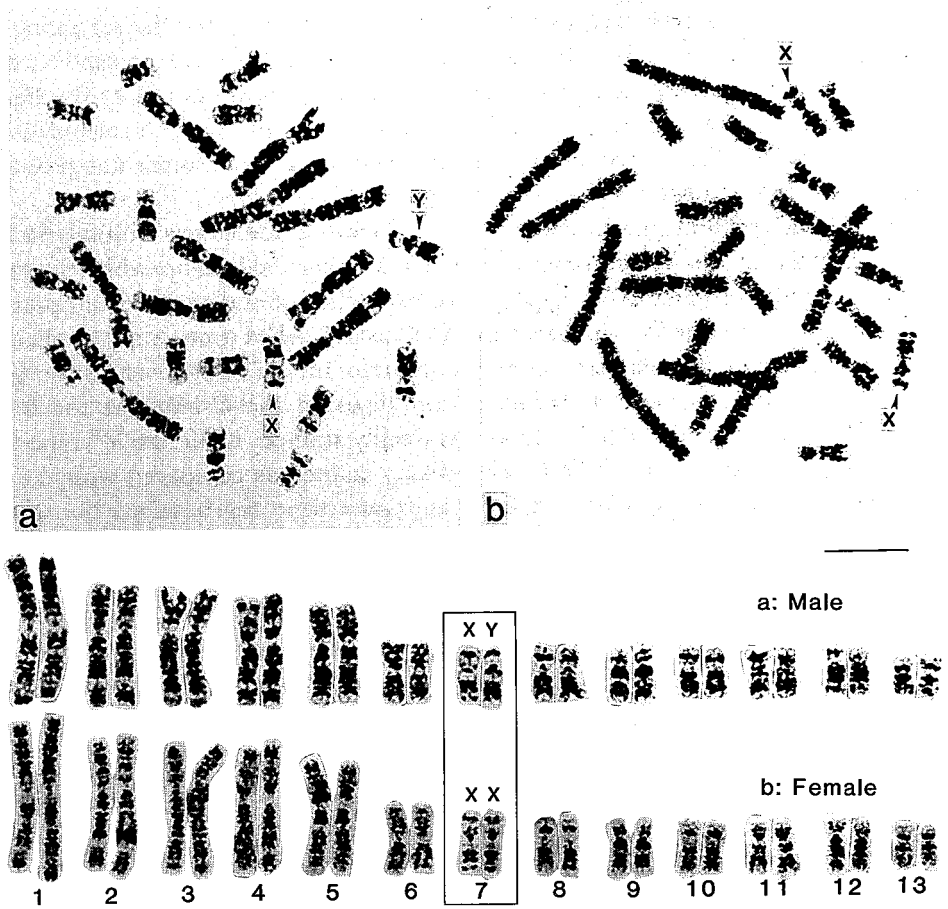

Fig. 8. Metaphase plates and karyotypes of *Rana rugosa* of the Hamakita population, stained by the late replication (LR)-banding method. Bar represents 10 μm.

type (Table 3; Fig. 8).

III. Third kind of populations (intermediate subgroup), obscure type whose sex-determining mechanism was unknown

1. Conventional GIEMSA staining

This group consisted of seven populations, the Daigo, Hitachiota, Ashikaga, Maebashi, Machida, Kamogawa and Isehara populations of the Kanto region, belonging to the intermediate subgroup of *R. rugosa*. Observations of chromosomes were made on 132 mitotic figures obtained from 21 females and on 113 mitotic figures obtained from 12 males of the foregoing five populations by the method of conventional GIEMSA staining. Each of the 13 pairs of chromosomes was homomorphic and no sex difference was found (Fig. 9). Chromosome pair

TABLE 5

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

Male (??)

Relative length (RL)				Numerical value of centromere position (NVC)				
Chromosome no.	Minimum	Maximum	Mean±SE	Chromosome no.	Minimum	Maximum	Mean±SE	Type
1	13.96	16.01	14.56±0.13	1	45.07	49.92	47.50±0.30	m
2	11.37	13.41	12.05±0.09	2	34.63	41.37	38.38±0.42	m
3	10.66	12.28	11.45±0.08	3	26.34	34.04	31.47±0.43	sm
4	9.96	11.26	10.85±0.08	4	40.41	47.00	43.13±0.44	m
5	9.14	10.57	9.82±0.09	5	37.61	43.27	40.60±0.34	m
6	5.92	6.84	6.33±0.05	6	44.26	50.33	47.39±0.38	m
7	5.17	6.05	5.58±0.05	7	14.00	21.69	18.37±0.54	st
8	4.83	5.92	5.42±0.06	8	34.99	45.14	41.43±0.53	m
9	4.53	5.76	5.16±0.06	9	26.13	34.82	30.45±0.43	sm
10	4.38	5.37	4.87±0.06	10	39.46	45.96	43.38±0.39	m
11*	4.77	5.62	5.17±0.05	11*	27.06	36.87	31.42±0.61	sm
12	4.23	5.25	4.56±0.06	12	23.10	32.22	28.76±0.60	sm
13	3.80	4.91	4.19±0.07	13	31.62	41.81	36.79±0.56	sm

Female (??)

Relative length (RL)				Numerical value of centromere position (NVC)				
Chromosome no.	Minimum	Maximum	Mean±SE	Chromosome no.	Minimum	Maximum	Mean±SE	Type
1	13.35	15.63	14.48±0.09	1	43.38	49.35	47.15±0.22	m
2	11.04	12.60	11.86±0.06	2	34.56	42.02	37.96±0.28	m
3	10.96	12.26	11.41±0.05	3	29.78	34.85	31.71±0.20	sm
4	10.26	12.30	11.01±0.06	4	39.87	46.45	43.19±0.23	m
5	9.38	10.85	9.96±0.04	5	35.49	45.27	39.99±0.29	m
6	5.82	6.97	6.23±0.04	6	44.42	49.73	47.26±0.19	m
7	5.45	6.14	5.80±0.03	7	13.67	23.52	18.91±0.38	st
8	4.80	5.75	5.30±0.04	8	36.16	45.97	41.92±0.37	m
9	4.84	5.69	5.27±0.04	9	22.20	36.04	31.29±0.43	sm
10	4.38	5.54	4.90±0.04	10	39.20	48.32	43.43±0.35	m
11*	4.34	5.55	5.02±0.04	11*	28.12	37.33	31.64±0.34	sm
12	3.95	5.05	4.58±0.03	12	24.07	35.62	30.03±0.41	sm
13	3.80	4.75	4.21±0.04	13	30.34	45.34	39.20±0.45	m

$$RL=\frac{\text{Chromosome length}}{\text{Genome size}}\times 100 \qquad NVC=\frac{\text{Short-arm length}}{\text{Chromosome length}}\times 100$$

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

SE: Standard error of the mean

*: secondary constriction

No. 7 was a pair of homozygous subtelocentric chromosomes. The numerical value of the centromere position (NVC) in females and males was 13.67~23.52, 18.64 on the average (Table 5). This value showed that the chromosomes were subtelocentric near telocentric, as the short arm was shorter than that of the chromosome pair No. 7 in populations of the western group.

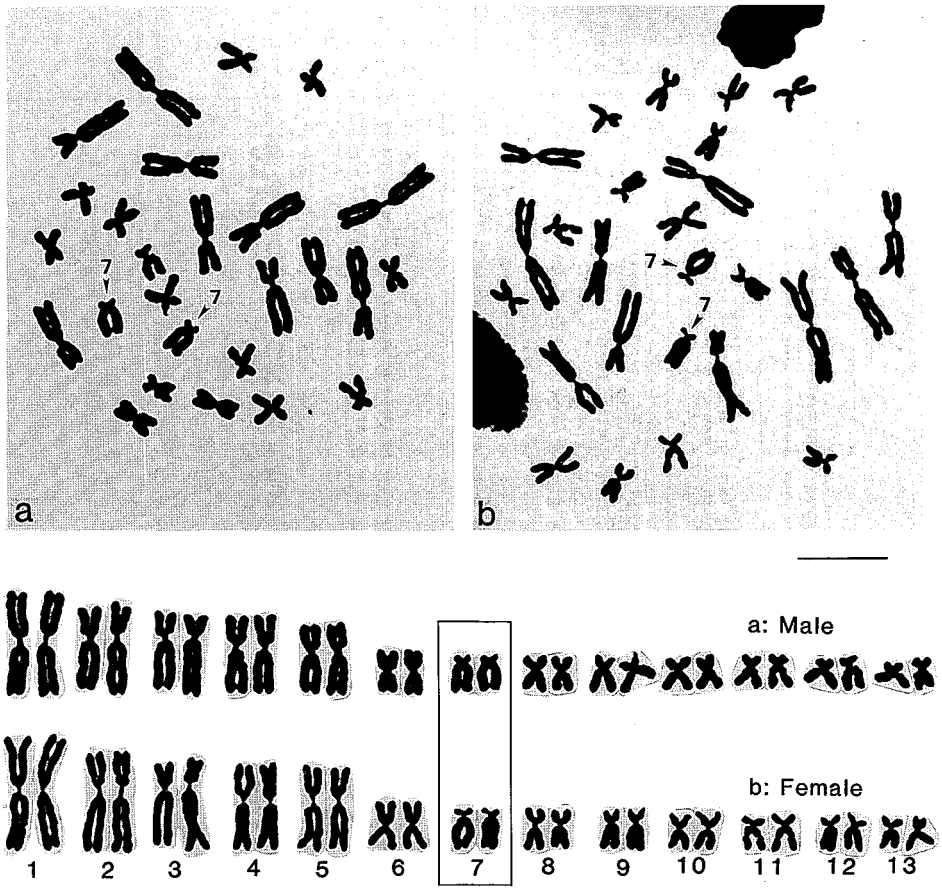

Fig. 9. Metaphase plates and karyotypes of *Rana rugosa* of the Daigo population, stained by the conventional GIEMSA staining method. Bar represents 10 μm.

2. C-band pattern

The C-band patterns were observed on 227 mitotic figures obtained from the foregoing 23 females and on 190 mitotic figures obtained from the foregoing 15 males of the seven populations belonging to the intermediate subgroup. In all the seven populations, the C-bands were observed at the centromere portions of all the 13 pairs of chromosomes, and at the basal, middle and distal portions of the long and short arms of some chromosomes. No sex difference was found in each of the 13 chromosome pairs, like those of the populations of the western group (Fig. 10). The C-band patterns of chromosome pair No. 7 in males and females were homologous, and there were no differences among different populations. While the C-band at the centromere portion was deeply stained, those at the long arm were weakly stained and there was no C-band on the short arm. Such a chromosome pair No. 7 was called the 7^C7^C type (Table 6; Figs. 2, 3).

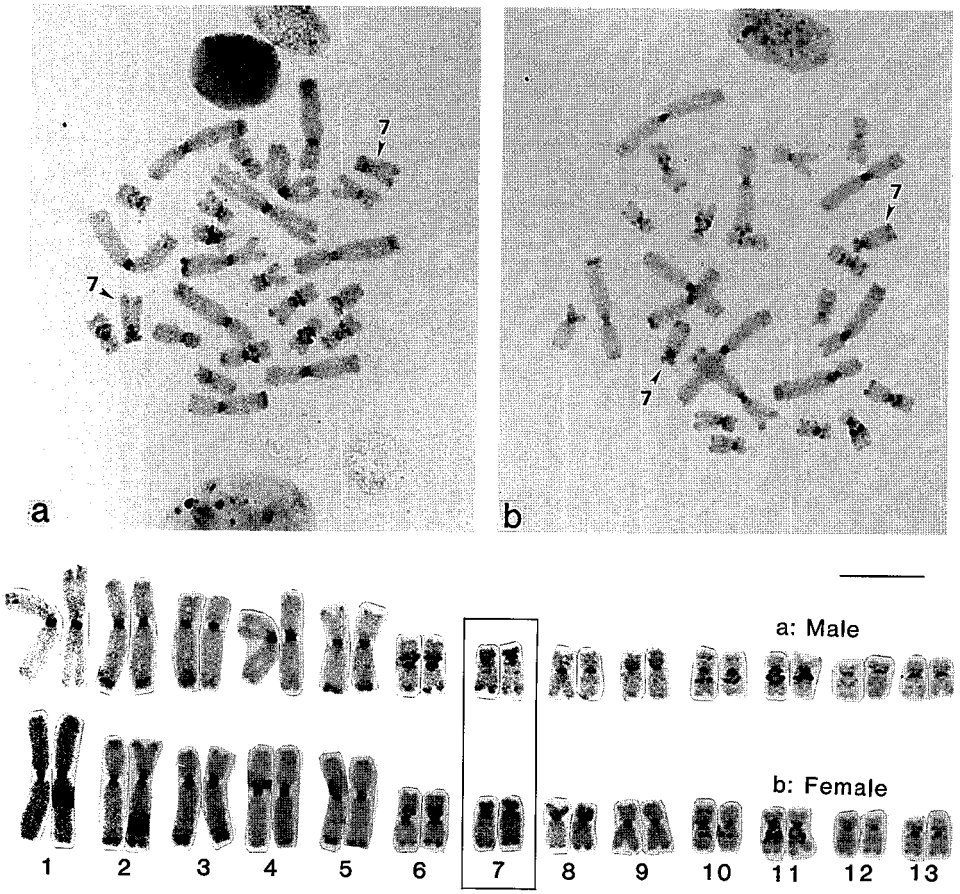

Fig. 10. Metaphase plates and karyotypes of *Rana rugosa* of the Daigo population, stained by the C-banding method. Bar represents 10 μm.

3. LR-band pattern

The LR-band patterns were observed on 130 mitotic figures obtained from the foregoing 22 females and on 133 mitotic figures obtained from the foregoing 13 males of the six populations belonging to the intermediate subgroup. In these populations, the LR-bands were observed at the centromere portions of all the 13 pairs of chromosomes. There were no sex differences in the LR-band pattern, like the C-band pattern. Each of the 13 chromosome pairs was homologous and there were no differences in the position, number and intensity of staining of the LR-bands between the homologous chromosomes (Fig. 11).

Chromosome pair No. 7 of the populations of the foregoing intermediate subgroup all consisted of subtelocentric chromosomes. There were one LR-band at the basal portion and four LR-bands at the middle and distal portions of the long arm. Of these LR-bands, the four at the middle and distal portions were deeply stained, while the second band at the middle portion was especially deeply stained.

TABLE 6

Number of mitotic figures used for chromosome analyses and banding patterns of chromosome pair No. 7 in the intermediate subgroup of the eastern group and the western group of *Rana rugosa*

Population	Sex	No. of frogs	No. of mitotic figures observed by the methods of			Bivalent chromosome No. 7
			GIEMSA staining	C-banding	LR-banding	
Daigo	Female	6	41	44	20	7^C7^C
	Male	2	26	24	3	7^C7^C
Hitachiota	Female	5	15	45	19	7^C7^C
	Male	1	11	12	5	7^C7^C
Ashikaga	Female	1	0	2	0	7^C7^C
	Male	1	0	11	0	7^C7^C
Maebashi	Female	1	0	18	12	7^C7^C
	Male	1	0	5	2	7^C7^C
Machida	Female	2	2	11	8	7^C7^C
	Male	1	0	2	0	7^C7^C
Kamogawa	Female	4	11	36	16	7^C7^C
	Male	5	34	75	63	7^C7^C
Isehara	Female	4	63	71	55	7^C7^C
	Male	4	42	61	60	7^C7^C
Total	Female	23	132	227	130	
	Male	15	113	190	133	
Okayama	Female	2	35	30	36	7^A7^A
	Male	2	29	22	13	7^A7^A
Kumano*	Female	5	19	47	36	7^A7^A
	Male	6	15	31	24	7^A7^A
Gotsu	Female	3	22	50	39	7^B7^B
	Male	4	51	66	37	7^B7^B
Nagayo	Female	3	46	25	21	7^B7^B
	Male	3	8	15	0	7^B7^B
Total	Female	13	122	152	132	
	Male	15	103	134	74	

* These frogs were reportd by NISHIOKA, MIURA and SAITOH (1993).

There was no LR-band on the short arm which was shorter than that of the 7^B chromosome. Thus, the 7^C chromosome of this group was considered to have been produced from the 7^B chromosome of the western group by a pericentric inversion (Table 6; Figs. 2, 3).

IV. Fourth kind of populations (western group), XX-XY type in which the X and Y chromosomes were not distinguished from each other

1. Conventional GIEMSA staining

This kind consisted of four populations, the Okayama, Kumano and Gotsu populations of the Chugoku region and the Nagayo population of the Kyushu

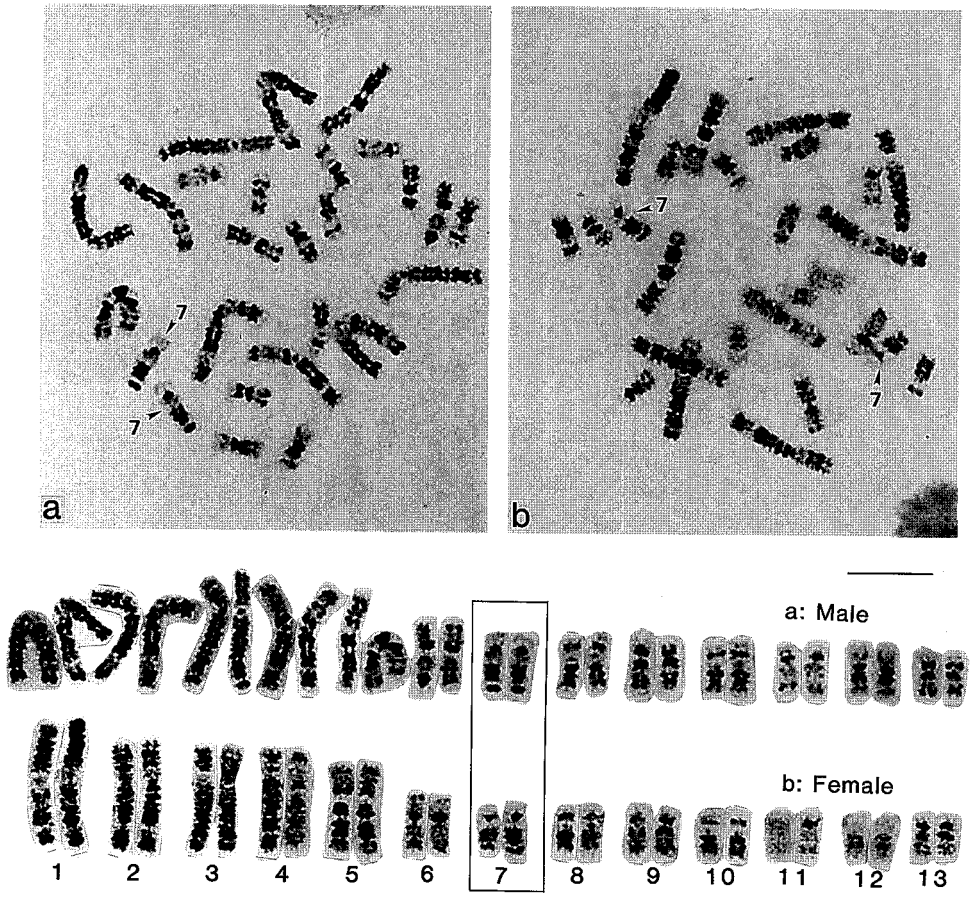

Fig. 11. Metaphase plates and karyotypes of *Rana rugosa* of the Daigo population, stained by the late replication (LR)-banding method. Bar represents 10 μm.

region, belonging to the western group of *R. rugosa*. Observations of chromosomes were made on 122 mitotic figures obtained from 13 females and on 103 mitotic figures obtained from 15 males by the conventional GIEMSA staining method (Fig. 12). In each population, all the 13 pairs of chromosomes were homomorphic and no sex difference was found between two homologous chromosomes, although the frogs of this group were of the XX-XY type in sex-determining mechanism (NISHIOKA, MIURA and SAITOH, 1993; KASHIWAGI, 1993). In the populations of the northern subgroup which was of the ZZ-ZW type in sex-determining mechanism and the population of the southern subgroup which was of the XX-XY type, the sex chromosomes were morphologically differentiated, and chromosome pair No. 7 was the sex chromosomes, that is, the Z and Y chromosomes were subtelo- or submetacentric, while the W and X chromosomes were metacentric. However, in the populations of the western group, chromosome pair No. 7 was a homologous pair of subtelocentric or submetacentric chromosomes in both females and males (Figs. 2, 3).

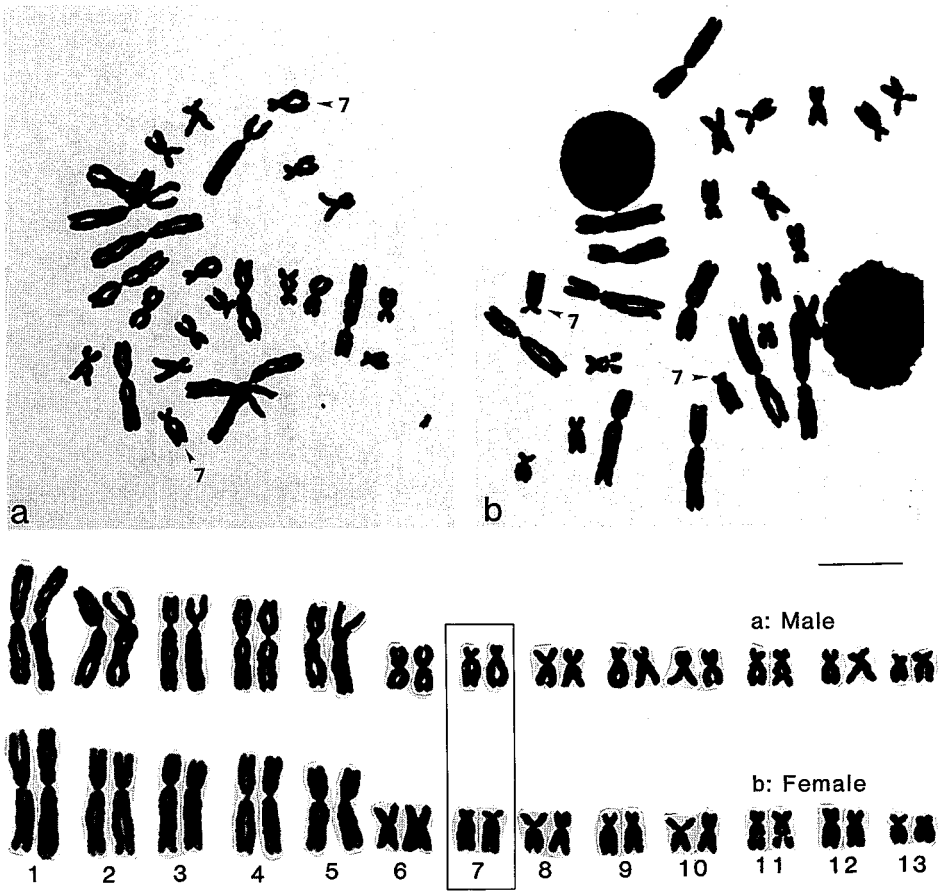

Fig. 12. Metaphase plates and karyotypes of *Rana rugosa* of the Gotsu population, stained by the conventional GIEMSA staining method. Bar represents 10 μm.

2. C-band pattern

The C-band patterns were observed on 152 mitotic figures obtained from the foregoing 13 females and on 134 mitotic figures obtained from the foregoing 15 males of the four populations. In all the four populations, C-bands were observed at the centromere portions of all the 13 pairs of chromosomes, and at the basal, middle and distal portions of long arms, and on the short arms of some chromosomes. However, there were no sex differences in each of the 13 pairs of chromosomes (Fig. 13). Chromosome pair No. 7 in the populations of this group consisted of subtelo- or submetacentric chromosomes in both males and females. While the C-bands were observed at the centromere portion and the basal portion of the long arm, in the C-bands at the basal portions of the long arms, there were two kinds. One kind of band was stained deeply (7^A), and the other kind of band was weakly stained (7^B). In both sexes of the Okayama and Kumano populations, chromosome pair No. 7 was of the 7^A7^A type, and was very similar to the C-band pattern of the Y^A chromosomes in the Hamakita, Oigawa, Miyakoda and

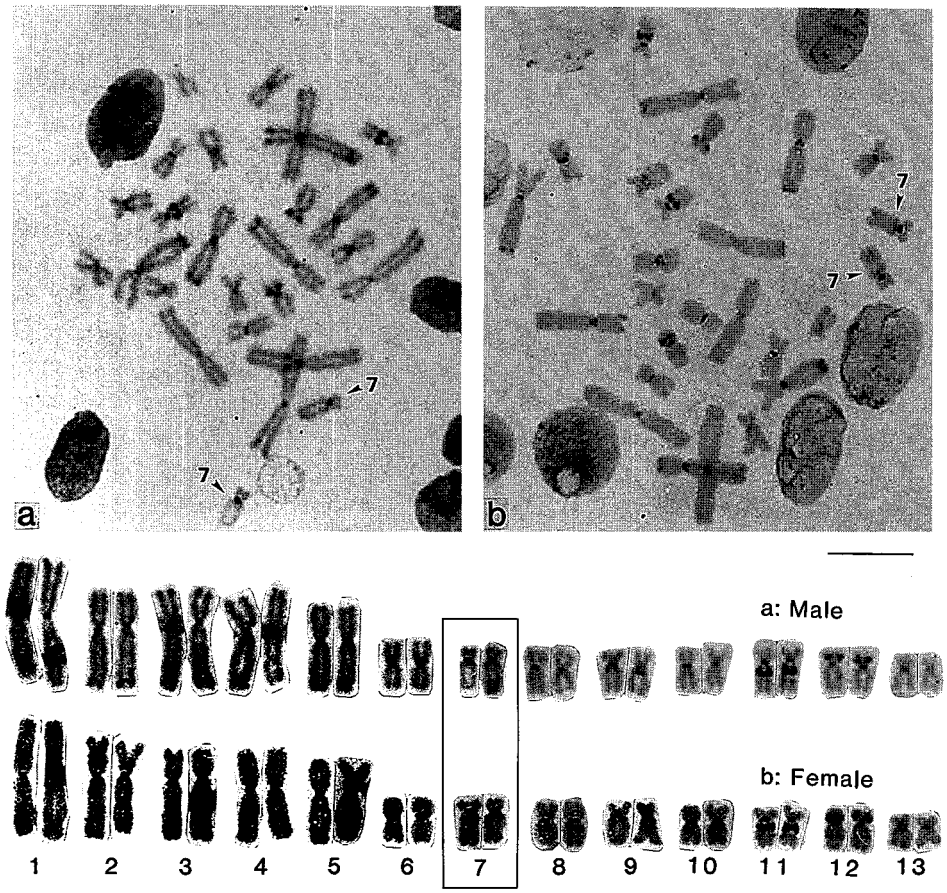

Fig. 13. Metaphase plates and karyotypes of *Rana rugosa* of the Gotsu population, stained by the C-banding method. Bar represents 10 μm.

Yonezu populations of the southern subgroup. In the Gotsu and Nagayo populations, chromosome pair No. 7 was of the 7^B7^B type, and very similar to the C-band pattern of the Y^B chromosome in the Hamakita, Oigawa and Toba populations of the southern subgroup (Table 6; Figs. 2, 3).

3. LR-band pattern

The LR-band patterns were observed on 132 mitotic figures obtained from the foregoing 13 females and on 74 mitotic figures obtained from the 12 males of the four populations. In all the four populations, LR-bands were observed at the centromere portions of all the 13 pairs of chromosomes. There were no sex differences in LR-band patterns. Each of the 13 pairs of chromosomes consisted of almost homologous chromosomes. There were no differences in the position, number and intensity of the LR-bands between the homologous chromosomes of each pair (Fig. 14). Chromosome pair No. 7 in the populations of this group consisted of subtelo- or submetacentric chromosomes, and the LR-band pattern

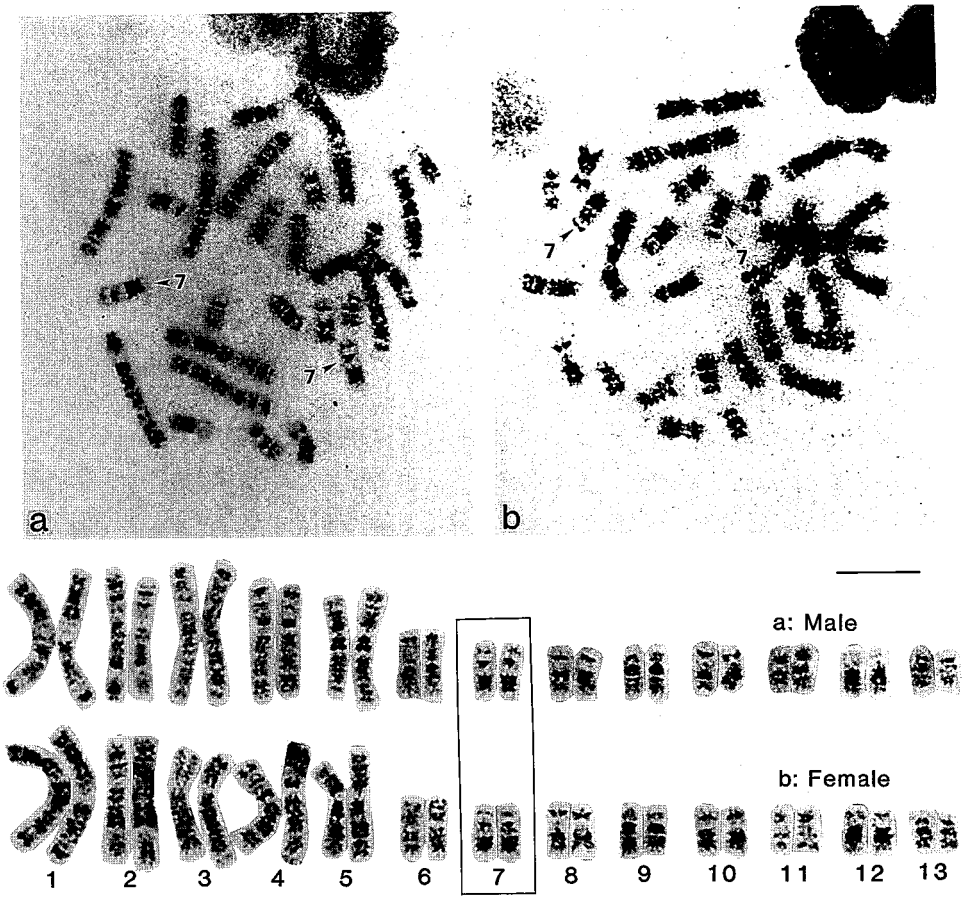

Fig. 14. Metaphase plates and karyotypes of *Rana rugosa* of the Gotsu population, stained by the late replication (LR)-banding method. Bar represents 10 μm.

was the same as that of the Y chromosome found in the Chubu region of the southern subgroup which was of the XX-XY type in sex-determining mechanism.

In the Okayama and Kumano populations in which chromosome pair No. 7 was always 7^A7^A type, the LR-band corresponding to the deeply stained C-band at the basal portion of the long arm was deeply stained. On the other hand, in the Gotsu and Nagayo populations which were of the 7^B7^B type, the LR-band corresponding to the weakly stained C-band at the basal portion of the long arm was extremely weak. The 7^A and 7^B chromosomes were very similar to the Y^A and Y^B chromosomes, respectively, found in the populations of the southern subgroup (Table 6; Figs. 2, 3).

DISCUSSION

The studies on sex chromosomes bearing sex-determining genes in amphibians have recently been remarkably developed by the progress in various banding

techniques and in analysis of the linkages between the genes controlling enzymes and proteins and the sex-determining genes. Various degrees of varieties in sex chromosomes have been found among different species or different populations of the same species in amphibians which are still in a very primitive state of sex differentiation.

It has been reported that chromosome pair No. 13 of 14 pairs was sex chromosomes in *Ambistoma mexicanum* by HAUSCHKA and BRUNST (1965) and that chromosome pair No. 14 was sex chromosomes in *A. laterale* by SESSIONS (1982). KEZER and SESSIONS (1979) and SESSIONS and KEZER (1987) made extensive cytogenetic studies on *Aneides ferreus* in North America and divided this species into two groups on the basis of the morphological differences in chromosome pair No. 13. In one group (*ferreus* II), chromosome pair No. 13 was sex chromosomes, which was constructed with a telocentric Z chromosome and a metacentric W chromosome, while there was one population which had two types of W chromosomes, metacentric and telocentric. In this population, the telocentric W chromosome could not be distinguished from the telocentric Z chromosome. On the other hand, chromosome pair No. 13 of the other group in *Aneides ferreus* (*ferreus* I) which was constructed with telocentric or subtelocentric chromosomes, and both male and female were homomorphic or heteromorphic, that is, in this group, chromosome pair No. 13 had no relation with sex. The same authors could not find morphologically differentiated sex chromosomes in four allied species of *Aneides ferreus*, *A. flavipunctatus*, *A. lugubris*, *A. hardii* and *A. aeneus*.

In *Crinia bilingua*, chromosome pair No. 12 was sex chromosomes, of which the W chromosome was large and subtelocentric and the Z chromosome was small and acrocentric (MAHONY, 1991). In *Xenopus laevis*, W chromosome was metacentric and Z chromosome was acrocentric (WEILER and OHNO, 1962). In *Buergeria buergeri*, chromosome pair No. 7 was sex chromosomes, of which the Z chromosome had a nucleolar organizer, while the W chromosome had none (OHTA, 1986). *Pyxicephalus adspersus* had sex chromosomes, of which the W chromosome was smaller than the Z chromosome (SCHMID, 1980; SCHMID and BACHMANN, 1981).

In five species of *Hydromantes*, *H. ambrosii*, *H. flavus*, *H. imperialis*, *H. italicus* and *H.* sp. nov, chromosome pair No. 14 was sex chromosomes (NARDI, ANDRONICO, de LUCCHINI and BATISTONI, 1986). In five species of *Necturus*, *N. alabamensis*, *N. beyeri*, *N. maculosus*, *N. punctatus* and *N. lewisi*, the Y chromosome was extremely smaller than the X chromosome, and in the former three species, chromosome pair No. 3 was sex chromosomes, while in the latter two species, chromosome pairs No. 6 and No. 4, respectively, were sex chromosomes (SESSIONS, 1980; SESSIONS and WILEY, 1985). Of eight European species of *Triturus*, in five species, *T. alpestris*, *T. karelini*, *T. marmoratus*, *T. cristatus* and *T. carnifex*, chromosome pair No. 4 was sex chromosomes, while in three species, *T. vulgaris*, *T. helveticus* and *T. boscai*, chromosome pair No. 5 was sex chromosomes (SCHMID, 1980; SCHMID, OLERT and KLETT, 1979; BALDWIN and MACGREGOR, 1985; MACGREGOR and SESSIONS, 1986).

In *Gastrotheca riobambae*, chromosome pair No. 4 was sex chromosomes, and the

Y chromosome was much larger than the X chromosome (SCHMID, 1983; SCHMID, HAAF, GEILE and SIMS, 1983; SCHMID, SIMS, HAAF and MACGREGOR, 1986), while in *G. walkeri* and *G. ovifera*, the X chromosomes were larger than the Y chromosomes. In these species, chromosome pairs Nos. 2 and 1 were sex chromosomes, respectively. In contrast, heteromorphic sex chromosomes were not found in *G. fissipes* (SCHMID, STEINLEIN, FEICHTINGER, de ALMEIDA and DUELLMAN, 1988). In *G. pseustes*, chromosome pair No. 5 was sex chromosomes, and there were two types in the Y chromosome. While one type could be distinguished from the X chromosome by the presence of C-band at the terminal portion of long arm, the Y chromosome of the other type could not be distinguished from the X chromosome (SCHMID, STEINLEIN, FREIEDL, de ALMEIDA, HAAF, HILLS and DUELLMAN, 1990).

By using a naturally sex-reversed XX male *Rana japonica* of the Wakuya population in Tohoku region, it was found that this species was male heterogametic (XY-type), and chromosome pair No. 4 was sex chromosomes. There was a C-band at the basal portion of the long arm of the X chromosome, and none on the Y chromosome (MIURA, 1984). On the other hand, by examining the linkages between sex-determining genes and 11 loci controlling eight enzymes and one blood protein, it was found that in the Ichinoseki and Toyama populations in eastern Japan of *R. japonica*, the sex-determining genes linked with the MPI locus, while in eight populations of western Japan, they linked with the Ab locus, and they did not link with the MPI locus. In the Akita population of eastern Japan, they did not link with the Ab locus nor with the MPI locus. However, it was assumed that the populations in eastern and western Japan were male heterogametic (SUMIDA and NISHIOKA, 1994).

In *Rana nigromaculata* group, NISHIOKA and SUMIDA (1994) reported that in the Konko population of *R. brevipoda*, the Hiro and Kumano populations and a part of the Kaita population of *R. nigromaculata*, the sex chromosomes could not be distinguished, although they were all male heterogametic, and the sex-determining genes linked with the SORDH, MPI, ENO, HK, Pep-B and LDH-B loci on chromosome No. 4. However, in the Maibara population of *R. brevipoda*, the foregoing six loci did not link with the sex-determining genes, although the latter linked with the ME-B locus on chromosome No. 3. NISHIOKA and SUMIDA, moreover, reported that the sex-determining genes in the remaining part of the Kaita population were situated on other than chromosomes Nos. 3 and 4.

In *R. rugosa* collected from the suburbs of Hiroshima city, KAWAMURA and NISHIOKA (1977) first reported that this species was male heterogametic on the basis of the femaleness of diploid gynogenetic frogs. In contrast, TOBISHIMA and SAITOH (1989) reported that *R. rugosa* collected from Hirosaki city, Aomori Prefecture and Yuda-cho, Iwate Prefecture were of the ZW-ZZ type by analyzing the karyotypes with the conventional staining and C-banding methods. The existence of these two types of sex-determining mechanisms found in the northeastern and western regions of Japan was reconfirmed soon thereafter by NISHIOKA, MIURA and SAITOH (1990, 1993). In order to clarify the distributions and origins of the two types of sex-determining mechanisms in Japan, NISHIOKA, KODAMA,

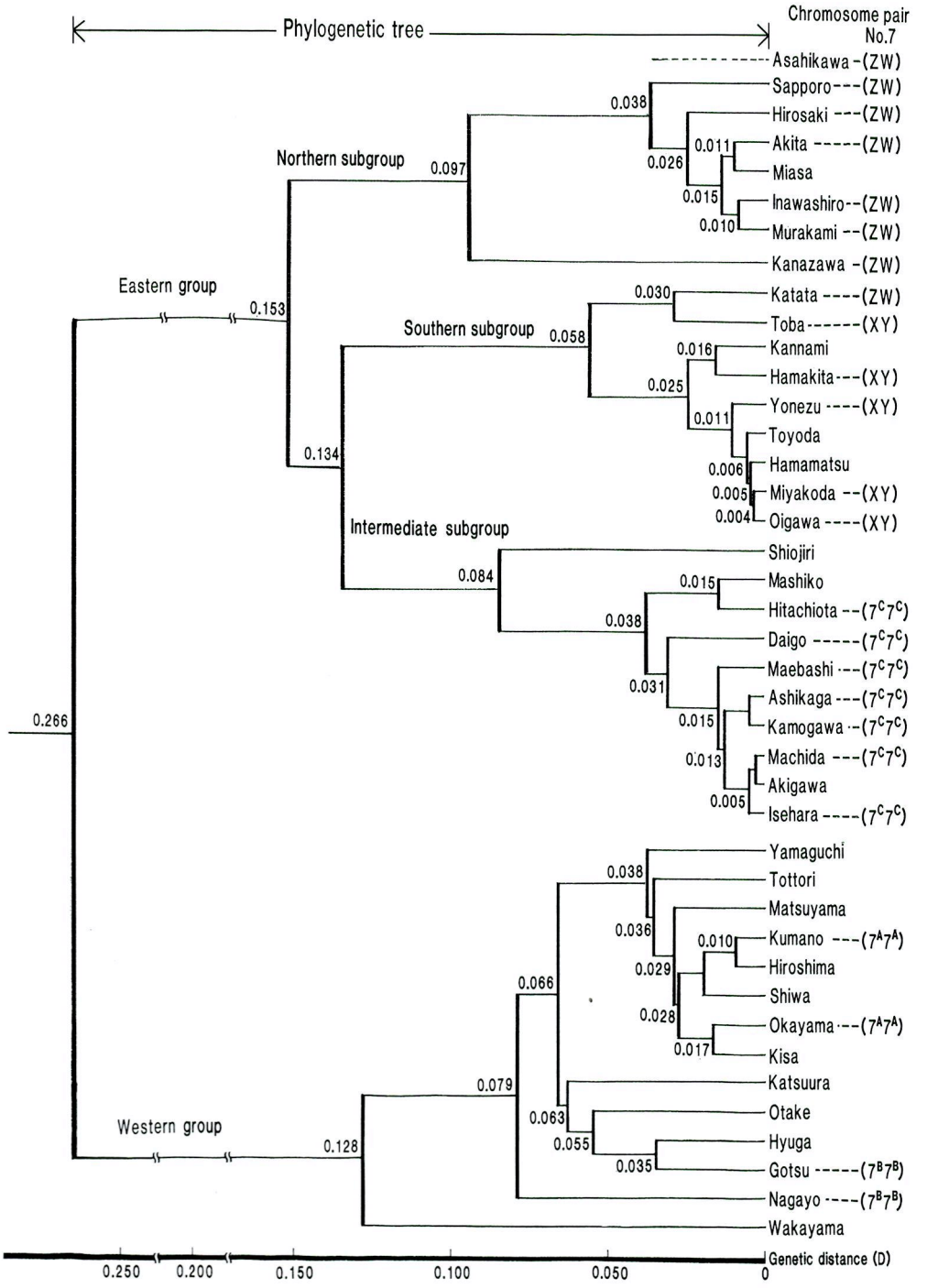

Fig. 15. Phylogenetic tree drawn for 40 populations of *Rana rugosa* by the UPGMA clustering method (NISHIOKA, KODAMA, SUMIDA and RYUZAKI, 1993), and the variations of chromosome pair No. 7 in 24 populations are shown.

SUMIDA and RYUZAKI (1993) drew a phylogenetic tree of *R. rugosa* from 40 populations distributed in Japan on the basis of the genetic distances by the UPGMA method. This phylogenetic tree showed that *R. rugosa* which invaded into Japan was first divided into the eastern and western groups. The eastern group contained 26 populations of eastern Japan, while the western group contained 14 populations of western Japan. The eastern group was then divided into three subgroups, northern subgroup containing seven populations, intermediate subgroup containing 10 populations, and southern subgroup containing nine populations.

The sex-determining mechanisms examined in these three subgroups of the eastern group and western group of *R. rugosa* were reported by NISHIOKA, MIURA and HANADA (1991). The populations of the northern subgroup seemed to be of the ZW-ZZ type in sex-determining mechanism. In the intermediate subgroup, the sex chromosomes were not morphologically distinguishable from each other and the sex-determining mechanism was obscure. In the southern subgroup, the sex chromosomes were examined in six of the nine populations. While one (Katata) population was of the ZW-ZZ type, five others were of the XX-XY type. In these five populations, the Y chromosomes morphologically differed from the X chromosomes. In the western group including 14 populations, sex chromosomes were examined only in five populations. NISHIOKA, MIURA and SAITOH (1993) and NISHIOKA, MIURA and HANADA (1991) have reported that these five populations of *R. rugosa* have no heterozygous chromosome pair. All the 13 chromosome pairs were completely homozygous.

In the present study, the sex chromosomes of *R. rugosa* distributed widely in Japan were analyzed by the methods of conventional GIEMSA staining, C-banding and late replication (LR)-banding. The 12 pairs other than chromosome pair No. 7 had no sex difference in all the populations. In chromosome pair No. 7, there were sex-specific changes among some local populations (Figs, 15, 16). Of the eastern group, seven populations belonging to the northern subgroup and one population of the southern subgroup had chromosome pair No. 7 which was the sex chromosomes of the ZW type. The Z chromosome was subtelo- or submetacentric, while the W chromosome was metacentric. By the C-banding and LR-banding patterns, the Z chromosome was divided into five types, while the W chromosome was divided into two types, W^1 and W^2. Five populations of the southern subgroup had chromosome pair No. 7 which was the sex chromosomes of the XY type. The X chromosome was metacentric and the Y chromosome was subtelo- or submetacentric. By the C-banding and LR-banding patterns, the Y chromosome was divided into two types, Y^A and Y^B, while the X chromosome was similar to the W^1 chromosome of the northern subgroup. However, the C-band at the distal portion of the long arm in the X chromosome was distinguished from that of the W^1 chromosome by weakness in staining, as found in W^1X chromosomes of the female hybrids between females of the Murakami population (W^1Z^B) and males of the Hamakita population (XY^A) (NISHIOKA and HANADA, 1994). Seven populations belonging to the intermediate subgroup had no sex differences

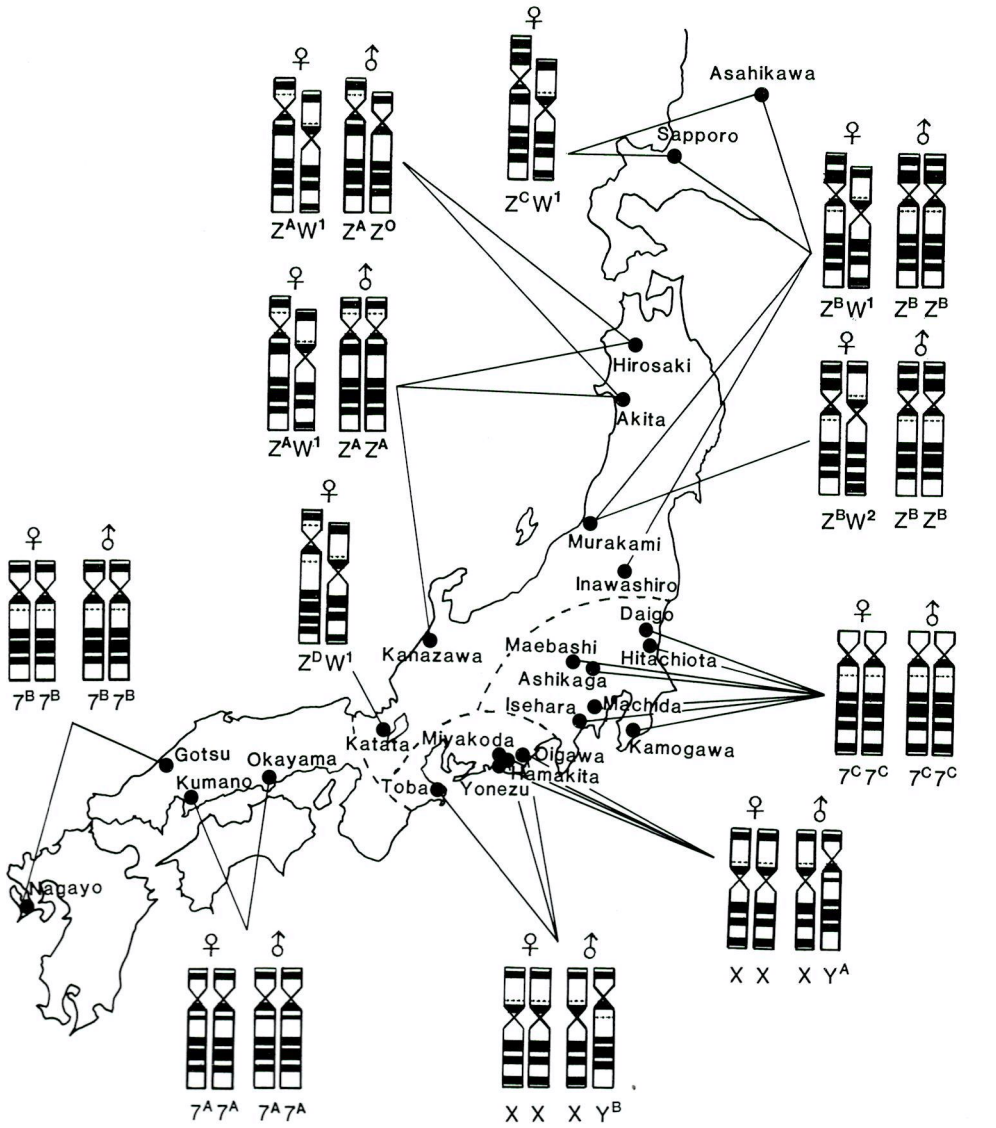

Fig. 16. Distribution of different types of chromosome pair No. 7 (sex chromosome) in 24 populations of *Rana rugosa*.

in all the 13 chromosome pairs. All these populations were obscure in sex-determining mechanism. Chromosome pair No. 7 consisted of homologous subtelocentric chromosomes in both males and females. Chromosome No. 7 was 7^C type which was considered to have been produced from the 7^B type chromosome of the western group by a pericentric inversion (Fig. 3). The X chromosome in the southern subgroup and the W^1 chromosome in the northern subgroup were considered to have been produced from the 7^C type chromosome of the intermediate subgroup by a pericentric inversion (Fig. 3).

Four populations of the western group had no sex difference in all the 13 chromosome pairs. However, this group was evidently of the XY type in sex-determining mechanism on the basis of the results of breeding experiments using males which were sex-reversed from genetic females by injection of androgen. Chromosome pair No. 7 consisted of homozygous submeta- or subtelocentric chromosomes in both males and females. Chromosome No. 7 was divided into two types, 7^A and 7^B, by the C-banding and LR-banding patterns. These two types were very similar to the Y^A and Y^B types, respectively, in the southern subgroup. In order to produce the X chromosome of the southern subgroup or the W^1 chromosome of the northern subgroup from the 7^B type chromosome of the western group, it is considered that two sequential pericentric inversions were necessary (Fig. 3).

Thus, it seems evident in *R. rugosa* that chromosome pair No. 7 produced different types of sex chromosomes in the western group and the northern and southern subgroups other than the intermediate subgroup of the eastern group at the beginning of sex differentiation after invasion into Japan.

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