between *Chaenogobius* species

7. *C.* sp. 1	8. *C.* sp. 2	9. *C. isaza*
1.286 (1.272–1.301)	1.570 (1.545–1.589)	1.529 (1.531–1.562)
1.310 (1.305–1.319)	1.577 (1.549–1.593)	1.554 (1.535–1.566)
1.290 —	1.574 (1.566–1.577)	1.549 —
1.146 —	1.376 (1.372–1.386)	1.352 —
1.301 (1.296–1.306)	1.593 (1.591–1.595)	1.567 —
0.237 (0.227–0.249)	0.225 (0.206–0.252)	0.103 (0.092–0.120)
—	0.414 (0.401–0.412)	0.358 —
8.	0.000 (0.000–0.001)	0.235 (0.232–0.242)
	9.	—

should supply significant information to establish the taxonomic relationship between them.

Three species of *C. urotaenia, C.* sp.1 and *C.* sp. 2 were confirmed that they were different species each other. Genetic distances between them ranged from 0.225 to 0.412 on average. These values are large, indicating that they are different species. *C. urotaenia* and *C.* sp. 2, distributing sympatrically at the four rivers in the Shimane Prefecture, had different alleles at the *Sod* locus and at the *Mdh* locus from each other and no heterozygote at these loci was observed through this study. Ecological study also showed that they are ethologically isolated [10].

This study revealed phylogenetic position of *C. isaza* which is specialized to land-locked freshwater and endemic to Lake Biwa. It was closely related to *C. urotaenia*, amphidromous species as previously suggested [11]. We used the estimation of divergence time from genetic distance proposed by Nei [18], $t=5\times10^6D$. It suggested that *C. isaza* was differentiated from *C. urotaenia* about 0.5 million years ago. This time agrees with the origin of other endemic species in Lake Biwa, which was suggested by fossil records [25, 26]

Isozyme polymorphisms supplied the information on population system in some species. The comparison between populations within three amphidromous species, *C. castaneus, C. urotaenia* and *C.* sp. 2, showed that the differentiation of populations did not reflect the geographic distance between populations from different rivers (data was not shown). This suggests that genetic mixture between populations occurs when larvae go down to the sea and they have no behavioral character to return to the river where they were born. This study indicates the effectiveness of application of isozyme polymorphism to ecological study of *Chaenogobius* species that have various life histories.

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Speciation of Japanese Pond Frogs Deduced from Lampbrush Chromosomes of their Diploid and Triploid Hybrids

HIROMI OHTANI

Laboratory for Amphibian Biology, Faculty of Science, Hiroshima University, Higashi-hiroshima 724, Japan

ABSTRACT—To examine the hybrid origin hypothesis of *Rana porosa porosa* cytogenetically, the lampbrush chromosomes of triparental allotriploids comprising the genomes of *R. nigromaculata*, *R. p. brevipoda*, and *R. p. porosa* were investigated together with those of their intra- and interspecific hybrids. The behavior of their homologous lampbrush chromosomes provided little evidence that chromosomal material from *R. nigromaculata* is present in the genomic chromosomes of *R. p. porosa*. On the contrary, it is suggested that *R. p. porosa* and *R. nigromaculata* are phylogenetically more distant than are *R. p. brevipoda* and *R. nigromaculata*.

INTRODUCTION

With respect to the differentiation of *Rana porosa porosa* Cope, Moriya [13] and Kawamura and Nishioka [4–6] proposed the hypothesis of hybrid origin between *R. nigromaculata* Hallowell and *R. p. brevipoda* Ito. This hypothesis was supported by Kuramoto [7], but questioned by Matsui and Hikida [12]. Recently, Nishioka *et al.* [17] offered support for the hypothesis from electrophoretic analysis, though the support was not without a tinge of interested consideration. Although I was a collaborator of the latter paper, I now question the hybrid origin hypothesis in view of the fact that the lampbrush chromosomes of *R. p. porosa* closely resemble those of *R. p. brevipoda*, and yet there are no landmarks derived from *R. nigromaculata* throughout their axes (unpublished).

The hybrid origin should be demonstrated by comparing the behavior of lampbrush chromosomes in diploid hybrids between the above-mentioned three taxa, because the number of chiasmata that control the behavior of lampbrush chromosomes changes in accordance with the extent of similarity between the homologues of parental species [9, 14, 20]. Similarly, when the genomic chromosomes of these three taxa are placed together in an oocyte, provided that *R. p. porosa* receives many dominant and recessive genes from *R. nigromaculata* as suggested by Kawamura and Nishioka [4], some of the chromosomes of *R. p. porosa* should join inevitably to those of *R. nigromaculata* and act as a mediator in formation of trivalents.

The lampbrush chromosomes of *R. nigromaculata* are easily distinguished from those of *R. p. brevipoda* [16] and *R. p. porosa* (unpublished) by size, type, and position of the landmarks. Thus, the lampbrush chromosomes of triparental allotriploid females were examined to cytogenetically determine the relative degree of synaptic affinity among the chromosomes of these taxa together with those of their intra- and interspecific hybrid females. This paper describes these results and a new hypothesis of the differentiation of *R. p. porosa* is proposed.

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MATERIALS AND METHODS

The female frogs studied are described in Table 1. The parental frogs for crosses were from the lineages of *R. p. brevipoda* collected from Konko, Okayama Prefecture, *R. p. porosa* from Machida, a city of Tokyo, and *R. nigromaculata* from Hiroshima. The frogs were crossed by artificial fertilization. Autotriploid frogs were produced by cooling the fertilized eggs of *R. p. brevipoda* to $\cong 1^{\circ}C$ for 2 hrs. Two kinds of allotriploid frogs were produced by inseminating a few diploid ova which *brevipoda* ♀ × *nigromaculata* ♂ hybrid females spawned with spermatozoa of the two subspecies. Tadpoles were fed on boiled spinach or chard, and frogs were fed on houseflies or tropical crickets.

Lampbrush chromosomes were removed from the ovarian eggs of two-year-old females just prior to hibernation (November) according to Gall's method with a slight modification [1, 20], and examined under a phase-contrast microscope. The abbreviations B, P, and N refer to *brevipoda*, *porosa*, and *nigromaculata* chromosomal sets, respectively. The letters in parentheses indicate the sources of cytoplasm. Chiasma frequencies per oocyte were compared using Student's or Aspin-Welch's *t*-test. Chi-square test also was used for the comparison of chiasma numbers in two kinds of allotriploids.

RESULTS

Autotriploid (B)BBB

Lampbrush chromosomes from 60 oocytes were analyzed in detail. All the oocytes contained 39 lampbrush chromosomes consisting of 13 triplets of homologues that belonged to five large chromosomes numbered 1 to 5 and eight small chromosomes numbered 6 to 13. These lampbrush chromosomes formed eight or more trivalents in all the oocytes; it was notable that those of six oocytes formed exclusively 13 trivalents (Table 2). All the chromosomes other than those

TABLE 1. Kind, origin, and number of female frogs used in the present study

Kind	Parental Origin		Number of females
	Female	Male	
Autotriploid (B)BBB (3n=39)	*R. p. brevipoda*	*R. p. brevipoda*	5
Allotriploid (B)BBN (3n=39)	*brevipoda* ♀ × *nigromaculata* ♂	*R. b. brevipoda*	5
Allotriploid (B)BPN (3n=39)	*brevipoda* ♀ × *nigromaculata* ♂	*R. b. porosa*	4
Diploid hybrid (P)PB (2n=26)	*R. p. porosa*	*R. p. brevipoda*	9
Diploid hybrid (P)PN (2n=26)	*R. p. porosa*	*R. nigromaculata*	9
Diploid hybrid (N)NP (2n=26)	*R. nigromaculata*	*R. p. porosa*	8
Non-hybrid (P)PP (2n=26)	*R. p. porosa*	*R. p. porosa*	11

TABLE 2. Number of oocytes having tri-, bi- and univalents in various combinations in the three kinds of triploids

No. of trivalents	No. of bivalents	No. of univalents	Kind (B)BBB	(B)BBN	(B)BPN
13 (39)	0	0	6		
12 (36)	1 (2)	1 (1)	16		
11 (33)	2 (4)	2 (2)	17		
10 (30)	3 (6)	3 (3)	13		
9 (27)	4 (8)	4 (4)	4		
8 (24)	5 (10)	5 (5)	4		
7 (21)	6 (12)	6 (6)			1
6 (18)	7 (14)	7 (7)			1
5 (15)	8 (16)	8 (8)		1	
4 (12)	9 (18)	9 (9)			2
3 (9)	10 (20)	10 (10)		8	2
2 (6)	11 (22)	11 (11)		12	1
1 (3)	12 (24)	12 (12)		12	4
0	13 (26)	13 (13)		11	6
0	0	39 (39)		2	8
	Total		60	46	25

Numbers in parentheses show numbers of lampbrush chromosomes forming tri-, bi-, or univalents.

of the trivalents formed bivalents and univalents of the same number.

The number of trivalents in chromosome Nos. 1 to 13 is presented in Table 3. Of the 655 trivalents, 189 joined three homologues in juxtaposition by four to 12 chiasmata and rarely by terminal fusions (Fig. 1A). In 359 other trivalents, two of the three homologues were joined by two to eight chiasmata and the rest was joined to one of them by one to three chiasmata, a terminal fusion, or both, in addition (Fig. 1B). In the remaining 107 trivalents, the three homologues were joined in tandem by one or two chiasmata or a terminal fusion (Fig. 1C).

In chromosome Nos. 1, 4, 6–9 and 12, the chiasma frequencies were about 1.5 times higher than those of diploid *R. p. brevipoda* (Table 4). Those of the remainder were slightly lower than 1.5 times. The number of chiasmata in each oocyte was between 35 and 76 (average, 55.0) in total.

Allotriploid (B)BBN

Lampbrush chromosomes from 57 oocytes were analyzed. Eleven of these oocytes, an aneuploid one (3n-2) lacking the *nigromaculata* chromosomes of Nos. 2 and 8, and 10 hexaploid ones, were omitted from the analysis. The remaining 46 oocytes contained 39 lampbrush chromosomes consisting of one *nigromaculata* and two *brevipoda* chromosomes in each of the 13 homologue triplets (Fig. 2). These chromosomes formed one to five trivalents in 33 oocytes (Table 2). The remaining 13 oocytes had no trivalents. All the chromosomes other than those of the trivalents formed bivalents and univalents of the same number, or simply univalents; two oocytes contained only 39 univalents.

In 64 of the 65 trivalents, a *nigromaculata* chromosome was always joined to one of the two *brevipoda* chromosomes by one or two chiasmata, a terminal fusion, or both, in addition (Table 3). The remaining one trivalent was seen in chromosome No. 6 and arranged three homologues of, in order, *brevipoda, nigromaculata* and *brevipoda*, which were each joined by one chiasma in tandem. On the other hand, in 507 triplets of homologues forming a bivalent and a univalent, the bivalent always consisted of two *brevipoda* chromosomes, and the univalent of a *nigromaculata* chromosome. In the remaining 26 triplets of homologues, they

TABLE 3. Behavior of homologous lampbrush chromosomes in each of the 13 triplets in the three kinds of triploids

Kinds	Chromosome behavior in a triplet	Chromosome no. 1	2	3	4	5	6	7	8	9	10	11	12	13	Total
(B)BBB	b-b-b	54	57	58	55	53	46	52	48	46	47	44	44	51	655
	b-b, b	6	3	2	5	7	14	8	12	14	13	16	16	9	125
	Total	60	60	60	60	60	60	60	60	60	60	60	60	60	780
(B)BBN	b-b-n	6	6	4	4	3	11	2	3	6	3	6	7	3	64
	b-n-b						1								1
	b-b, n	38	38	40	40	41	32	42	41	38	41	38	37	41	507
	b, b, n	2	2	2	2	2	2	2	2	2	2	2	2	2	26
	Total	46	46	46	46	46	46	46	46	46	46	46	46	46	598
(B)BPN	b-p-n or p-b-n	3	4	3	4		2	2	4	1	2	1	3	2	31
	b-n-p											1	1		2
	b-p, n	14	12	14	13	17	14	14	12	16	15	15	13	14	183
	b-n,p or p-n,b		1				1	1*	1					1*	5
	b, p, n	8	8	8	8	8	8	8	8	8	8	8	8	8	104
	Total	25	25	25	25	25	25	25	25	25	25	25	25	25	325

The abbreviations b, p, and n indicate *brevipoda, porosa,* and *nigromaculata* lampbrush chromosomes, respectively.
"-" indicates a connection between two homologues.
* Joining was effected between *brevipoda* and *nigromaculata* chromosomes.

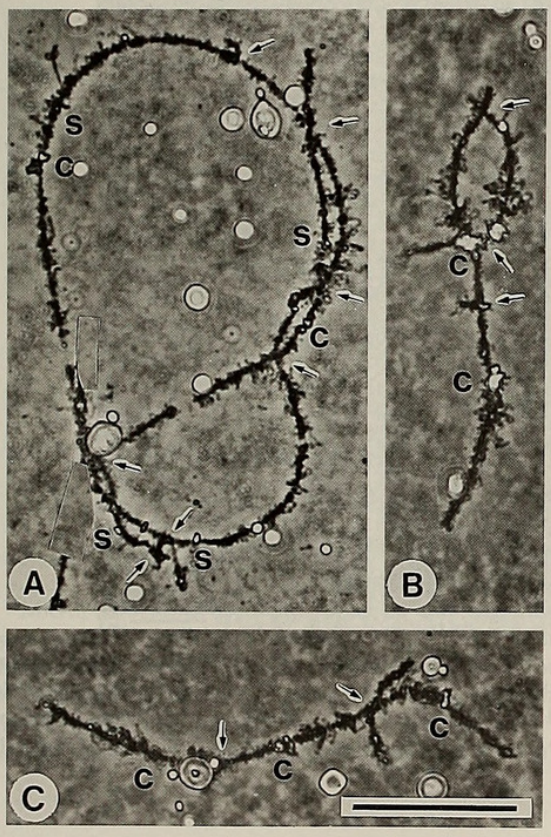

FIG. 1. Microphotographs of trivalents of chromosome Nos. 3 (A) and 10 (B and C) in an autotriploid, (B)BBB. Arrows indicate the positions of chiasmata. c and s represent compound- and simple-type giant loops, respectively. Bar=50 μm.

remained as univalents.

In all the trivalents, *nigromaculata* and *brevipoda* chromosomes were joined by 57 (3%) chiasmata in total except for the terminal fusions. By contrast, joining of two *brevipoda* chromosomes in the bivalents and trivalents was by 2033 (97%) chiasmata in total except for the terminal fusions.

The chiasma frequencies in chromosome Nos. 1 to 13 were about 1.2 times higher than those of diploid *R. p. brevipoda* except in chromosome Nos. 11 and 13, though the chiasmata for *nigromaculata* and *brevipoda* chromosomes accounted for no more than 3% of the total (Table 4). The number of chiasmata in each oocyte was between 0 and 60 (average, 45.4) in total. This average value was different from that of the diploid *R. p. brevipoda* ($t=3.7$, $P<0.001$) or the autotriploid (B)BBB ($t=4.4$, $P<0.0001$).

Allotriploid (B)BPN

Lampbrush chromosomes from 41 oocytes were analyzed. Sixteen of the 41 oocytes were seven aneuploid oocytes of 3n-1 (three), 3n-2 (two), and 3n-5 (two), and nine hexaploid oocytes; all of which were omitted from the analysis. In the remaining 25 normal triploid oocytes in which each triplet of homologues consisted of one *brevipoda*, one *porosa*, and one *nigromaculata* chromosomes, the lampbrush chromosomes were somewhat similar in behavior to those of the other allotriploid (B)BBN (Table 2). In 11 oocytes they formed one to seven trivalents, while all the chromosomes other than those of the trivalents formed bivalents and univalents of the same number (Fig. 3). In six other oocytes they formed 13 bivalents and 13 univalents. The remaining eight oocytes had only 39 univalents.

Of the 33 trivalents, 31 joined a *nigromaculata* chromosome to either of the *brevipoda* and *porosa* chromosomes by one or two chiasmata, a terminal fusion, or both, in addition (Table 3). The remaining two trivalents arranged three

TABLE 4. Frequency of chiasmata in chromosome Nos. 1 to 13

Type	Chromosome no. 1	2	3	4	5	6	7	8	9	10	11	12	13	Total
(P)PP	5.3	4.2	4.0	4.2	3.7	2.3	2.6	2.2	2.1	2.1	2.0	2.1	2.0	39.0
(B)BB*	4.8	4.7	4.1	4.1	3.6	2.4	2.3	2.3	2.0	2.2	2.1	1.9	2.0	38.7
(N)NN*	5.0	4.5	3.7	4.4	3.6	2.3	2.5	2.3	2.0	2.0	2.0	2.1	2.0	38.6
(B)BBB	7.2	6.4	5.7	6.1	5.1	3.6	3.5	3.3	3.1	2.8	2.7	2.8	2.8	55.0
(B)BBN	6.0	5.9	4.6	4.9	4.3	2.7	2.9	2.7	2.7	2.3	2.1	2.3	2.0	45.4
(B)BPN	5.0	4.3	3.6	4.0	3.7	2.3	2.0	1.8	2.1	1.6	1.9	1.6	1.6	35.7
(P)PB	5.0	3.6	3.2	4.0	3.4	2.5	2.4	2.2	1.6	2.1	1.9	2.1	1.9	35.8
(P)PN	2.6	2.6	1.9	2.5	2.1	1.7	1.7	1.9	1.4	1.9	1.7	1.6	1.6	25.2
(N)NP	2.9	2.8	2.3	2.1	2.1	1.9	1.7	2.0	1.5	2.0	1.6	1.6	1.3	26.0
(B)BN*	3.7	2.3	2.5	3.0	2.4	2.3	1.8	2.0	1.6	1.9	1.8	1.8	1.8	28.9
(N)NB*	3.4	2.3	2.6	3.0	2.7	2.0	1.7	1.9	1.5	1.8	1.6	1.6	1.6	27.6

* Data from [20]

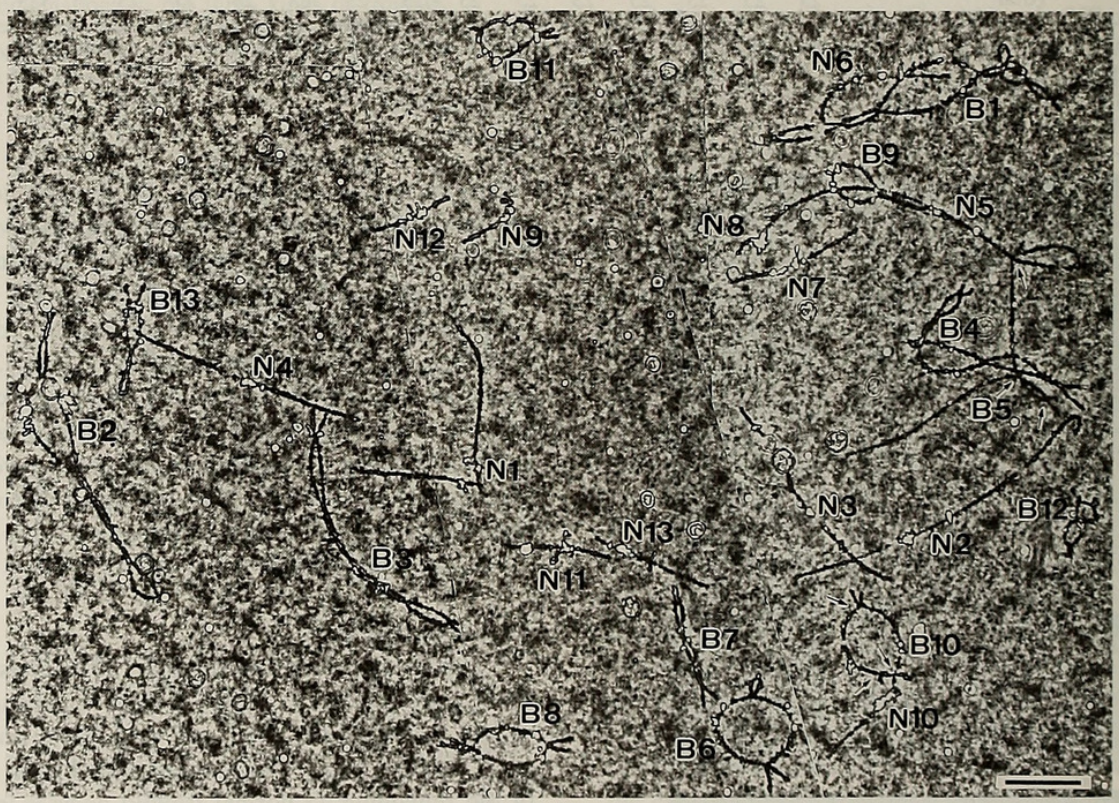

FIG. 2. Microphotographs of the lampbrush chromosomes in an oocyte of an allotriploid, (B)BBN. Number represents the chromosome number. B and N represent *brevipoda* and *nigromaculata* chromosomes, respectively. Two *brevipoda* chromosomes form a bivalent and one *nigromaculata* chromosome forms a univalent in chromosome Nos. 1 to 13 except for 5 and 10. The three homologues of chromosome Nos. 5 and 10 form a trivalent which joins a *nigromaculata* chromosome to one of the two *brevipoda* chromosomes by a single chiasma. Arrows indicate the positions of chiasmata. Bar=50 μm.

homologues of, in order, *brevipoda, nigromaculata* and *porosa*; the three homologues of chromosome No. 11 were each joined by one chiasma in tandem, and those of chromosome No. 12 also were joined by two chiasmata and by a terminal fusion. In 183 of the 188 triplets of homologues forming a bivalent and a univalent, the bivalents consisted of *brevipoda* and *porosa* chromosomes, and the univalents of *nigromaculata* chromosomes. In the remaining five triplets of homologues, bivalents included a *nigromaculata* chromosome.

In the bivalents and trivalents, joining of *nigromaculata* and *brevipoda* or *porosa* chromosomes was effected by 35 (4%) chiasmata in total except for terminal fusions. In contrast, *brevipoda* and *porosa* chromosomes were joined by 857 (96%) chiasmata in total except for the terminal fusions.

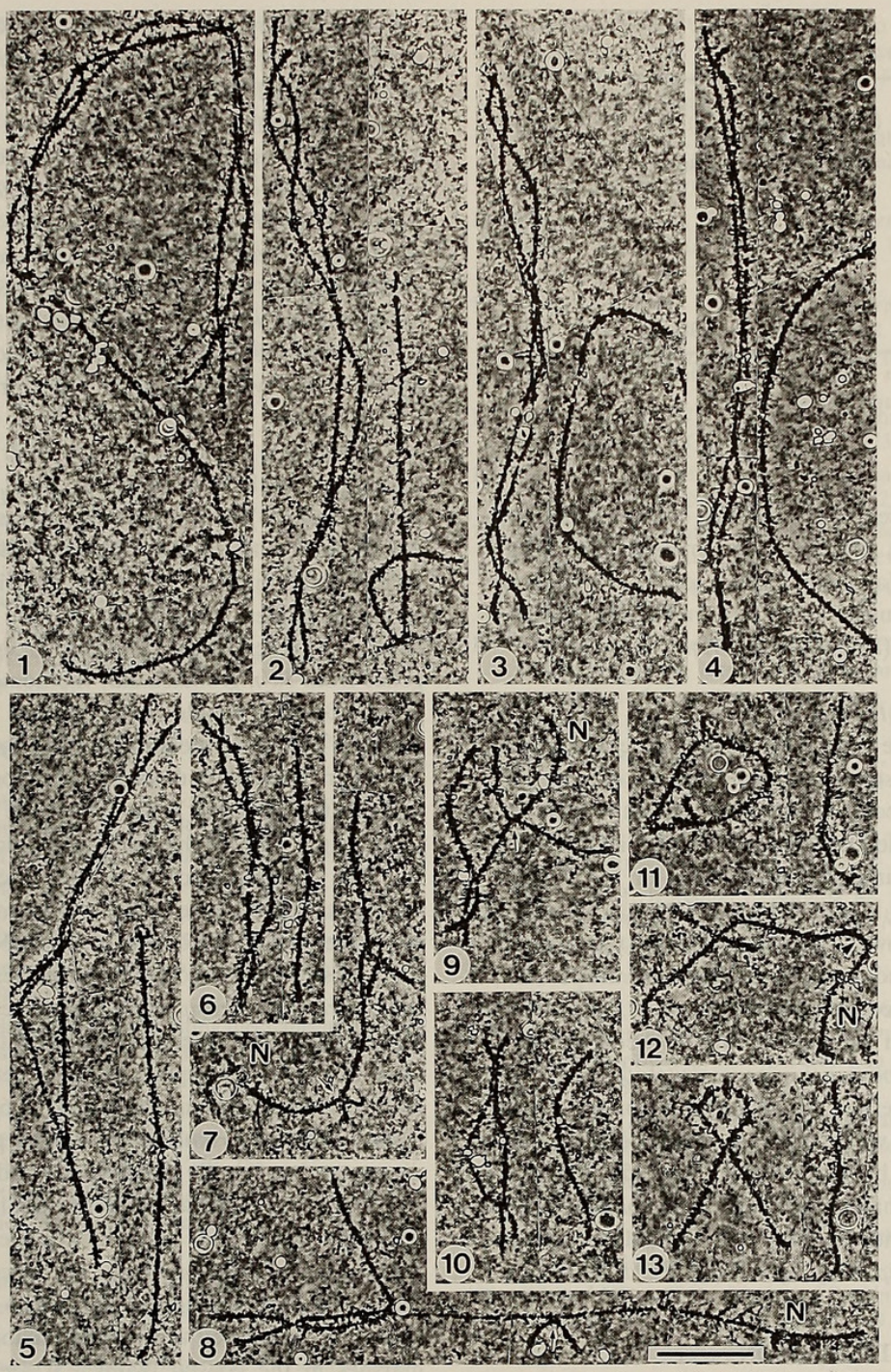

FIG. 3. Microphotographs of the lampbrush chromosomes in an oocyte of a triparental allotriploid, (B)BPN. The number in each photograph represents the chromosome number. The abbreviation N in photographs 7, 8, 9 and 12 represents a *nigromaculata* chromosome. Although landmarks did not develop very much in this preparation, *nigromaculata* chromosomes are infallibly distinguished from those of *brevipoda* and *porosa*. Two *brevipoda* and *porosa* chromosomes form a bivalent and one *nigromaculata* chromosome forms a univalent, in chromosome Nos. 1 to 13 except 7, 8, 9, and 12. The homologues of chromosome Nos. 7, 8, 9, and 12 form a trivalent which joins a *nigromaculata* chromosome by a single chiasma (arrow, in Nos. 7, 8, and 9), and by a terminal fusion (arrow head, in No. 12). Bar=50 μm.

These values were not different from those in the other allotriploid (B)BBN ($\chi^2=3.0$, $P=0.08$).

The chiasma frequencies in chromosome Nos. 1 to 13 were generally much lower than in another allotriploid (B)BBN (Table 4), but the total number of chiasmata in each oocyte (0–67, average=35.7) was not different from that of (B)BBN ($t=1.8$, $P=0.09$).

Intraspecific hybrid (P)PB

In the 30 oocytes examined, all the lampbrush chromosomes formed 13 bivalents like those of parental subspecies *R. p. brevipoda* and *R. p. porosa* (Table 5). The bivalents had one to eight chiasmata. When the chiasma frequency in chromosome Nos. 1 to 13 was compared with those of the two parental subspecies, those of chromosome Nos. 2, 3 and 9