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The Karyotypes of the Two Sibling Species of Japanese Pond Frogs, with Special Reference to Those of the Diploid and Triploid Hybrids

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(With 10 Text-figures)

INTRODUCTION

The fact that the chromosomes of Japanese pond frogs, *Rana nigromaculata,* are 26 in diploid number has been reported by IRIKI (1932) and ascertained by KAWAMURA (1937, '39a). The same number has been counted by TING (1939) in specimens collected in China. While the frogs used by KAWAMURA as material were collected from Hiroshima, those of IRIKI were from Kyoto. In those days, the Japanese pond frogs were generally considerd to belong to one subspecies, *Rana nigromaculata nigromaculata.* However, it later became clear that there are two sympatric species, *Rana nigromaculata* and *Rana brevipoda* in a wide area including Kyoto district (ITO, 1941; MORIYA, 1951, '54, '59a, b, c; KAWAMURA, 1962). These two species are distinguishable from each other by differences in morphological, embryological and ecological characters. They are usually isolated from each other by ecological differences.

Although the two species are also isolated from each other by hybrid sterility, the latter is in a incomplete status. While male hybrids are nearly completely sterile, female hybrids are fertile and can produce their progeny by mating with males of the parental species. It has been known that natural hybridization frequently occur in Niigata district bordering the Sea of Japan and that the areas from this district to the eastern and north-eastern plains are occupied by a subspecies, *Rana brevipoda porosa,* which is intermediate in morphological character (KAWAMURA, 1962). Moreover, some traces of the morphological characteristics of *Rana nigromaculata* are found in the populations of *Rana brevipoda brevipoda* distributed in the western districts, such as Nagoya and Kyoto. In the most western districts of distribution including Okayama, the populations of this species appear to have been kept most purely in morphological character. In Hiroshima district, there is solely *Rana nigromaculata.*

The present authoress compared the karyotypes of the two species by making use of typical specimens, that is, *Rana brevipoda* from Okayama and *Rana nigromaculata* from Hiroshima district. She also observed the chromosomes of reciprocal diploid as well as triploid hybrids between the two species, as it was supposed that the chromosomes of each species are easily and accurately detectable in the

metaphase plates of such hybrids. The main results of these observations are presented in this paper.

MATERIAL AND METHODS

Tadpoles of the two frog species, *Rana nigromaculata* HALLOWELL and *Rana brevipoda* ITO, were utilized for examining the karyotypes of these species. The tadpoles of each species developed from eggs of five females by artificial fertilization with sperm of five males. The males and females of *Rana nigromaculata* were collected from the suburbs of Hiroshima, while those of *Rana brevipoda* were from the suburbs of Okayama. The ovulation of each female was accelerated by an injection of a mixture of a frog pituitary and 0.1 cc. of progesterone at the concentration of 10 mg./cc.

Triploid tadpoles can be easily produced from fertilized eggs by suppression of the second polar body formation. In the present research, autotriploids of *Rana nigromaculata* and reciprocal allotriploids of *R. nigromaculata* and *R. brevipoda* were utilized for karyotype analysis. The autotriploids were obtained by refrigeration of eggs of two female *nigromaculata* at 1～2°C for three hours, 20 minutes after insemination with sperm of two males of the same species. It was found that more than 80% of the tadpoles developed from these eggs were triploids.

Allotriploid tadpoles were produced from eggs of three female *nigromaculata* inseminated with sperm of three male *brevipoda* by the same method as stated above. The cell nuclei of these triploids consisted of two *nigromaculata* and one *brevipoda* genomes. The reciprocal allotriploids were also produced from eggs of a female *brevipoda* inseminated with sperm of a male *nigromaculata* by refrigeration at 1～3°C for 2～2.5 hours, 20 minutes after insemination. The cell nuclei of these allotriploids consisted of two *brevipoda* and one *nigromaculata* genomes.

As the control series of the autotriploid *nigromaculata,* eggs of the same females inseminated with sperm of the same males were reared up to the tadpole stage. On the other hand, reciprocal diploid hybrids produced from the same females and males as those of the experimental series of reciprocal allotriploids were reared as the respective controls.

A preparation was made from the tail-tip of each tadpole by the squash method after water pretreatment, according principally to MAKINO and NISHIMURA (1952). The procedure of this method is as follows: tadpoles were kept alive for 15～18 hours at room temperature, in a 0.005% colchicine (Merck) solution. Then their tail-tips were cut off, immersed in distilled water for 60～90 minutes and stained for 30～60 minutes on slide glasses with 1% orcein (Chroma) in 45% acetic acid. Lastly, they were squashed under cover glasses after being heated for 20～30 seconds and mounted with PVLB*.

Karyotype analysis was carried out by making use of 4500-times-enlarged

* PVLB: paraffin, vaselinum, lanolin and Canada balsam=2: 1: 1: 1

photographs of metaphase spreads. In each of the photographs, the length of each chromosome was measured and then an average length of each pair of homologous chromosomes was calculated. The sum total of such average lengths calculated on all the kinds of chromosome pairs was treated as the length of one genome. A hundred-times quotient of the average length of each pair of homologous chromosomes divided by the length of the genome was called the relative chromosome length of this pair. The position of the centromere of each chromosome was presented by a hundred-times quotient of the short arm length divided by the chromosome length.

The chromosomes of *Rana nigromaculata* were analysed on 407 metaphase spreads of 105 tadpoles obtained from five females. Out of these metaphase spreads a hundred were selected and utilized to calculate the relative chromosome length and the position of the centromere of each chromosome. In *Rana brevipoda*, 393 metaphase spreads of 100 tadpoles obtained from five females were used for karyotype analysis. A hundred of them were especially selected in order to calculate the same as those in the other species (Table 1).

The karyotype of *Rana nigromaculata* was compared with that of *Rana brevipoda* in terms of relative chromosome length and centromere position by HUBBS and HUBBS' method (1953).

TABLE 1

Number of tadpoles and their mitoses examined for chromosome analysis

Species	Locality	Series	No. of analysed tadpoles	No. of analysed mitoses	No. of measured mitoses
Rana nigromaculata	Hiroshima	(N)NN.No. 1	20	59	14
		(N)NN.No. 2	20	85	19
		(N)NN.No. 3	20	76	23
		(N)NN.No. 4	20	94	23
		(N)NN.No. 5	25	93	21
		Total	105	407	100
Rana brevipoda	Okayama	(B)BB.No. 1	20	82	20
		(B)BB.No. 2	20	64	16
		(B)BB.No. 3	20	76	15
		(B)BB.No. 4	20	73	24
		(B)BB.No. 5	20	98	25
		Total	100	393	100

The following abbreviations are used in the present paper.

(N)NB—— Diploid hybrid between a female *nigromaculata* and a male *brevipoda*.

(B)BN—— Diploid hybrid between a female *brevipoda* and a male *nigromaculata*.

(N)NNN— Autotriploid of *Rana nigromaculata*.

(N)NNB— Triploid hybrid (Allotriploid) consisting of two *nigromaculata* and one *brevipoda* genomes.

(B)BBN— Triploid hybrid (Allotriploid) consisting of two *brevipoda* and one *nigromaculata* genomes.
(N)——— *Rana nigromaculata* cytoplasm.
(B)——— *Rana brevipoda* cytoplasm.

OBSERVATION

I. Karyotypes of two Japanese pond frog species

1. *Rana nigromaculata* HALLOWELL

The chromosomes are 26 in diploid number, as established by IRIKI (1932). The karyotype is shown in Fig. 1, where the chromosomes are arranged in the order of length. There are 13 pairs, each of which consists of two homologous chromosomes of quite the same size and shape. They are divided into two groups according to their size. Group 1 consists of five large chromosomes, Nos. 1 to 5, while group 2 of eight small ones, Nos. 6 to 13. The relative length and the numerical value of the centromere position of each chromosome are presented in Tables 2 and 3, respectively. On the other hand, all the chromosomes are divided into three types, the median, submedian and subterminal, in accordance with the numerical values of their centromere positions, 50.0~37.5, 37.5~25.0 and 25.0~12.5, respectively.

Among the chromosomes of group 1 there are four pairs of the median and one pair of the submedian type, while there are four pairs of the median, two pairs of the submedian, one pair of the submedian or subterminal and one pair of the subterminal type among those of group 2, as presented in Table 3.

TABLE 2

Relative length of each chromosome in 100 metaphase plates of *Rana nigromaculata*

Chromosome no.	Relative length		
	Minimum	Maximum	Mean
1	13.1	17.1	14.8±0.08
2	11.2	14.0	12.6±0.07
3	10.4	13.0	11.6±0.05
4	9.8	12.7	11.4±0.05
5	9.0	11.0	9.8±0.03
6	4.9	7.0	6.2±0.05
7	4.4	6.8	5.5±0.04
8	4.4	6.6	5.4±0.06
9	4.0	6.3	5.3±0.05
10	4.0	5.4	5.0±0.03
11	4.0	5.8	4.8±0.03
12	3.2	5.1	4.1±0.03
13	2.8	4.7	3.5±0.03

$$\text{Relative chromosome length} = \frac{\text{Each chromosome length}}{\text{Genome length}} \times 100$$

± Standard error of the mean

TABLE 3

Centromere position of each chromosome in 100 metaphase plates of *Rana nigromaculata*

Chromosome no.	Numerical value of the centromere position			Type
	Minimum	Maximum	Mean	
1	47.1	49.6	46.7±0.15	m
2	35.3	46.9	38.4±0.15	m
3	26.2	37.0	31.5±0.25	sm
4	37.5	46.2	42.2±0.19	m
5	39.3	46.4	41.5±0.14	m
6	39.6	47.7	43.6±0.19	m
7	20.6	28.4	24.6±0.18	st
8	33.5	43.9	39.6±0.18	m
9	21.3	27.8	24.9±0.17	sm or st
10	39.6	46.5	42.2±0.16	m
11	35.1	45.5	40.0±0.22	m
12	25.0	34.5	30.0±0.22	sm
13	29.0	41.8	35.4±0.31	sm

$$\text{Numerical value of the centromere position (N.V.C.)} = \frac{\text{Short-arm length}}{\text{Chromosome length}} \times 100 \pm \text{Standard error of the mean}$$

Type of a chromosome:

N.V.C.	Type
50.0~37.5	m
37.5~25.0	sm
25.0~12.5	st
12.5~ 0.0	t

Chromosome No. 1 is the largest and of the median type. Chromosome No. 2 is remarkably shorter than No. 1 and of the median near the submedian type. Chromosomes Nos. 3 and 4 are very similar to each other in length, although they are easily distinguishable in shape, since No. 3 is of the submedian type, differing from No. 4 which is of the median type. Between No. 2 and No. 3 or No. 4 there is a clear difference in length. Chromosome No. 5 is of the median type and similar to No. 4 in shape. However, it is somewhat distinctly shorter than the latter.

The chromosomes of group 2 are remarkably smaller than those of group 1: there is a distinct difference in length between No. 5, the shortest of group 1 and No. 6, the longest of group 2. Chromosome No. 6 is of median type. Chromosomes Nos. 7, 8 and 9 are very similar to one another in relative length, although No. 8 clearly differs from Nos. 7 and 9 in the position of the centromere. In the latter respect there is no significant difference between the chromosomes Nos. 7 and 9. Accordingly, these two chromosomes can not be distinguished. Chromosomes Nos. 10 and 11 are much similar to each other in relative length and of median type, while they differ from Nos. 9 and 12 in the position of the centromere: in contrast with the latter two of the submedian type, they are of the median. On the other hand, chromosomes Nos. 10 and 11 are easily distinguishable from each other by the presence of a secondary constriction in the long arm of No. 11. As stated above, chromosome No. 12 can be distin-

guished from Nos. 10 and 11 by its smaller size and the difference in the position of the centromere. Chromosome No. 13 is also easily distinguishable from the others, owing to its smallest size and the position of the centromere, although there is wide deviation in the latter.

2. *Rana brevipoda* Ito

The chromosomes of *Rana brevipoda* are 26 in diploid number, being the same as those of *Rana nigromaculata*. The karyotype of this species arranged corresponding to that of the latter shows that both karyotypes are quite similar at a glance (Fig. 2). The relative length and the numerical value showing the position of the

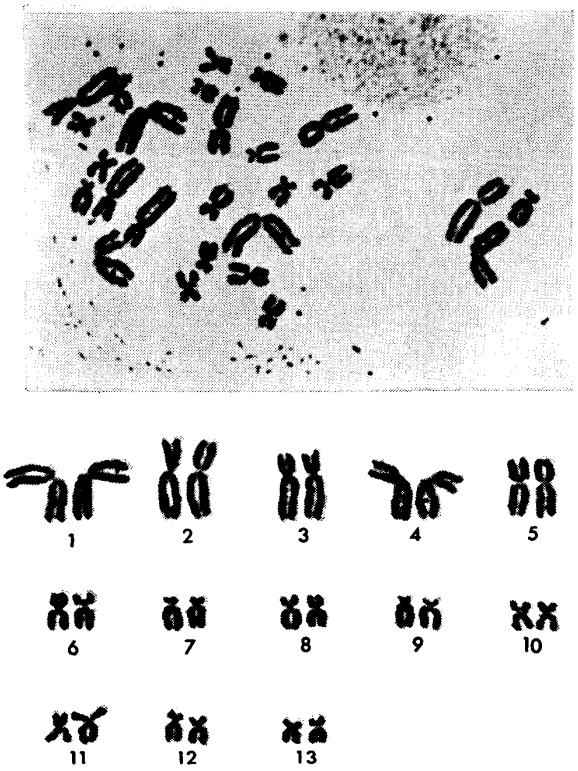

Fig. 1 Metaphase plate and the karyotype of an epidermal cell from a *Rana nigromaculata* tadpole. ×1500.

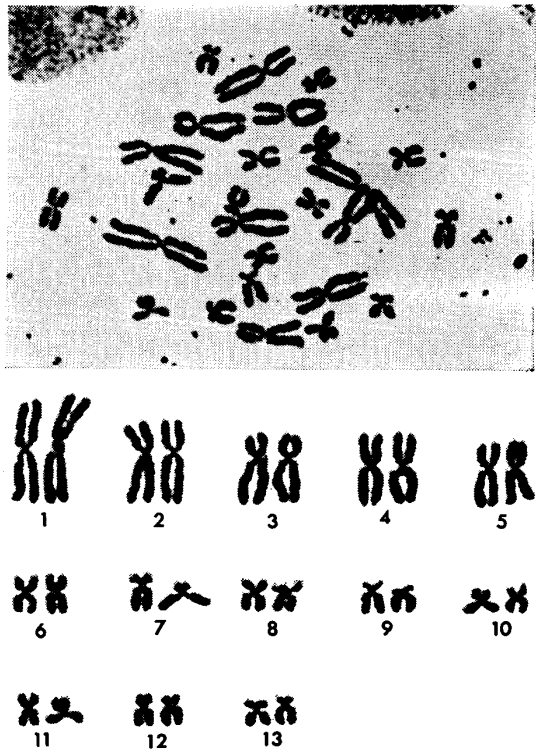

Fig. 2 Metaphase plate and the karyotype of an epidermal cell from a *Rana brevipoda* tadpole. ×1500.

centromere of each chromosome are presented in Tables 4 and 5, respectively.

The 13 pairs of chromosomes of this species are divided into two groups, as those of *R. nigromaculata* are. Group 1 consists of five pairs of large chromosomes. Chromosome No. 1 is the largest and of the median type, while No. 2 is clearly shorter than No. 1 and of the median or submedian type. Chromosomes Nos. 3 and 4 are nearly the same in relative length, although they are of the submedian and median types, respectively. These two chromosomes are much smaller than No. 2 and clearly larger than No. 5 which is the smallest in group 1. Chromosome No. 5 is the same as No. 4 in the point that they are of the median type.

Group 2 consists of eight pairs (Nos. 6~13) of small chromosomes: there is

TABLE 4

Relative length of each chromosome in 100 metaphase plates of *Rana brevipoda*

Chromosome no.	Relative length		
	Minimum	Maximum	Mean
1	13.6	18.0	15.3±0.08
2	12.0	14.5	13.0±0.06
3	10.0	12.5	11.0±0.05
4	9.6	12.4	11.2±0.07
5	8.7	11.0	9.7±0.04
6	5.6	6.8	6.2±0.03
7	4.8	7.3	5.9±0.05
8	4.8	6.7	5.6±0.04
9	4.2	6.3	5.1±0.05
10	3.9	5.5	4.7±0.03
11	4.1	5.2	4.6±0.03
12	3.1	4.9	4.0±0.03
13	3.2	5.2	4.1±0.03

$$\text{Relative chromosome length} = \frac{\text{Each chromosome length}}{\text{Genome length}} \times 100$$

± Standard error of the mean

TABLE 5

Centromere position of each chromosome in 100 metaphase plates of *Rana brevipoda*

Chromosome no.	Numerical value of the centromere position			Type
	Minimum	Maximum	Mean	
1	42.6	48.8	46.6±0.16	m
2	33.8	40.8	37.8±0.15	m or sm
3	29.0	38.0	32.5±0.26	sm
4	38.1	47.2	42.3±0.18	m
5	38.6	45.1	41.9±0.12	m
6	38.4	47.2	43.0±0.23	m
7	26.3	34.8	30.0±0.32	sm
8	35.9	45.4	40.3±0.28	m
9	22.6	28.7	25.4±0.11	sm or st
10	38.7	46.0	42.0±0.16	m
11	36.0	44.0	40.5±0.18	m
12	27.0	36.3	31.6±0.27	sm
13	33.4	46.2	37.6±0.21	m or sm

$$\text{Numerical value of the centromere position (N.V.C.)} = \frac{\text{Short-arm length}}{\text{Chromosome length}} \times 100$$

± Standard error of the mean

Type of a chromosome:

N.V.C.	Type
50.0~37.5	——m
37.5~25.0	——sm
25.0~12.5	——st
12.5~ 0.0	——t

a very distinct difference in the size of chromosomes between groups 1 and 2. Chromosomes Nos. 6 and 7, and Nos. 7 and 8 are respectively somewhat similar to each other in relative length. However, No. 7 is of the submedian type, while Nos. 6 and 8 are of the median. Nos. 6 and 8 are usually distinguishable

from each other by a difference in relative length. Chromosome No. 9 is of the submedian or subterminal type and significantly shorter than No. 7 which is of the submedian type. Moreover, Nos. 7 and 9 are significantly different in the position of the centromere. Chromosomes Nos. 10 and 11 are very similar to each other in relative length and centromere position. No. 11 has a distinct secondary constriction in the long arm, as found in the same chromosome of *Rana nigromaculata*. Nos. 10 and 11 chromosomes are somewhat shorter than No. 9 and much longer than Nos. 12 and 13. Chromosome No. 12 is the smallest among the 13 pairs of chromosomes and of the submedian type. Differing from No. 12, No. 13 chromosome is of the median or submedian type. There is no significant difference in relative length between these two chromosomes.

3. Comparison of the karyotypes of the two species

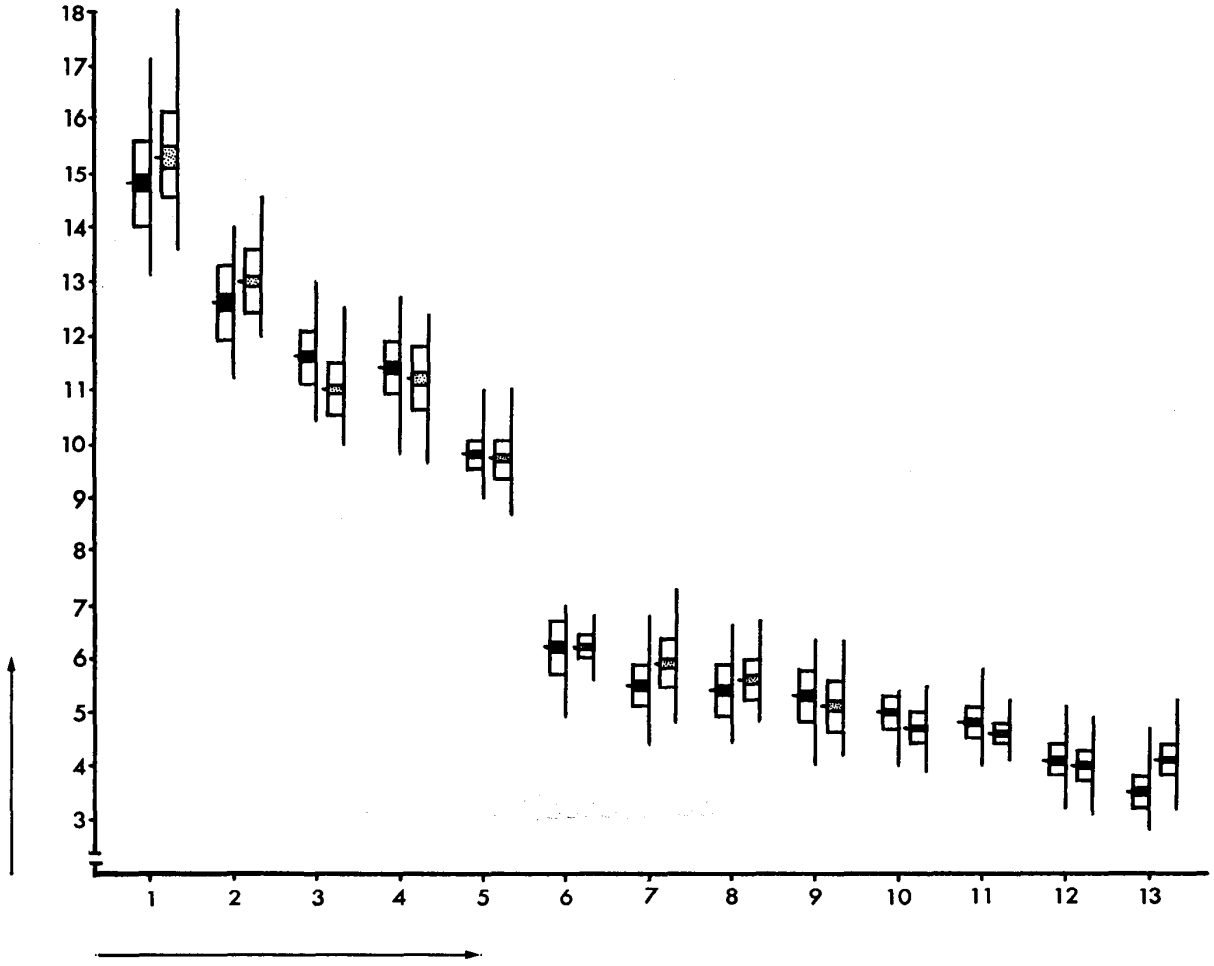

Fig. 3 A graph showing differences in relative chromosome length between *Rana nigromaculata* and *Rana brevipoda*.

The left and right of each pair represent a *nigromaculata* and a *brevipoda* chromosome, respectively. A vertical line shows the range of relative chromosome lengths; a short horizontal line, the mean of the latter; an open rectangle on each side of the horizontal line, the standard deviation of the mean; a black or dotted rectangle on each side of the horizontal line, two times the standard error of the mean. In general, if a black and a dotted rectangles do not overlap, the difference between the two chromosomes is statistically significant.

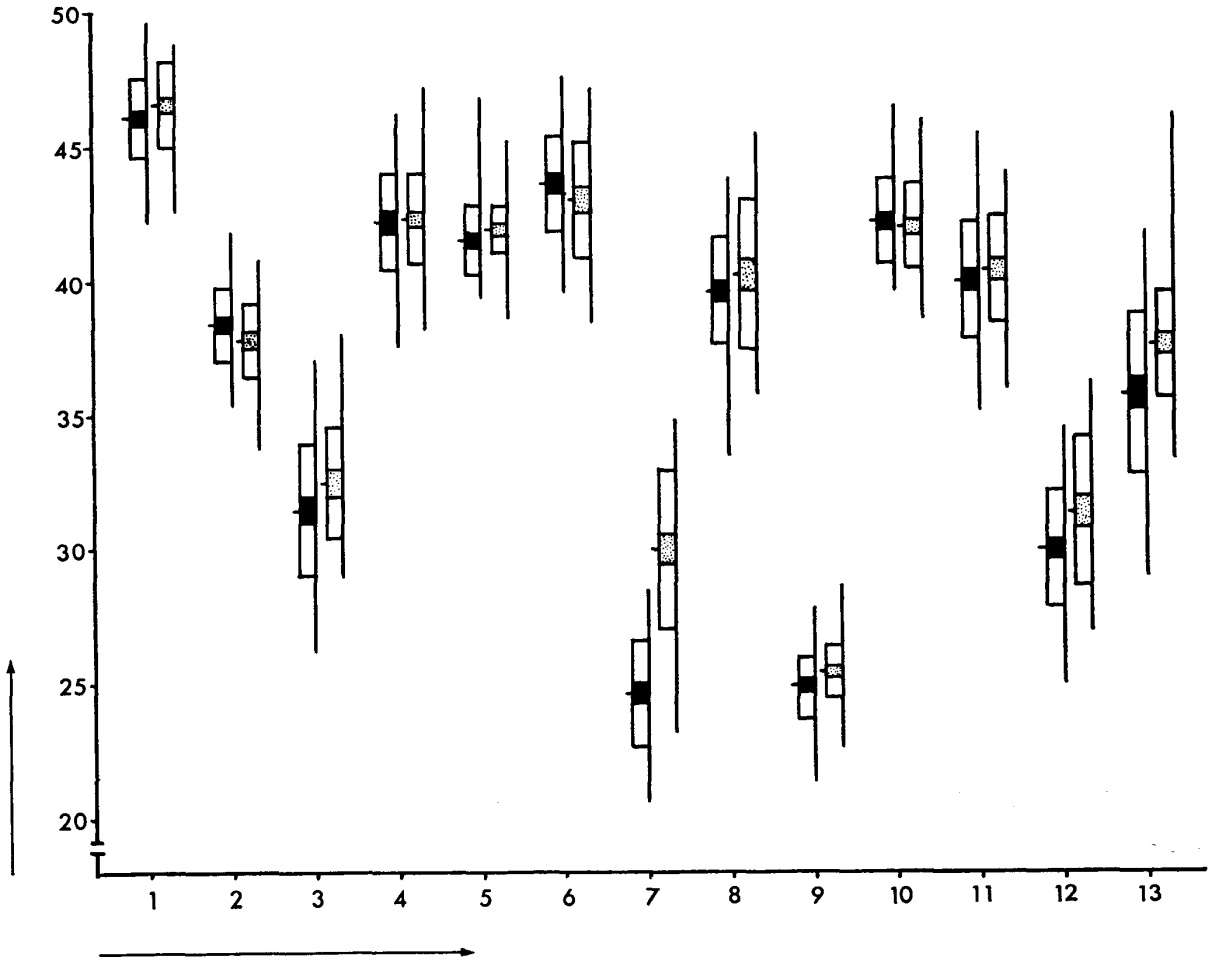

Fig. 4 A graph showing differences in centromere position between *Rana nigromaculata* and *Rana brevipoda*.

The left and right of each pair represent a *nigromaculata* and a *brevipoda* chromosome, respectively. A vertical line shows the range of numerical values of the centromere position; a short horizontal line, the mean of the numerical values; an open rectangle on each side of the horizontal line, the standard deviation of the mean; a black or dotted rectangle on each side of the horizontal line, two times the standard error of the mean. In general, if a black and a dotted rectangles do not overlap, the difference between the two chromosomes is statistically significant.

The karyotypes of *Rana nigromaculata* and *Rana brevipoda* are quite the same in the points that they consist of five large and eight small chromosomes, and that No. 11 has a secondary constriction. However, they are clearly different from each other in the type of the centromere position of chromosome No. 7. While this chromosome of *Rana nigromaculata* is of the subterminal type, that of *Rana brevipoda* is of the submedian (Tables 3, 5).

The results of statistic examination of the relative lengths and the numerical values of the centromere positions by the HUBBS and HUBBS' method clearly show minute differences between the karyotypes of both species as found in Figs. 3 and 4. Figure 5 shows the composite idiograms of *Rana nigromaculata* and *Rana brevipoda* drawn from data of Tables 2~5. Concerning the relative chromo-

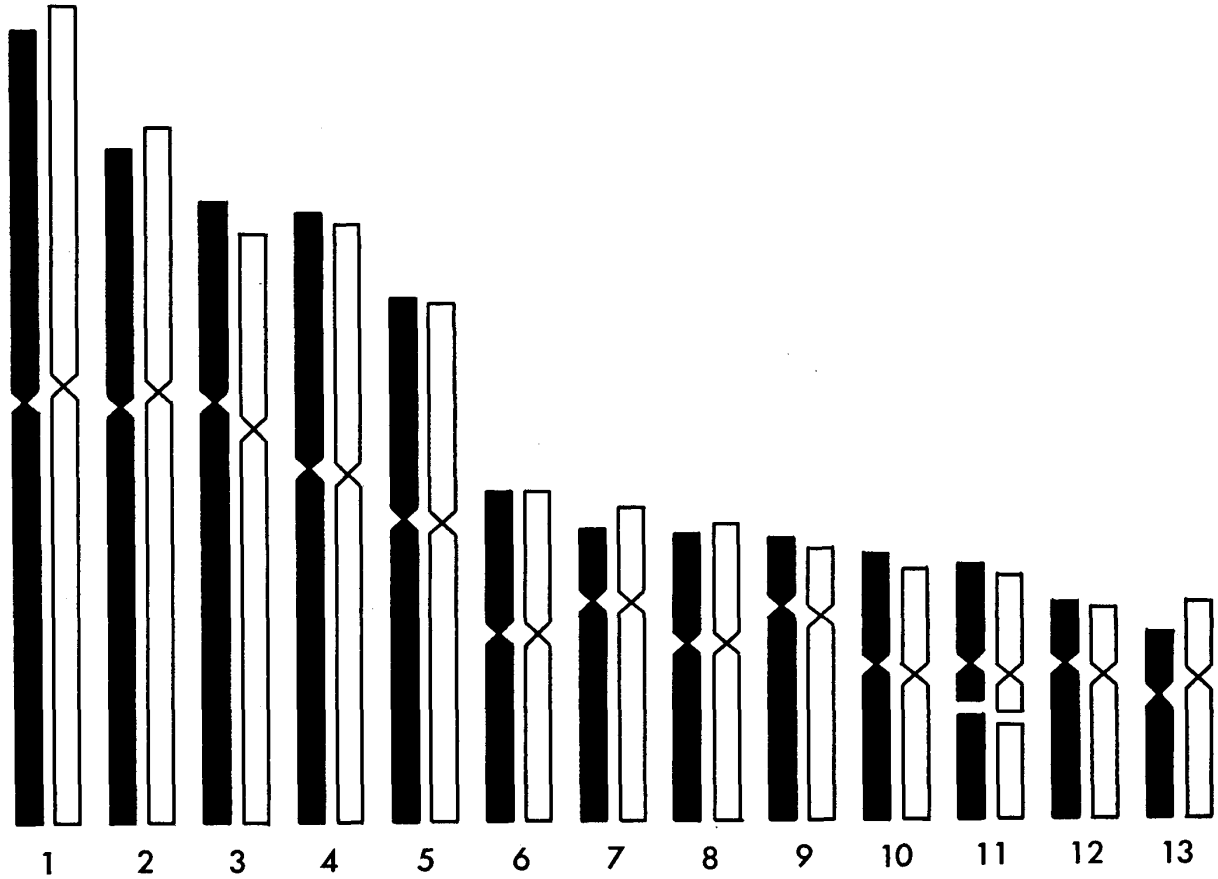

Fig. 5 Composite idiogram showing differences in relative chromosome length and centromere position between *Rana nigromaculata* and *Rana brevipoda*.

Black and white bars represent *nigromaculata* and *brevipoda* chromosomes, respectively. Constrictions indicate the centromeres. A gap in chromosome No. 11 indicates the secondary constriction.

some length, it is found that there are significant differences in chromosomes Nos. 1, 2, 3, 7, 10 and 13 between the two species: Nos. 1, 2, 7 and 13 of *Rana brevipoda* are larger than those of *Rana nigromaculata*, while Nos. 3 and 10 of the latter species are larger than the former. In *Rana brevipoda*, moreover, chromosomes Nos. 3 and 12 are rather smaller than Nos. 4 and 13, respectively. Concerning the numerical value of the centromere position, there is a significant difference in chromosomes Nos. 7, 12 and 13 between the two species: the centromere in *Rana nigromaculata* is situated nearer to the terminal of the short arm of each chromosome than that in *Rana brevipoda*.

II. Karyotypes of reciprocal diploid hybrids

In the karyotypes of the *nigromaculata* or *brevipoda* controls there is scarcely a significant difference in size between the two homologous chromosomes of each of the 13 pairs. Only a few percent of metaphase plates have one or two pairs of homologous chromosomes which are unequal in size. In two of 80 metaphase plates obtained from 50 *nigromaculata* tadpoles of three series, there was one pair

of somewhat unequal chromosomes. There was also one pair of unequal chromosomes in two of 65 metaphase plates obtained from 40 *brevipoda* tadpoles of one series.

In contrast with the karyotypes of *nigromaculata* and *brevipoda*, the two homologous chromosomes of each pair in the karyotypes of reciprocal hybrids between *nigromaculata* and *brevipoda* are usually different in size (Figs. 6, 7). Such comparison between two homologous chromosomes was made in the five pairs (Nos. 1~5) of large chromosomes for convenience' sake. As a result, among 100 metaphase plates obtained from 70 (N)NB tadpoles of three series as well as 50 metaphase plates from 32 (B)BN tadpoles of one series, there were no ones, in which the two homologous chromosomes of each of the five pairs were equal

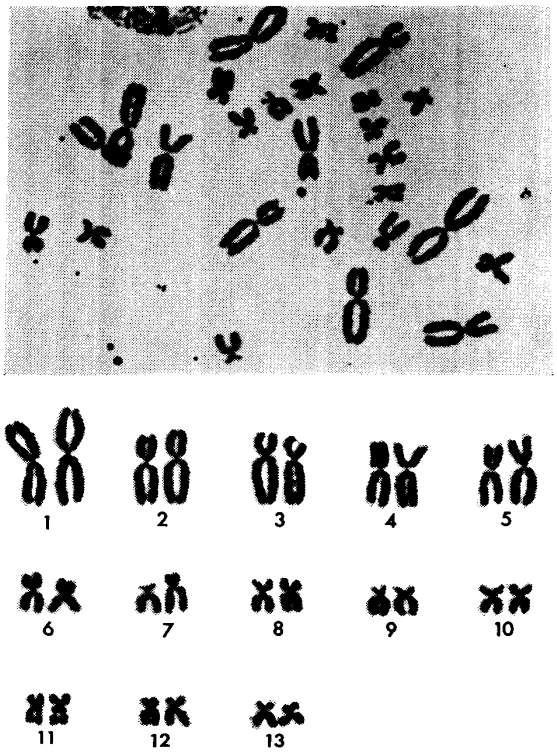

Fig. 6 Metaphase plate and the karyotype of an epidermal cell from a hybrid (N)NB tadpole. ×1500.

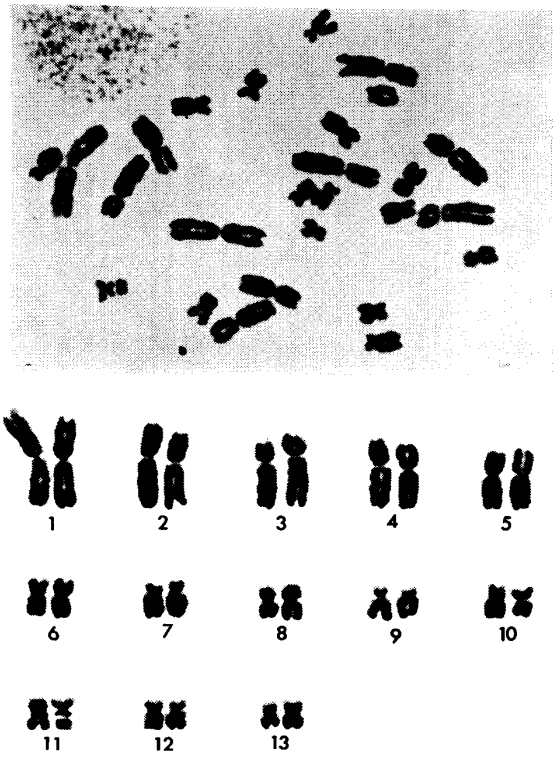

Fig. 7 Metaphase plate and the karyotype of an epidermal cell from a hybrid (B)BN tadpole. ×1500.

in size and shape. In most of the metaphase plates there were some distinct differencies in all the five pairs, while in the others such differences were found in three or four pairs.

III. Karyotypes of auto- and allotriploids

1. Autotriploids

In autotriploid *nigromaculata*, the three homologues of each of the five triplets of large chromosomes were usually equal in size. This fact was found in 18 of

20 analysed metaphase plates obtained from 15 tadpoles of two series (Fig. 8). One of the remaining two had three triplets, Nos. 3, 4 and 5, in which there was a slight difference in size among the three homologous chromosomes. Such a difference was also found in two triplets, Nos. 4 and 5, of the other metaphase plates.

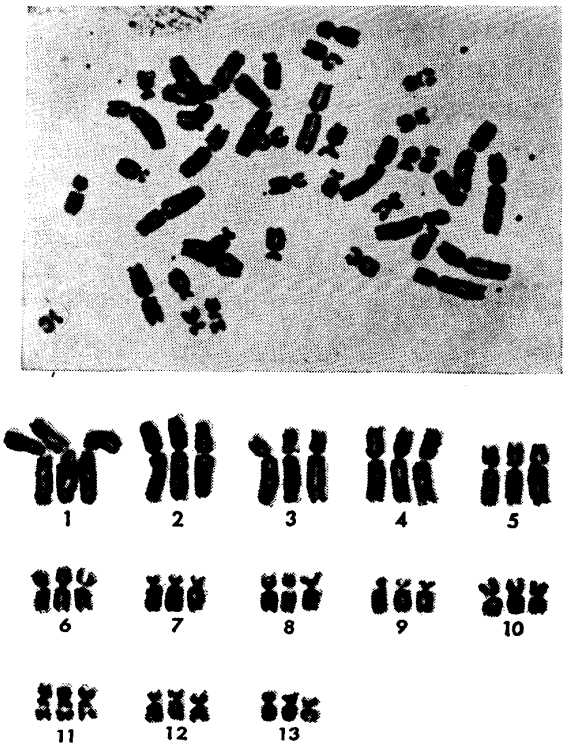

Fig. 8 Metaphase plate and the karyotype of an epidermal cell from an autotriploid (N)NNN tadpole. ×1500.

2. Allotriploids

Differing from the state of affairs in the karyotypes of the above autotriploids, two chromosomes of each triplet are different in size from the remaining in those of allotriploids. In allotriploids (N)NNB, for example, the two chromosomes of equal size in each triplet are reasonably considered to be of *nigromaculata*, while the other is of *brevipoda*. From such a point of view, the *nigromaculata* and *brevipoda* chromosomes in the five triplets of large chromosomes were examined in 50 karyotypes of metaphase plates obtained from 30 allotriploid (N)NNB tadpoles of three series as well as 50 karyotypes from 41 (B)BBN tadpoles of one series (Figs. 9, 10). The results of such examinations are presented in Tables 6 and 7, together with the five triplets of large chromosomes in the 20 karyotypes of the 15 autotriploid (N)NNN tadpoles.

In the allotriploid (N)NNB tadpoles, each of the five *nigromaculata* chromosomes Nos. 1～5 was smaller than the *brevipoda* homologue in 34～41 out of 50 metaphase plates. In the remaining, the former were nearly equal to or larger than the latter. On the other hand, there were 32 metaphase plates, in which all the

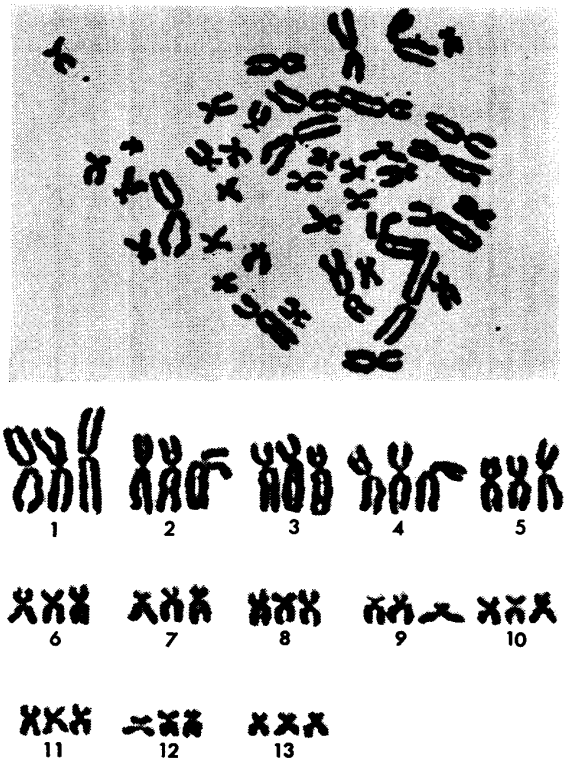

Fig. 9 Metaphase plate and the karyotype of an epidermal cell from an allotriploid (N)NNB tadpole. ×1500.

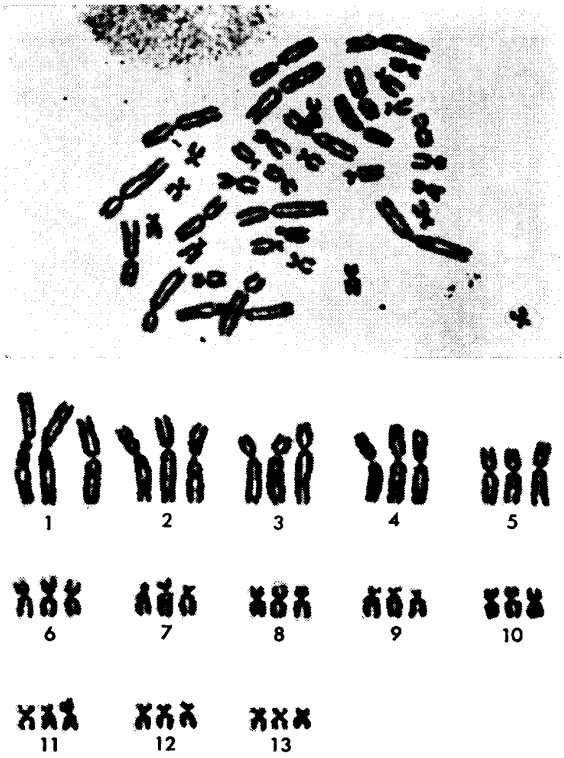

Fig. 10 Metaphase plate and the karyotype of an epidermal cell from an allotriploid (B)BBN tadpole. ×1500.

nigromaculata chromosomes Nos. 1～5 were smaller than the *brevipoda* homologues, three in which Nos. 1, 2, 4 and 5 were smaller, two in which Nos. 1, 3 and 5 were smaller, seven in which only one chromosome, No. 1, 2 or 5, was smaller, and six in which no *nigromaculata* chromosomes were smaller.

In the allotriploid (B)BBN tadpoles, each of the three *nigromaculata* chromosomes Nos. 1, 2 and 4 was smaller than the *brevipoda* homologue in 32～40 out of 50 metaphase plates, while two *nigromaculata* chromosomes Nos. 3 and 5 were smaller than the *brevipoda* in only 6 and 13 metaphase plates, respectively. Among these 50 metaphase plates, there were 23 in which the *nigromaculata* chromosomes Nos. 1, 2 and 4 were smaller than the *brevipoda* homologues, three in which all the *nigromaculata* chromosomes were smaller, five in which Nos. 1, 2, 4 and 5 were smaller, six in which two chromosomes, Nos. 1 and 2, Nos. 1 and 5, or Nos. 4 and 5, were smaller, nine in which only one chromosome, No. 1, 3 or 4, was smaller, and four in which no *nigromaculata* chromosomes were smaller.

TABLE 6

Number of karyotypes with a kind of relation in size between *nigromaculata* and *brevipoda* chromosomes in each of the five triplets of group 1 in reciprocal triploid hybrids and autotriploid

Kind	Chrom. no. / Relation in size	No. 1	No. 2	No. 3	No. 4	No. 5
(N)NNB	N>B	9	9	10	12	9
	N≒B	2	5	6	3	0
	N<B	39	36	34	35	41
	Total	50	50	50	50	50
(B)BBN	N>B	6	10	25	9	32
	N≒B	4	8	19	7	5
	N<B	40	32	6	34	13
	Total	50	50	50	50	50
(N)NNN	N≦N≧N	0	0	1	2	2
	N≒N≒N	20	20	19	18	18
	Total	20	20	20	20	20

TABLE 7

Number of karyotypes with a kind of combination of the five triplets of group 1 in the relation of size between *nigromaculata* and *brevipoda* chromosomes in reciprocal triploid hybrids

Kind / Comparison in size	N<B	N≒B	N>B	No. of karyotypes
(N)NNB	Nos. 1, 2, 3, 4 and 5	None	None	32
	Nos. 1, 2, 4 and 5	None	No. 3	3
	Nos. 1, 3 and 5	No. 2	No. 4	2
	No. 1	Nos. 2, 3 and 4	No. 5	1
	No. 1	Nos. 3 and 4	Nos. 2 and 5	1
	No. 2	Nos. 3 and 4	Nos. 1 and 5	1
	No. 5	No. 1	Nos. 2, 3 and 4	2
	No. 5	No. 2	Nos. 1, 3 and 4	2
	None	No. 3	Nos. 1, 2, 4 and 5	3
	None	None	Nos. 1, 2, 3, 4 and 5	3
			Total	50
(B)BBN	Nos. 1, 2, 3, 4 and 5	None	None	3
	Nos. 1, 2, 4 and 5	No. 3	None	5
	Nos. 1, 2 and 4	None	Nos. 3 and 5	23
	Nos. 1 and 2	Nos. 3, 4 and 5	None	1
	Nos. 1 and 5	Nos. 2 and 3	No. 4	4
	No. 1	Nos. 3 and 4	Nos. 2 and 5	3
	No. 1	Nos. 3 and 5	Nos. 2 and 4	1
	No. 3	No. 1	Nos. 2, 4 and 5	2
	No. 3	Nos. 2, 4 and 5	No. 1	1
	Nos. 4 and 5	Nos. 2 and 3	No. 1	1
	No. 4	Nos. 1, 2 and 3	No. 5	2
	None	Nos. 3, 4 and 5	Nos. 1 and 2	2
	None	None	Nos. 1, 2, 3, 4 and 5	2
			Total	50

DISCUSSION

Since about fifty years ago, it has been clarified by many investigators that the chromosomes of most of the *Rana* species examined are 26 in diploid number and consist of 10 large and 16 small ones (WITSCHI, 1922, '24, '25, in *R. temporaria*; IRIKI, 1932, in *R. nigromaculata* and *R. rugosa*; GALGANO, 1933, in *R. esculenta*; SATO, 1933, in *R. limnocharis*; KAWAMURA, 1939b, '43, in *R. japonica*; WICKBOM, 1945 in *R. esculenta* and *R. temporaria*, and others.). However, it was the matter attained about ten years after the time when a new staining technique with aceto-orcein was devised by TJIO and LEVAN (1954) that the karyotype analysis was made in clear-cut metaphase plates of amphibians. In fact, the shape and structure of each chromosome became clearly observable by application of prefixation treatment in addition to the new technique or by the introduction of some other means.

During the latest ten years, the karyotypes of frogs well-known in North America or Europe were reported by DIBERARDINO (1962, *R. pipiens*), HENNEN (1963, '64, *R. pipiens* and *R. sylvatica*), KILEY and WOHNUS (1968, *R. pipiens*), GUILLEMIN (1967, *R. temporaria* and *R. dalmatina*), ULLERICH (1967, *R. esculenta, R. temporaria* and *R. arvalis*), MORESCALCHI (1962, '67, *R. esculenta, R. ridibunda, R. temporaria, R. graeca* and *R. arvalis*) and GÜNTHER (1970, *R. esculenta* and *R. ridibunda*). While the chromosomes of *Rana arvalis* are 24 in diploid number and there are six pairs of large chromosomes, those of the other brown or wood frogs as well as pond or water frogs stated above are 26 and consist of 10 large and 16 small chromosomes. All these investigators described the relative length and centromere position of each of the chromosomes at the metaphase and made possible to compare the karyotypes of the species studied by them with those of the others more acculately than in former days.

The two Japanese pond frog species, *R. nigromaculata* and *R. brevipoda*, are very similar to each other in karyotype. Needless to say, their chromosomes are 26 in diploid number and divided into two groups of 10 large and 16 small ones. The centromeres of the chromosomes Nos. 1, 4 and 5 of group 1 are median, while that of No. 3 is submedian and No. 2 is median near submedian or nearly intermediate between median and submedian. The chromosomes Nos. 6, 8, 10, and 11 of group 2 are median in centromere position, while the others are submedian, subterminal, nearly intermediate between median and submedian or between submedian and subterminal. In the centromere positions of the chromosomes of group 1, the two Japanese species are quite the same as European pond frog species, *R. esculenta* and *R. ridibunda* studied by MORESCALCHI (1967) and by GÜNTHER (1970) as well as European brown frog species, *R. temporaria* and *R. graeca* by MORESCALCHI and *R. temporaria* by GUILLEMIN (1967). They are aslo very similar to American *R. pipiens* studied by DIBERARDINO (1962) and KILEY and WOHNUS (1968) and *R. sylvatica* by HENNEN (1964). In the point that the largest chromosome No. 6 of group 2 has a median centromere, all

the above-stated frogs together with *R. dalmatina* studied by GUILLEMIN are quite the same. Among the next three chromosomes Nos. 7~9, there are a median and two submedian or subterminal chromosomes in the two Japanese species. In this point, the latter are also quite the same as the above European and American frog species. The next two chromosomes, Nos. 10 and 11 of the two Japanese species are median in centromere position, differing from the European pond and brown frog species, in which Nos. 10 and 11 are submedian and median, respectively. In *R. pipiens*, DIBERARDINO observed two submedian chromosomes, while KILEY and WOHNUS did two median chromosomes. Of the two smallest chromosomes Nos. 12 and 13 of the two Japanese species, one is submedian and the other is submedian or nearly intermediate between median and submedian. There is no difference in this point between the Japanese and all the European species except for *R. dalmatina*.

The two Japanese species, *R. nigromaculata* and *R. brevipoda*, are closely related to the two European species, *R. esculenta* and *R. ridibunda*, as viable hybrids are easily obtained by artificial fertilization between *R. brevipoda* and *R. esculenta* or *R. ridibunda*, just as between *R. brevipoda* and *R. nigromaculata* (KAWAMURA, NISHIOKA and KURAMOTO, 1972; KAWAMURA and NISHIOKA, unpublished). As stated above, the Japanese species are different in the centromere position of the chromosome No. 10 from the European species. Moreover, the median chromosome No. 11 of the Japanese species has a distinct secondary constriction in the long arm, differing from the European species which have such a constriction in the long arm of the submedian chromosome No. 11.

When the two Japanese species are compared with each other in the relative length and the centromere position of each chromosome, there are some minute differences between them. In the metaphase plates of reciprocal hybrids, such differences seem to appear more remarkably, as the chromosomes of both species are kept in the same circumstances. However, the determination of the origin of each of two homologous chromosomes in the metaphase plates of reciprocal diploid hybrids is almost impossible, owing to the similarity of the karyotypes of the two species.

Such identification seems to be possible in the metaphase plates of reciprocal triploid hybrids, since two and one chromosomes of each triplet derived from the egg and spermatozoon, respectively. As a matter of fact, each triplet usually consisted of two chromosomes of equal size and a larger or smaller chromosome, differing from that of autotriploids which usually consisted of three chromosomes of equal size. Accordingly, the two chromosomes of equal size were considered to be those of the mother species. Although the *brevipoda* chromosome was larger than the *nigromaculata* in each of the five triplets of group 1 in more than two-thirds of the metaphase plates of (N)NNB tadpoles, a different state of affairs was found in the reciprocal allotriploids (B)BBN. In the latter, the *nigromaculata* chromosome was larger than the *brevipoda* in each of the triplets of chromosomes Nos. 3 and 5 in more than half of the metaphase plates, while the *brevipoda* chromosomes were larger in each of the remaining three triplets in most metaphase plates, as

in the reciprocal triploid hybrids (Tables 6 and 7). This seems to show that the chromosomes Nos. 3 and 5 are more labile in size than the others on one hand, and that there is some difference in cytoplasm between *Rana nigromaculata* and *R. brevipoda* on the other hand.

SUMMARY

1. The karyotypes of the two sibling species, *Rana nigromaculata* and *Rana brevipoda*, were compared by making use of tadpoles of typical specimens collected from the field and of reciprocal diploid and triploid hybrid tadpoles produced in the laboratory.

2. The chromosomes of the two species are 26 in diploid number. They are divided into two groups: group 1 consisting of five large chromosomes and group 2 of eight small ones. Chromosome No. 11 has a secondary constriction in the long arm. Although the karyotypes of the two species are very similar to each other, there are minute differences between them in the numerical values of the centromere positions as well as the relative lengths of some chromosomes.

3. While there is scarcely a significant difference in size between the two homologous chromosomes of each of the 13 pairs in the karyotype of *nigromaculata* or *brevipoda*, the two homologues of each pair in the karyotypes of reciprocal diploid hybrids are usually different in size.

4. In reciprocal triploid hybrids, two chromosomes of each triplet in metaphase plates are usually equal to each other and differ from the remaining in size. The *brevipoda* chromosomes of group 1 seem to be larger than the *nigromaculata* in most cases, although the chromosomes Nos. 3 and 5 are somewhat labile in size.

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