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Sex Determining Mechanism in *Buergeria buergeri* (SCHLEGEL) I. Heterozygosity of Chromosome Pair No. 7 in the Female

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(With 8 Text-figures)

INTRODUCTION

The common bell-ring frog, *Buergeria buergeri*, belonging to Rhacophoridae is a unique frog which has been reared from old times in Japan to respond the beautiful mating call of males. SATO (1934), YAMAMOTO and MASUDA (1973) and OKUMOTO (1977) have reported that the chromosomes of this species are 26 in diploid number, consisting of 10 large and 16 small ones. However, they did not notice the existence of a heteromorphic pair of chromosomes.

In amphibians, it has been a conflicting problem for a long time whether sex chromosomes can be morphologically identified or not. While WITSCHI (1922, 1929, 1933) and YOSIDA (1957) reported a pair of sex chromosomes, X- and Y-chromosomes, in *Rana temporaria* and *Hyla arborea japonica*, respectively, and WEILER and OHNO (1962) reported a pair of Z- and W-chromosomes in *Xenopus laevis*, many other investigators have never recognized a heteromorphic pair of sex chromosomes in these species. However, SCHMID (1980) recently reported that an African frog, *Pyxicephalus adspersus*, belonging to Ranidae has highly differentiated sex chromosomes of ZW-type. This report was immediately followed by another of SCHEMPP and SCHMID (1981) who demonstrated heteromorphic sex chromosomes of XY-type in *Rana esculenta*.

The present author has recently observed that one of the lampbrush bivalents in the oocytes of *Buergeria buergeri* is evidently heteromorphic. The female heterozygosity of this species was also confirmed by examining the chromosomes of somatic cells. The results of observations on lampbrush chromosomes as well as on somatic chromosomes will be reported in this paper.

MATERIALS AND METHODS

Five female *Buergeria buergeri* (SCHLEGEL) from Togouchi-cho, Hiroshima Prefecture (3), Omogo, Ehime Prefecture (1) and Kurama, Kyoto Prefecture (1) were used in examining lampbrush chromosomes. The preparations of lampbrush chromosomes were principally made by the method of GALL (1966).

A mixture of 5 parts of 0.075 M KCl, 1 part of 0.075 M NaCl and 0.1 part of 10% formalin was used in order to disperse lampbrush chromosomes. Lampbrush chromosomes of each female were analyzed in 20 or 30 oocytes which were 2.0 mm in mean diameter.

The preparations of somatic chromosomes were obtained from 22 female adult frogs and 41 male adult frogs collected from Togouchi-cho, Hiroshima Prefecture. The chromosomes were analyzed in 401 metaphase plates from the females and in 1704 metaphase plates from the males. The preparations were principally made by the method of OMURA (1967).

Somatic chromosomes were also observed in the tail-tips of 612 tadpoles collected from Togouchi-cho, Hiroshima Prefecture. The preparations were principally made by the method of MAKINO and NISHIMURA (1952) and NISHIOKA (1972). However, colchicine was not applied to the tadpoles, as it was necessary to rear them continuously after examining their chromosomes.

The number of nucleoli in the nucleus of each cell was counted in the tail-tips of 342 of the above 612 tadpoles whose karyotypes were determined. The tail-tips were fixed in NAVASHIN's fluid and stained with HEIDENHAIN's iron hematoxylin. The volume of each nucleolus was calculated from the area measured in a photograph.

OBSERVATION

I. Lampbrush chromosome

The oocytes of female *Buergeria buergeri* contained 13 lampbrush bivalents. The homologues of each bivalent were joined with each other by chiasmata or terminal fusions. The bivalents were divided into two groups in size. Group 1 included five large bivalents, Nos. I to V, while group 2 included eight small bivalents, Nos. VI to XIII (Fig. 1). Landmarks of lampbrush chromosomes were extremely few in *Buergeria buergeri*, that is, only four bivalents, Nos. II, VII, XII and XIII, possessed one or two landmarks (Fig. 2). Bivalent No. VII carried a large sphere which was $8 \sim 16\ \mu$ in diameter. This sphere was very similar in morphology to the free floating nucleoli abundantly existing in the germinal vesicle of an oocyte (Fig. 3). Bivalent No. VII in the oocytes of females is heteromorphic, as this sphere is situated on one of the homologues of the bivalent. It is well-known that the nucleolar organizer is recognized as a secondary constriction in a somatic chromosome. Then, the present author compared lampbrush bivalent No. VII with somatic chromosome No. 7, as the latter has a satellite which is connected to the long arm of the chromosome with a kind of secondary constriction. It is noteworthy that the pair of somatic No. 7 chromosomes in males is homomorphic, as each of the two homologues has a satellite, while that is heteromorphic in females, as the satellite exists only in one of the two homologues. It was found that the position of the sphere in lampbrush chromosome No. VII almost agreed with that of this secondary constriction. Consequently, it is evident that

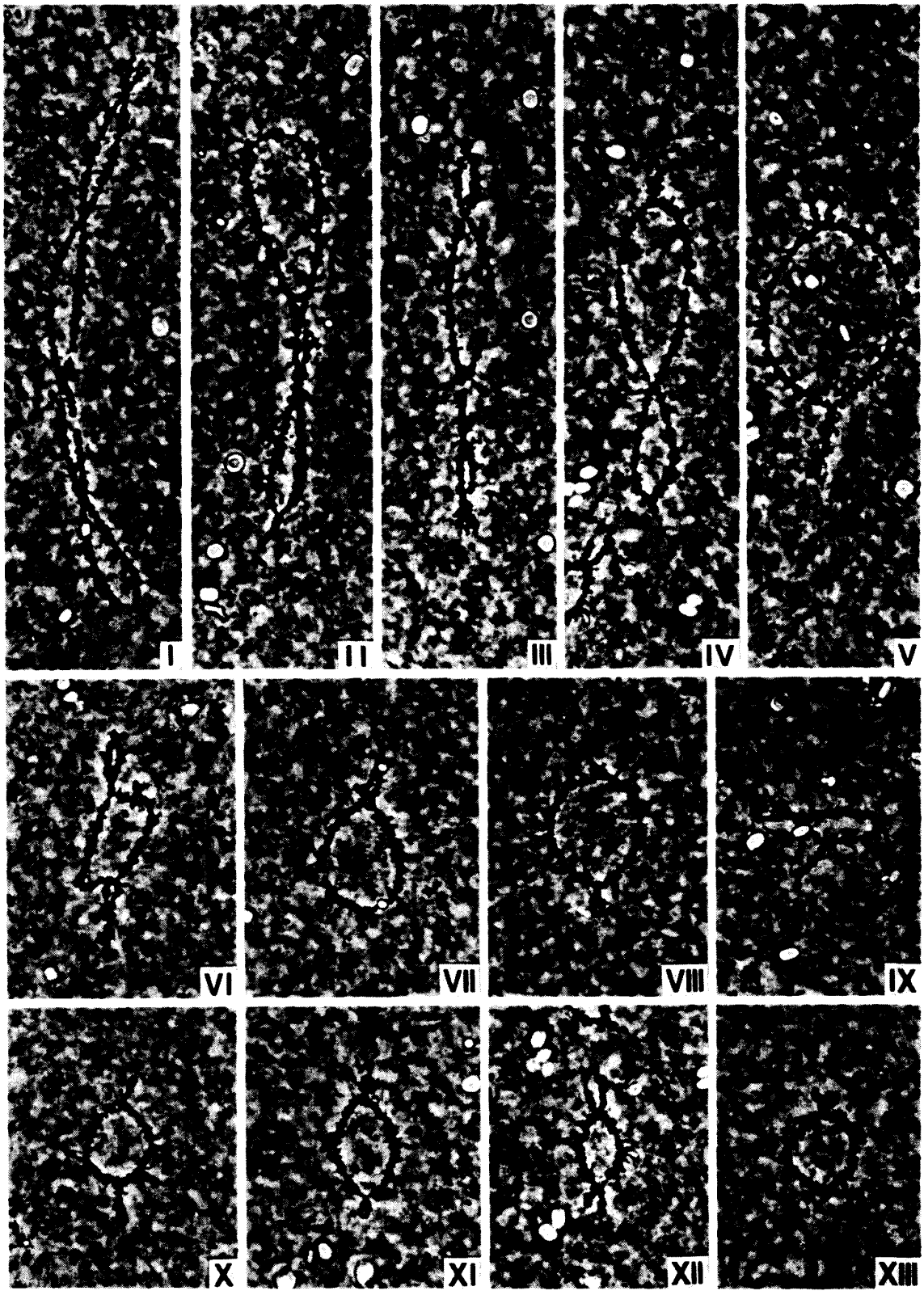

Fig. 1. Microphotographs of bivalent chromosomes Nos. I~XIII in an oocyte of a female *Buergeria buergeri*. ×600

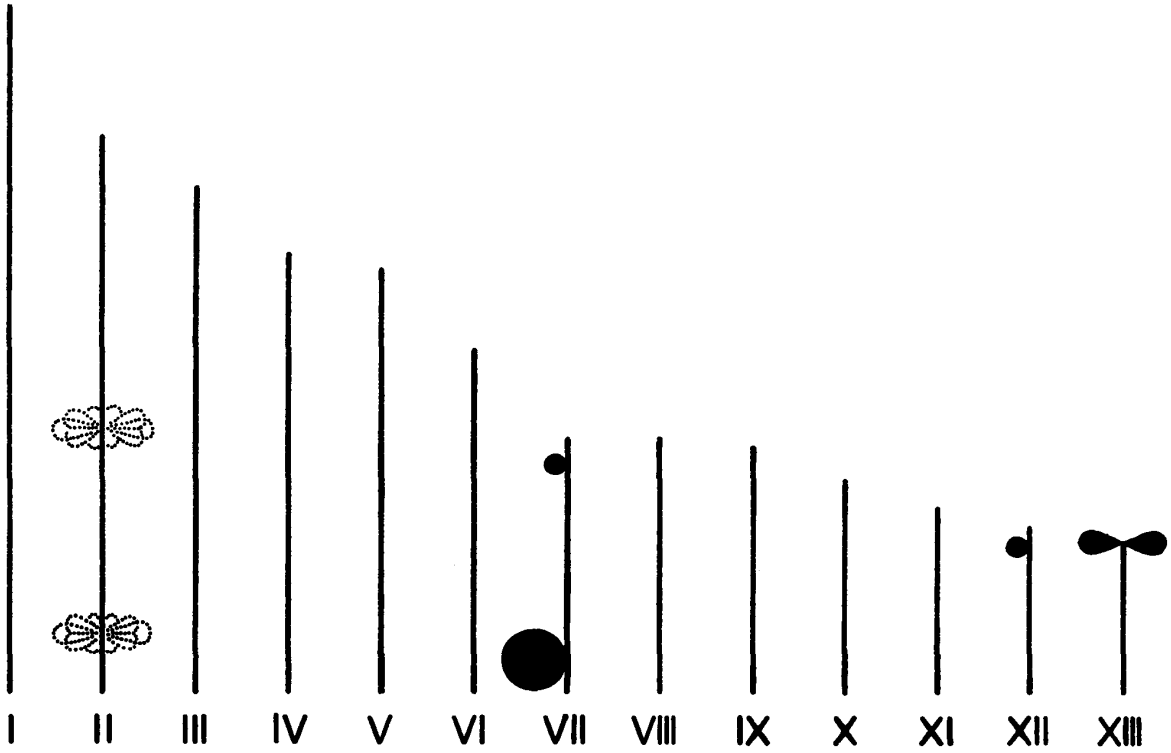

Fig. 2. Diagram showing the relative lengths and landmarks of thirteen bivalent chromosomes of *Buergeria buergeri*.

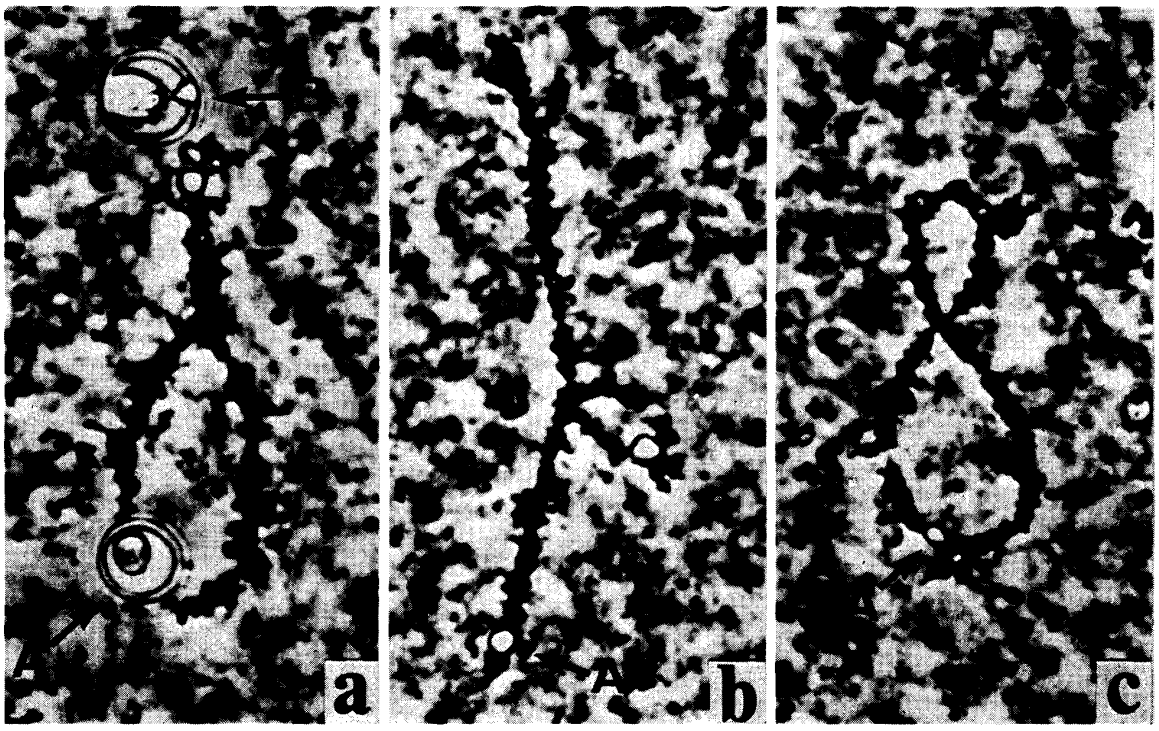

Fig. 3. Microphotographs of bivalent chromosome No. VII carrying a large sphere. ×1200
a. Hiroshima population. b. Ehime population. c. Kyoto population.
A, sphere B, free nucleolus

this sphere is a nucleolus. The segment having the sphere corresponded to a part of the long arm of somatic chromosome No. 7, and the sphere was located at 13.3% of lampbrush chromosome No. VII.

Each of lampbrush bivalent chromosomes Nos. I~XIII had 0~9 chiasmata. The frequency distribution of chiasmata in each bivalent is presented in Tables 1~3. The largest bivalent (No. I) mostly had 4~7 chiasmata. The other four large bivalents (Nos. II~V) generally had 2~5 chiasmata, while the largest (No. VI) of the eight small bivalents usually had 2~4. Most of the six smallest bivalents (Nos. VIII~XIII) had two chiasmata. Bivalent No. VII was exceptional in chiasma frequency. It was evidently distinguished from the other 12 bivalents by paucity of chiasmata (Tables 1~3). As the centromere could not be observed in the lampbrush chromosomes of *Buergeria buergeri*, the distribution of chiasmata was examined in bivalents Nos. II, VII, XII and XIII which had landmarks for

TABLE 1

Number of chiasmata in the 13 lampbrush chromosomes in the Hiroshima population

Chromosome no.	0	1	2	3	4	5	6	7	8	9	Total	Fusion	Mean
I			1	2	5	10	5	5	1	1	160	4	5.33 (5.47)
II			5	9	6	8		1	1		116	4	3.87 (4.00)
III			2	5	14	6	2	1			124	4	4.13 (4.27)
IV			3	8	15	3		1			112	4	3.73 (3.87)
V			2	12	11	3	2				111	6	3.70 (3.90)
VI			8	13	7	2					93	1	3.10 (3.13)
VII	17	13									13	22 (+21)*	0.43 (1.87)
VIII			20	9	1						71	1	2.37 (2.40)
IX			20	9		1					72	1	2.40 (2.43)
X		2	20	6	2						68	4	2.27 (2.40)
XI		1	22	6	1						67		2.23
XII		2	24	3	1						63	(+1)*	2.10 (2.13)
XIII		2	24	3	1						63	1 (+3)*	2.10 (2.23)

* The number in parentheses represents the joins at the landmarks.

TABLE 2

Number of chiasmata in the 13 lampbrush chromosomes in the Ehime population

Chromosome no.	0	1	2	3	4	5	6	7	8	Total	Fusion	Mean
I					1	10	5	3	1	113	2	5.65 (5.75)
II			1	7	9	3				74	3 (+3)*	3.70 (4.00)
III				3	12	4	1			83		4.15
IV			2	7	9	1	1			72		3.60
V				8	9	3				75		3.75
VI			10	8	2					52	1	2.60 (2.65)
VII	1	16	3							22	12 (+19)*	1.10 (2.65)
VIII			14	5	1					47		2.35
IX			15	5						45		2.25
X			18	1	1					43		2.15
XI			19	1						41		2.05
XII		1	19							39	1	1.95 (2.00)
XIII		1	18	1						40	1	2.00 (2.05)

* The number in parentheses represents the joins at the landmarks.

TABLE 3

Number of chiasmata in the 13 lampbrush chromosomes in the Kyoto population

Chromosome no.	1	2	3	4	5	6	7	8	Total	Fusion	Mean
I				4	9	6		1	105		5.25
II		1	10	6	3				71	3	3.55 (3.70)
III		1	3	9	7				82	1	4.10 (4.15)
IV			8	10	2				74		3.70
V		3	9	7	1				66		3.30
VI		5	13	2					57		2.85
VII	12	6	2						30	8 (+12)*	1.50 (2.50)
VIII		18	2						42		2.10
IX		16	4						44	1	2.20 (2.25)
X		18	2						42		2.10
XI	1	17	2						41	1	2.05 (2.10)
XII		20							40	1	2.00 (2.05)
XIII		20							40	1	2.00 (2.05)

* The number in parentheses represents the joins at the landmarks.

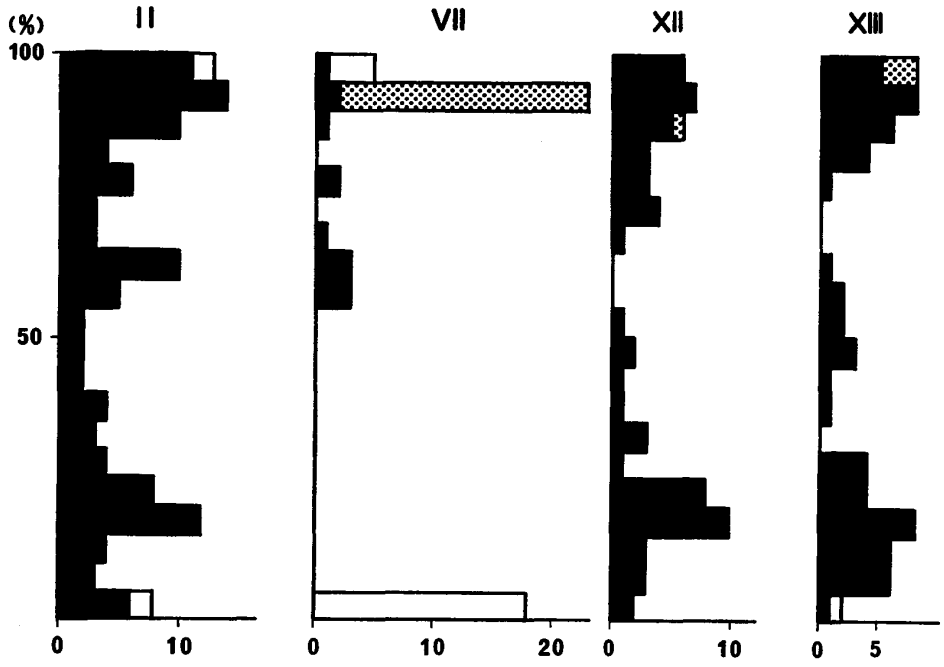

Fig. 4. Graphs of bivalent chromosomes Nos. II, VII, XII and XIII showing chiasma distributions in the Hiroshima population. The relative lengths of chromosomes and the numbers of chiasmata are shown on the ordinate and abscissa, respectively. A black area represents the number of chiasmata, a white area represents the number of terminal fusions, and a dotted area represents the number of gene product fusions.

the purpose of assuming the position of the centromere. It was found that chiasmata were nearly one-sided in distribution in bivalent No. VII, while they were almost evenly distributed along the total length in the other three bivalents, Nos. II, XII and XIII (Fig. 4).

In 30 oocytes obtained from three females of the Hiroshima population, 17 of 30 No. VII bivalents had no chiasma, while the other 13 had a single chiasma.

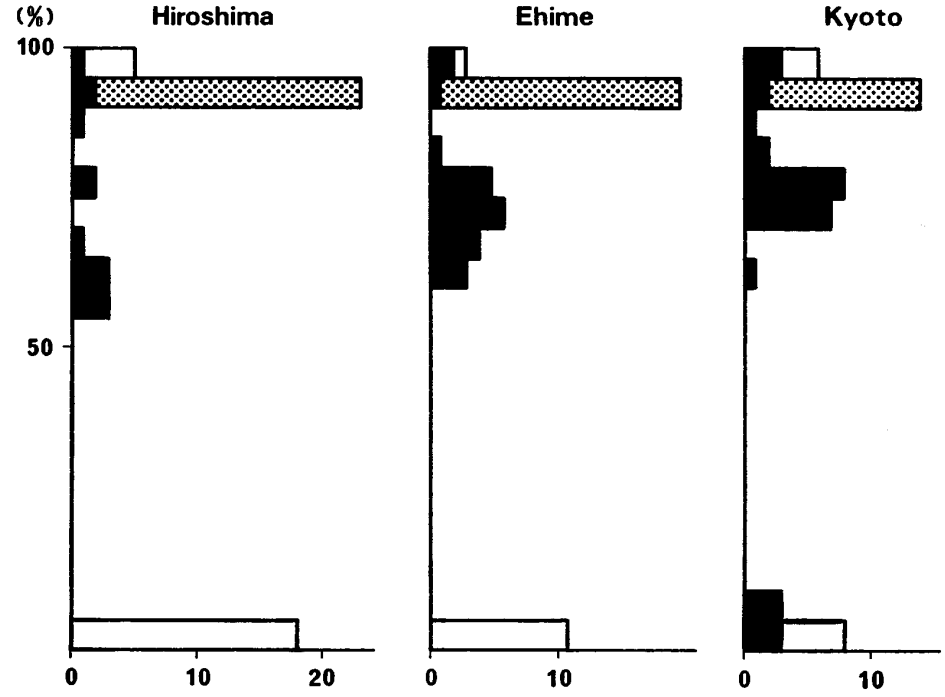

Fig. 5. Graphs of bivalent chromosome No. VII showing chiasma distributions in three populations.

Bivalent No. VII was 0.43 in chiasma frequency, whereas the other seven small bivalents were 2.10~3.10. The homologues of each of the bivalents lacking a chiasma were joined with each other by one or two terminal fusions or at the landmark situated at the site of 90.4% of the bivalent. Some of the bivalents having one chiasma were also joined by one or two terminal fusions or at the landmark. Bivalent No. VII had a total of 22 terminal fusions and a total of 21 joins situated at the landmark in the 30 bivalents. In contrast, each of the other bivalents had no or a few terminal fusions. Besides, one of bivalents No. XII and three of bivalents No. XIII had one join at the landmark. In any case, no chiasmata were observed in the range between the tip (0%) and the middle (about 55%) of each bivalent No. VII (Fig. 5).

In 20 oocytes removed from a female of the Ehime population, only one of 20 No. VII bivalents had no chiasma. Of the remaining 19, 16 and three had one and two chiasmata, respectively. Bivalent No. VII was 1.10 in chiasma frequency, while the other seven small bivalents were 1.95~2.60. The homologues of the single bivalent lacking chiasmata were joined with each other by terminal fusions and a join situated at the landmark. The other 19 bivalents having one or two chiasmata had usually one or two terminal fusions or a join at the landmark. Bivalent No. VII had a total of 12 terminal fusions and a total of 19 joins at the landmark in the 20 bivalents. In all the 20 bivalents, no chiasmata were observed in the range between the tip (0%) and a site (about 60%) slightly apart from the middle of each bivalent (Fig. 5).

In 20 oocytes obtained from a female of the Kyoto population, 12, 6 and 2 of

20 No. VII bivalents had one, two and three chiasmata, respectively. Bivalent No. VII was 1.50 in chiasma frequency, while the other seven small bivalents were 2.00~2.85. The 20 bivalents had a total of eight terminal fusions and a total of 12 joins at the landmark. In all of these bivalents, no chiasmata were observed in the range between a site (about 10%) near the tip and another site (about 60%) near the middle of each bivalent (Fig. 5). In contrast with bivalent No. VII of the Hiroshima and Ehime populations, that of the Kyoto population had a chiasma near the tip in six of the 20 bivalents.

II. Somatic chromosome

The somatic chromosomes of both males and females were all 26 in diploid number. Like the lampbrush bivalents, they were divided into two groups in size. Group 1 included five pairs of large chromosomes, Nos. 1 to 5, while group 2 included eight pairs of small chromosomes, Nos. 6 to 13. In male frogs, each of the 13 chromosome pairs was always homomorphic. In female frogs, all the pairs except the pair of No. 7 chromosomes were also homomorphic. However, it was remarkable that the pair of No. 7 chromosomes in females was heteromorphic (Figs. 6 and 7). In detail, one homologue of this pair had a satellite, while the other had none. In males, both homologues of this pair were satellite chromosomes.

Then, the present author observed chromosomes in epidermal cells of the tail-tips of 612 tadpoles of *Buergeria buergeri*. It was found that 333 of these

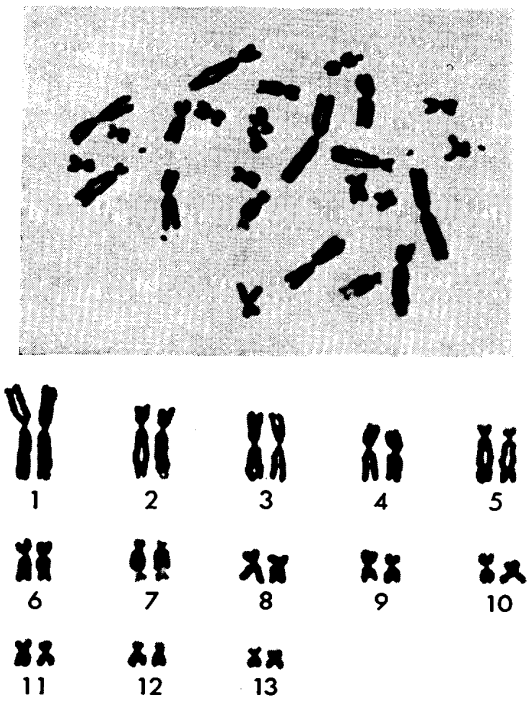

Fig. 6. Metaphase plate and the karyotype of a bone marrow cell from a male *Buergeria buergeri*. ×900

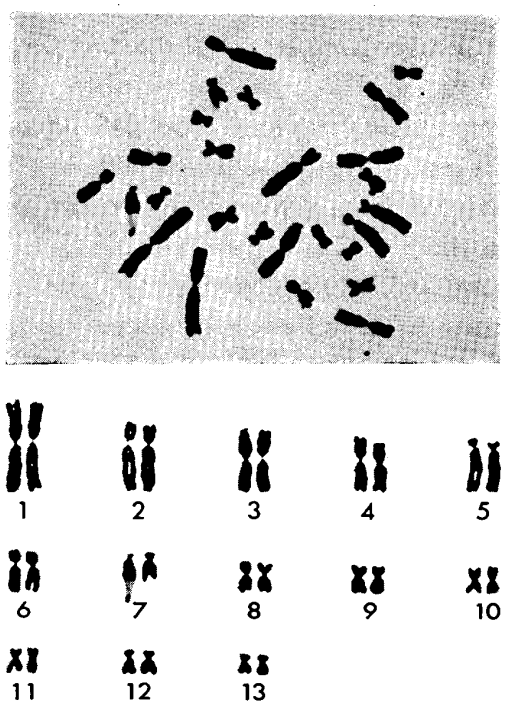

Fig. 7. Metaphase plate and the karyotype of a bone marrow cell from a female *Buergeria buergeri*. ×900

TABLE 4
The relationship between the zygosity of No. 7 chromosomes and the sex of juveniles

	Zygosity		
	Total	Hetero	Homo
No. of tadpoles	612	333	279
No. of juveniles	214	145	69
♀		136	0
♂		9	69

tadpoles were heteromorphic in the pair of No. 7 chromosomes, while the other 279 were homomorphic in this pair (Table 4).

All the tadpoles, whose chromosomes were elucidated, were continuously reared. When they attained completion of metamorphosis and grew further for a few months, their sex was identified by observing their gonadal structure. The results showed that 136 of 145 heterozygous tadpoles became females with well-differentiated ovaries, while the remaining nine became males with testes. On the other hand, all of 69 homozygous tadpoles became males with well-differentiated testes. Thus, it seems evident that the female is heterozygous in *Buergeria buergeri* and that chromosome No. 7 is a sex chromosome.

III. Nucleolus

The number of nucleoli in the nucleus of each epidermal cell was counted in

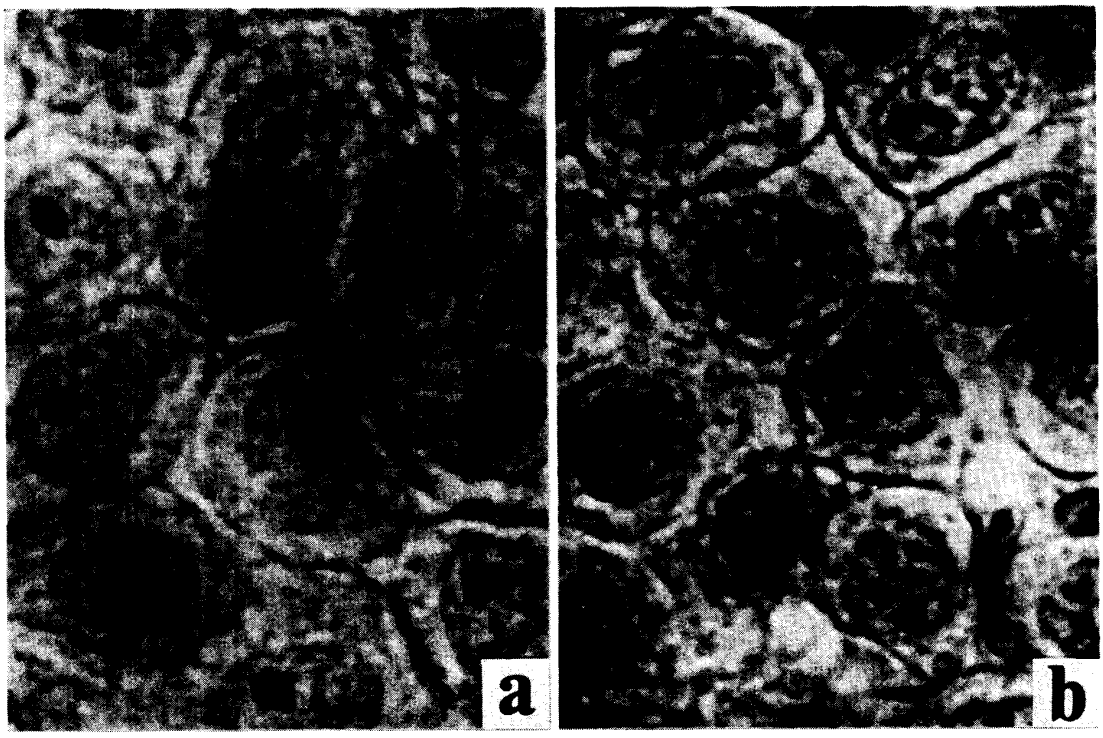

Fig. 8. Microphotographs of epidermal cells of tadpoles. ×2000

a. Tadpole having heterozygous No. 7 chromosomes.
b. Tadpole having homozygous No. 7 chromosomes.

168 homozygous and 174 heterozygous tadpoles. It was found that 164 of the homozygous tadpoles had two nucleoli, while the remaining four possessed only one. On the other hand, 167 of the heterozygous tadpoles possessed only one nucleolus, while the remaining seven had two. The single nucleolus contained in each cell of the heterozygous tadpoles were evidently larger than one of the two nucleoli in each cell of the homozygous tadpoles (Fig. 8). Then, the volume of nucleoli was measured in both homozygous and heterozygous tadpoles in order to compare both kinds of nucleoli more accurately. The results showed that the nucleoli of 100 nuclei of 10 heterozygous tadpoles were $3.76 \pm 0.122\ \mu^3$ on the average in volume, while the nucleoli of 100 nuclei of 10 homozygous tadpoles were $4.20 \pm 0.151\ \mu^3$ on the average in sum total of the two nucleoli in each nucleus. The difference between these two figures was not statistically significant by T-test. In the homozygous tadpoles, the two nucleoli of each nucleus were frequently fused into a single nucleolus. Such a fused nucleolus was $4.21 \pm 0.171\ \mu^3$ on the average in volume. In this case, there was no statistically significant difference in nucleolar size between heterozygous and homozygous tadpoles. Nuclei of heterozygous and homozygous tadpoles were also compared with each other in dimensions of the largest cross-section. No significant difference was found in this respect between the two kinds of tadpoles.

DISCUSSION

In amphibians, WITSCHI (1922, 1929, 1933) is the first investigator who has observed a heteromorphic chromosome pair in the meioses of spermatocytes of males belonging to the differentiated race of *Rana temporaria*. According to WITSCHI, X- and Y-chromosomes are separated in the second meiotic division. However, sex chromosomes with such a behavior during meioses subsequently have never been observed in amphibians, although abundant cytological studies have been made by many investigators. In anurans, heteromorphic chromosomes were not described by IRIKI (1932) in *Rana nigromaculata* and *Rana rugosa*, by MAKINO (1932) in *Rana chensinensis*, by SATO (1933) in *Rana limnocharis* and by GALGANO (1933) in *Rana temporaria*, by SAEZ, ROJAS and ROBERTIS (1936) in *Bufo arenarum* and by WICKBOM (1945) in 10 species. No sex chromosomes were also cytologically observed by NATARAJAN (1958) in *Bufo melanostictus*, by SAEZ and BRUM (1960) in seven anuran species of South America, by BIANCHI and LAGUENS (1964) in *Bufo arenarum*, by MORESCALCHI (1963a) in *Bufo viridis*, by SETO (1964) and SETO and MAKINO (1964) in *Hyla arborea japonica*, by MIKAMO and WITSCHI (1966) in *Xenopus laevis*, by GUILLEMIN (1967) in *Rana temporaria* and *Rana dalmatina*, by ULLERICH (1967) in *Rana temporaria*, *Rana arvalis*, *Rana esculenta*, *Bufo marinus* and *Limnodynastes tasmaniensis*, by COLE (1971) in *Phyllomedusa dacnicolor*, by DENARO (1972) in eight species of Leptodactylidae anurans, by KANG and SUNWOO (1973) in *Bufo kangii*, by WU (1978) and WU and YANG (1980) in *Bufo bufo gargarizans*, *Rana nigromaculata* and *Rana plancyi* and by SCHMID (1978) in 12 species belonging to Ranidae, Microhylidae and Rhacophoridae.

On the other hand, a pair of two unequal chromosomes has been observed by YOSIDA (1957) in diploid mitoses of male *Hyla arborea japonica*. According to him, the X-chromosome ranks in size between chromosomes Nos. 6 and 7 and the Y-chromosome is nearly of the same size as No. 8. YOSIDA's finding in the Japanese tree-frog was confirmed by MATSUDA (1963) in cultured kidney cells. A distinct heteromorphic pair of sex chromosomes has been observed by WEILER and OHNO (1962) and MORESCALCHI (1963b) in *Xenopus laevis*. This species showed female heterogamety. While the W-chromosome is the largest element in the chromosome complement, the Z was the smallest. SANDERS and CROSS (1963) reported that the male had 21 chromosomes in *Bufo houstonensis*, although no claim was made for the unpaired chromosome to be a sex chromosome. This chromosome number of male *Bufo houstonensis* was thereafter corrected and changed into 22 by BOGART (1968). KURAMOTO (1980) has reported that male *Rana narina* has two pairs of heteromorphic chromosomes, Nos. 1 and 8. The heteromorphism of pair No. 8 was much more distinct than that of pair No. 1. The sum of relative lengths of the smaller component of No. 1 and the larger component of No. 8 was roughly equal to the sum of the other components of Nos. 1 and 8. Thus, it was indeterminate to him whether the heteromorphism was an indication of sex chromosome or a result of a simple translocation.

SCHMID (1980) has found by using various banding methods highly differentiated heteromorphic sex chromosomes in the karyotype of female *Pyxicephalus adspersus*. In this species, chromosomes No. 8 are sex chromosomes, the W-chromosome being considerably smaller than the Z. SCHEMPP and SCHMID (1981) reported in the following year that chromosomes No. 4 in the karyotype of *Rana esculenta* could be identified as sex-specific chromosomes of the XX/XY-type by a banding technique devised by them. Although Y-chromosome is equal to X-chromosome in size and shape, the Y is distinguished from the X by possessing an extremely late replicating region, which is lacking in the X. In the meioses of the male, bivalent No. IV consisting of heteromorphic X- and Y-chromosomes behaves exactly the same as the autosomal bivalents do.

In contrast to anurans, heteromorphic chromosomes have often been observed in the females of urodeles. CALLAN and LLOYD (1960) have reported that the homologues of the largest lampbrush bivalent (No. I) in oocytes of the four subspecies of *Triturus cristatus* are heteromorphic, although they did not identify them to be sex chromosomes. The heteromorphism of lampbrush bivalent No. I has also been observed by MANCINO and NARDI (1971) in *Triturus marmoratus*. Chiasmata were not usually observed within the heteromorphic region of this bivalent. From these findings, they have assumed that the heteromorphic bivalent (No. I) consists of W- and Z-chromosomes. In the axolotl, HAUSCHKA and BRUNST (1964) have suggested that primary sexual dimorphism of the ZW-type is found in chromosome pair No. 13 in mitotic divisions of females but not of males. LACROIX (1968) has reported that lampbrush bivalent No. IV is heteromorphic in *Pleurodeles waltl* and *P. poireti*. LACROIX (1970) has also confirmed that bivalent No. IV is homomorphic in the oocytes of sex-reversed genetic males

of *Pleurodeles poireti*. From these findings, he has assumed the female of this species to be heterogametic (ZW). LEÓN and KEZER (1974) have reported that No. XIII of the 23 lampbrush bivalents contained in oocytes of *Siren intermedia* is heteromorphic. Recently, SCHMID, OLERT and KLETT (1979) showed that chromosomes No. 4 of *Triturus a. alpestris* and chromosomes No. 5 of *Triturus v. vulgaris* and *Triturus h. helveticus* are sex-specific. In the former two species, the male has one mitotic chromosome pair which is heteromorphic in distribution of constitutive heterochromatin. Chromosomes No. 5 of male *Triturus h. helveticus* showed a greatly reduced frequency of chiasma-formation in the long arms, although no sex-specific heteromorphism of the constitutive heterochromatin could be observed.

It has been reported by SATO (1934), YAMAMOTO and MASUDA (1973) and OKUMOTO (1977) that *Buergeria buergeri* is 26 in diploid chromosome number, consisting of 10 large and 16 small chromosomes. No heteromorphic pair of chromosomes was observed by these authors, although YAMAMOTO and MASUDA (1973) and OKUMOTO (1977) have described that chromosome No. 7 has a satellite. The present author first noticed the existence of a heteromorphic bivalent in the lampbrush chromosomes of oocytes from female *Buergeria buergeri*. Lampbrush bivalent No. VII is heteromorphic, as one of the homologues has a distinct sphere which is very similar to a nucleolus in morphology. In accord with this finding, chromosome pair No. 7 appearing at the metaphase in the mitotic division of bone marrow cells removed from female adults is heteromorphic, as one of the homologues has a satellite at the tip of the long arm, while the other has none. Chromosome pair No. 7 in the mitotic division of bone marrow cells obtained from male adults evidently differs from the finding stated above in that each homologue has a satellite. It was also found that chromosome pair No. 7 of tadpoles determined to become females is heteromorphic, while that of tadpoles determined to become males is homomorphic. These findings seem to indicate that the female is heterozygous and the male is homozygous in *Buergeria buergeri* and chromosome pair No. 7 is sex chromosomes of ZW-type. Although a few tadpoles being heteromorphic in chromosome pair No. 7 became males, this seems to be attributable to incomplete observations of chromosomes. The fact that lampbrush bivalent No. VII has no chiasmata within the range between the tip and about the middle seems to confirm that chromosome pair No. 7 is a sex chromosome pair. Besides, it is noteworthy that female tadpoles have one nucleolus in the nucleus of each somatic cell, while male tadpoles have two nucleoli. This seems to show that Z-chromosome having a satellite brings a nucleolar organizer. Such a characteristic is very useful in assuming the sex of tadpoles.

SUMMARY

1. The lampbrush chromosomes in oocytes and the mitotic chromosomes in somatic cells of tadpoles as well as in bone marrow cells of male and female

adults were observed in the common bell-ring frog, *Buergeria buergeri*. The chromosomes of this species are 26 in diploid number, consisting of five pairs of large and eight pairs of small chromosomes.

2. Lampbrush bivalent No. VII is heteromorphic, as a distinct sphere is situated at one of the homologues. Chiasma frequency is extremely low in bivalent No. VII. No chiasmata are found within the range between the tip and about the middle of this bivalent.

3. Chromosome pair No. 7 appearing in mitoses of bone marrow cells removed from female adults is heteromorphic. One of the homologues has a satellite at the tip of the long arm, while the other has none. Each of the homologues of chromosome pair No. 7 appearing in mitoses of male adults has a satellite.

4. Chromosome pair No. 7 of tadpoles determined to become females is heteromorphic, while that of tadpoles determined to become males is homomorphic.

5. It seems evident that *Buergeria buergeri* is female heterozygous in sex determination and that chromosome pair No. 7 is a pair of sex chromosomes of ZZ/ZW-type.

6. The somatic cells of male tadpoles have two nucleoli in the nucleus, while those of female tadpoles have a single nucleolus. This seems to show that the nucleolus is derived from the nucleolar organizer of Z-chromosome.

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