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Abnormalities of meioses in male reciprocal hybrids between the Hiroshima and Ichinoseki populations of *Rana japonica*

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Abstract. In order to elucidate cytogenetically the cause of male sterility in intraspecific hybrids of *Rana japonica*, the behavior of chromosomes in the first meiosis was observed in spermatocytes from male reciprocal hybrids between two populations from Hiroshima and Ichinoseki. In the parental Hiroshima and Ichioseki populations, 2530 (96.7%) meiotic spreads had 13 bivalents and 78 (3.0%) contained 12 bivalents and two univalents, whereas in reciprocal hybrids only 337 (7.0%) contained 13 bivalents and the other 4445 (93.0%) had 2–26 univalents. A total of 31647 (93.4%) bivalents was ring-shaped and the other 2234 (6.6%) were rod-shaped in both parental populations, whereas in reciprocal hybrids 26352 (57.1%) and 19819 (42.9%) bivalents were ring- and rod-shaped, respectively. These results show that meiotic chromosomes of reciprocal hybrids are characterized by a remarkable increase in univalents and rod-shaped bivalents.
Key words. Cytogenetics; meiosis; spermatocytes; intraspecific hybrids; amphibia; anura; *Rana japonica*.

The cytogenetic study of meiosis in F_1 hybrids between closely related species or subspecies is important to elucidate the mechanisms of speciation and evolution. Chromosome behavior at spermatogenesis in amphibian F_1 hybrids at different taxonomic levels (species, subspecies or races) has been observed by several investigators[1–5] over the past five decades. According to them, the meiotic chromosomes of these hybrids were characterized by aberrations such as a drastic reduction in chiasma frequency and an increase in univalents resulting from the failure of bivalent formation. It seems likely that there may be different degrees of meiotic aberrations depending on the taxonomic levels at which hybridization was observed, although meiotic chromosomes of hybrids at populational level have not been reported. Thus, it is interesting to compare the meiotic aberrations of hybrids at different taxonomic levels (species, subspecies, race, population).

Sumida[6] reported that the Hiroshima population of *Rana japonica* is genetically separated from the Ichinoseki population of the same species by the sterility of male hybrids. Though the two populations are not morphologically distinct, they are easily distinguishable both karyologically and biochemically. When hybridization experiments were carried out between them, it was found that the sex ratio was skewed in favor of males in the reciprocal hybrids, and that these male hybrids showed differing degrees of testicular abnormality[6].

The present paper observes the behavior of meiotic chromosomes during spermatogenesis using the testes of male reciprocal hybrids between the Hiroshima and Ichinoseki populations of *Rana japonica*, in order to elucidate the cause of male sterility in intraspecific hybrids at populational level and to compare it with other hybrids in urodeles and anurans.

Materials and methods

Specimens of *Rana japonica* Günther were collected from the suburbs of Hiroshima City, Hiroshima Prefecture in western Japan and Ichinoseki City, Iwate Prefecture in eastern Japan. Reciprocal crossings were carried out during the breeding seasons of 1979–1983 by artificial insemination[7]. Ovulation was induced by injecting bullfrog pituitaries into the body cavity. Tadpoles were fed on boiled spinach, and metamorphosed froglets were fed on two-spotted crickets. The testes of mature males were used for observation of spermatogenesis. One testis was fixed in Navashin's solution, sectioned at 10 μm and stained with Heidenhain's iron hematoxylin for histological observation, while the other was used to make chromosome preparations.

Meiotic chromosomes were prepared according to the techniques described by Schmid et al.[8] with a slight modification. 0.12 ml of a 0.1% colchicine solution was injected i.p. 2 h before frogs were sacrificed. The testis was removed and minced finely in a Petri dish. The cells were vigorously resuspended into 5 ml of 0.075 M KCl solution for 30 min at room temperature. 1 ml of fixative (3:1 methanol: glacial acetic acid) was added and the mixture agitated gently. The suspension was then centrifuged at 800 rpm for 5 min. The supernatant was discarded and replaced slowly by 5 ml of fixative. The cell suspension was again gently agitated and centrifuged at 800 rpm for 5 min. This procedure was repeated two or three times. One or two drops of the concentrated cell suspension were dropped onto a clean

slide and quickly dried by igniting the alcohol contained in the suspension. The chromosomes were stained for 10 min with a 4% Giemsa solution. Germ cells at various stages of spermatogenesis from spermatogonia to sperm were observed in the preparations. Mitoses of the spermatogonia and meioses of the spermatocytes indicated the presence of a few aneuploid cells and polyploid cells (4n, 6n and 8n) in addition to the normal diploid cells. Chromosome analysis was carried out using only diploid cells at diakinesis and metaphase of the first reduction division, when bivalent and univalent chromosomes could easily be distinguished from each other.

The abbreviations of H and I refer to a genome of the Hiroshima population and a genome of the Ichinoseki population, respectively. The letters in parentheses indicate sources of cytoplasm.

Results

Inner structure of testes

Testes could be classified into five types (Types 1–5) on the basis of inner structural abnormality[6]. In the parental Hiroshima and Ichinoseki populations, the testis was normal, and the seminiferous tubules were filled with dense bundles of normal spermatozoa (Type 1, fig. 1a). In reciprocal hybrids, the testes showed various degrees of abnormality (Types 2–5, figs 1b–1d). In Type 2, bundles of spermatozoa were small and coarse, and sparsely distributed abnormal spermatozoa and pycnotic nuclei were observed (fig. 1b). In Type 3, there were considerable numbers of abnormal spermatozoa and pycnotic nuclei, in addition to a few sparsely distributed normal bundles of spermatozoa (fig. 1c). In Type 4, seminiferous tubules were filled with abnormal spermatozoa and pycnotic nuclei; however, a few normal spermatozoa were present. No normal spermatozoa were seen in the seminiferous tubules of Type 5, which were filled with numerous abnormal spermatozoa and pycnotic nuclei (fig. 1d).

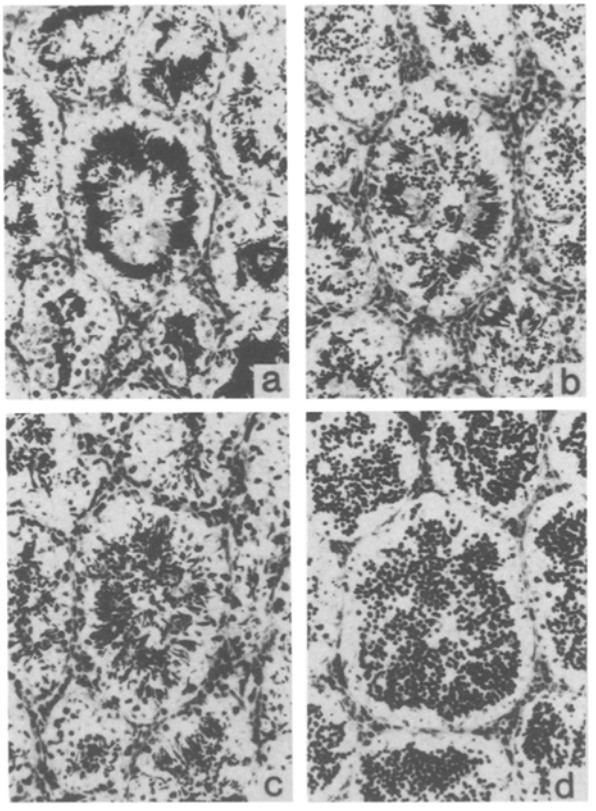

Figure 1. Cross-sections of the testes of the parental Ichinoseki population and the reciprocal hybrids between the Hiroshima and Ichinoseki populations of *Rana japonica*. ×150.
a Type 1 testis of an Ichinoseki male.
b Type 2 testis of a hybrid between Ichinoseki female and Hiroshima male.
c Type 3 testis of a hybrid between Ichinoseki female and Hiroshima male.
d Type 5 testis of a hybrid between Hiroshima female and Ichinoseki male.

Meiosis

Variation in meiotic spreads. *Rana japonica* was reported to have 13 mitotic chromosome pairs, five large and eight small, in diploid sets[6,9–12]. The karyotypes of the Hiroshima and Ichinoseki populations are very similar, but they differ slightly in the centromere position of chromosomes nos 6 and 9[6].

Several variations were observed in the first meiotic spread of the parental populations. Bivalents showed two distinct shapes: a ring shape, in which two homologous chromosomes were joined at their two ends, and a rod shape, in which two homologous chromosomes were joined at one end (fig. 2). Most meiotic spreads were observed to have 13 rings, five large and eight small (fig. 2a). Some spreads contained 12 rings and one rod (figs 2b and 2c). Still others had 10 rings and three rods (fig. 2d). Other spreads showed 10 rings, two rods and two univalents (fig. 2e) or nine rings, three rods and two univalents (fig. 2f). One clear distinction between the parental populations and the reciprocal hybrids was that numerous rods and univalents were observed in the latter (fig. 3). The reciprocal hybrids showed high variability in the number of univalents per spread. Some spreads showed 13 bivalents consisting of seven rods and six rings (fig. 3a), whereas others, which contained 2–26 univalents, could be classified into 13 types on the basis of the number of their univalents (figs. 3b–3n).

Frequency of spread types. A total of 1230 meiotic spreads from 10 males, (H)HH, nos 1–10, having testes of Type 1, was observed in the Hiroshima population (tables 1 and 2). Of these, 1202 (97.7%) contained 13 bivalents, whereas 26 (2.1%) had 12 bivalents and two univalents (table 1, fig. 4). In the Ichinoseki population, a total of 1385 meiotic spreads from 15 males, (I)II, nos 1–15, having testes of Type 1, was analyzed

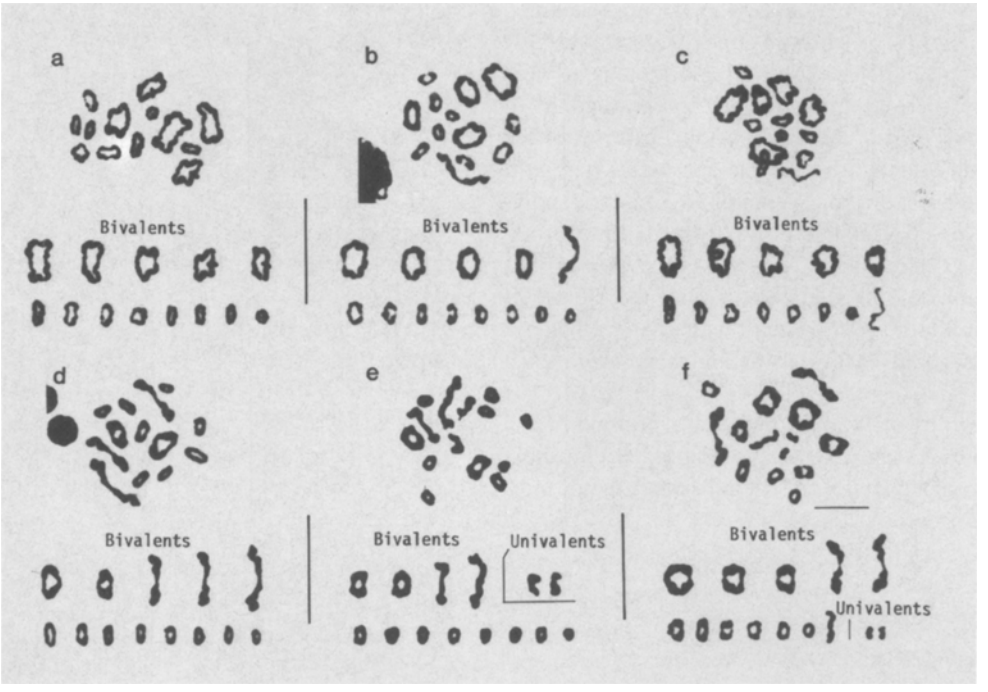

Figure 2. Spermatocytes at the first meiosis and chromosome complements in the parental Hiroshima and Ichinoseki populations of *Rana japonica*; *a*–*d* contain 13 bivalents, whereas *e* and *f* contain 12 bivalents and two univalents. The horizontal bar represents 10 µm.

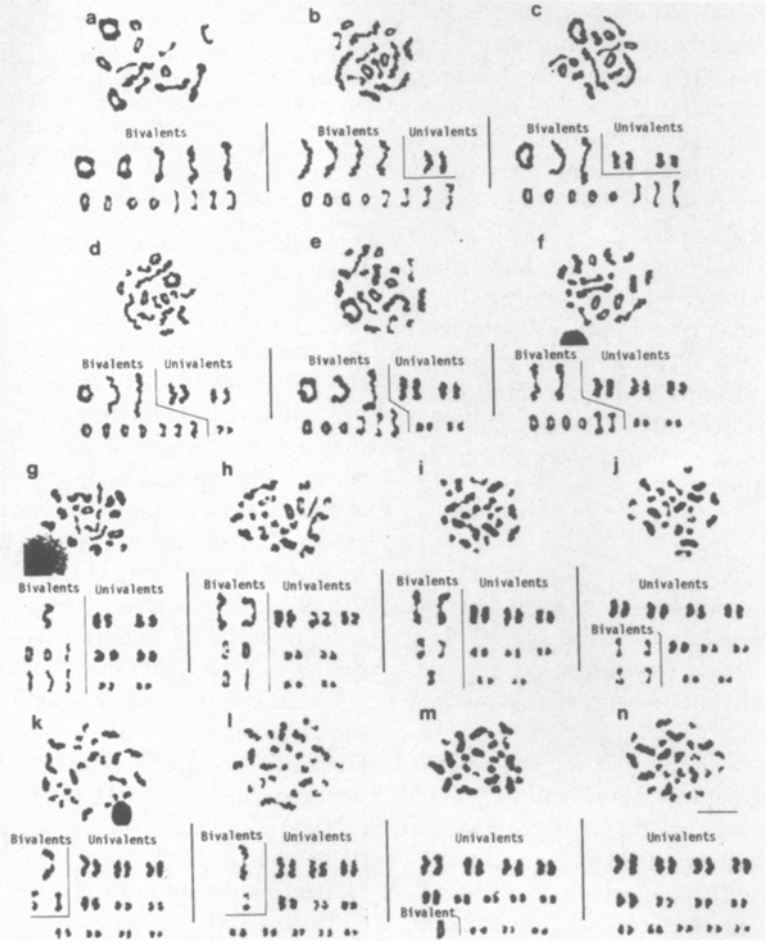

Figure 3. Spermatocytes at the first meiosis and chromosome complements in the reciprocal hybrids between the Hiroshima and Ichinoseki populations of *Rana japonica*; *a* contains 13 bivalents, whereas *b*–*n* contain 2–26 univalents. The horizontal bar represents 10 µm.

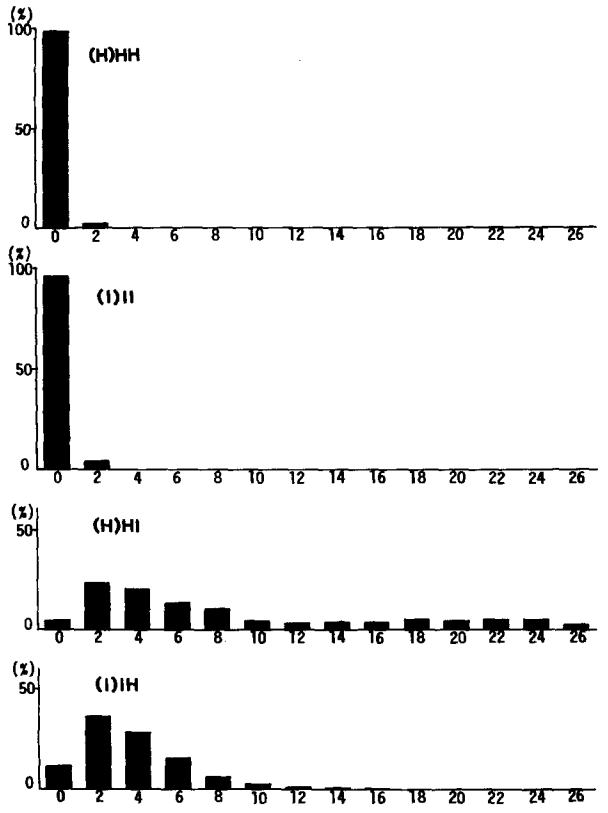

Figure 4. Frequencies of meiotic spreads differing in the number of univalents in males of the Hiroshima and Ichinoseki populations, (H)HH and (I)II, of *Rana japonica* and their reciprocal hybrids, (H)HI and (I)IH. The figures on the horizontal axis show the numbers of univalents.

(tables 1 and 2). 1328 (95.9%) of these contained 13 bivalents, whereas another 52 (3.8%) showed 12 bivalents and two univalents (table 1, fig. 4). The mean numbers of univalents per spermatocyte were 0.06 and 0.11 in the Hiroshima and Ichinoseki populations, respectively. The proportions of univalents to all chromosomes in the Hiroshima and Ichinoseki populations were 0.2% and 0.4%, respectively.

In the hybrid between Hiroshima females and Ichinoseki males, a total of 2901 meiotic spreads from 18 males, (H)HI, nos 1–18, having testes of Types 3–5, was analyzed (tables 1 and 2). Only 122 (4.2%) contained 13 bivalents, whereas the remaining 2779 (95.8%) contained 2–26 univalents (table 1, fig. 4). Meiotic spreads containing two univalents and 12 bivalents were most numerous, at 618 (21.3%), followed by 578 (19.9%) having four univalents and 11 bivalents, 383 (13.2%) containing six univalents and 10 bivalents, and 238 (8.2%) comprising eight univalents and nine bivalents (table 1, fig. 4). Meiotic spreads containing 10, 12, 14, 16, 18, 20, 22 and 24 numbered about 100 each, for a total of 898 (31.0%). Meiotic spreads containing 26 univalents were least common, at 64 (2.2%). Of the 18 male hybrids, five (nos 2, 6, 8, 9 and 11), having testes of Types 4 and 5, were different from the other 13 in the frequency of their univalents (table 2). In the 13 male hybrids, spreads containing 2–6 univalents were most numerous, and those having more than 12 univalents were very rare, whereas in the other five hybrids (nos 2, 6, 8, 9 and 11) most spreads contained more than 12 univalents (table 2). The mean number of univalents per spermatocyte was 8.68, and the proportion of univalents to all chromosomes was 33.4%.

Of 1881 meiotic spreads analyzed from 12 male hybrids between Ichinoseki females and Hiroshima males, (I)IH, nos 1–12, having testes of Types 2–4, 215 (11.4%) consisted of 13 bivalents, 678 (36.0%) contained 12 bivalents and two univalents, 532 (28.3%) contained 11 bivalents and four univalents, 286 (15.2%) comprised 10 bivalents and six univalents, 110 (5.8%) showed nine bivalents and eight univalents and 35 (1.9%) had eight bivalents and 10 univalents (tables 1 and 2, fig. 4). Meiotic spreads having 2–10 univalents numbered 1641 (87.2%) in total, whereas another 25 (1.3%) contained 12–24 univalents. The mean number of univalents per spermatocyte was 3.62, and the proportion of univalents to all chromosomes was 13.9%.

Increase in rod-shaped bivalents in the reciprocal hybrids. The numbers of ring- and rod-shaped bivalents seen in the parental populations and their reciprocal

Table 1. Numbers of meiotic spreads differing in number of univalents in males of the Hiroshima and Ichinoseki populations of *Rana japonica* and their reciprocal hybrids.

Type of frog	No. of meioses	No. of univalents (%)														Mean no. of univalents per cell
		0	2	4	6	8	10	12	14	16	18	20	22	24	26	
(H)HH No. 1–10	1230	1202 (97.7)	26 (2.1)	1 (0.1)								1 (0.1)				0.06
(I)II No. 1–15	1385	1328 (95.9)	52 (3.8)	2 (0.1)	1 (0.1)					1 (0.1)	1 (0.1)					0.11
(H)HI No. 1–18	2901	122 (4.2)	618 (21.3)	578 (19.9)	383 (13.2)	238 (8.2)	119 (4.1)	84 (2.9)	89 (3.1)	89 (3.1)	131 (4.5)	116 (4.0)	131 (4.5)	139 (4.8)	64 (2.2)	8.68
(I)IH No. 1–12	1881	215 (11.4)	678 (36.0)	532 (28.3)	286 (15.2)	110 (5.8)	35 (1.9)	9 (0.5)	6 (0.3)	3 (0.2)	3 (0.2)	2 (0.1)		2 (0.1)		3.62

(H) HH: Hiroshima population. (I) II: Ichinoseki population.
(H) HI: Hybrid between Hiroshima female and Ichinoseki male.
(I) IH: Hybrid between Ichinoseki female and Hiroshima male.

Table 2. Numbers of meiotic spreads differing in number of univalents and types of testes in each of males of the Hiroshima and Ichinoseki populations of *Rana japonica* and their reciprocal hybrids.

Type of frog	No. of meioses	No. of univalents														Type of testes
		0	2	4	6	8	10	12	14	16	18	20	22	24	26	
(H)HH, No. 1	102	101	1													1
(H)HH, No. 2	122	119	3													1
(H)HH, No. 3	118	118														1
(H)HH, No. 4	129	128	1													1
(H)HH, No. 5	124	118	6													1
(H)HH, No. 6	129	127	1									1				1
(H)HH, No. 7	120	114	6													1
(H)HH, No. 8	122	119	2	1												1
(H)HH, No. 9	139	135	4													1
(H)HH, No. 10	125	123	2													1
(I)II, No. 1	126	126														1
(I)II, No. 2	111	108	3													1
(I)II, No. 3	108	103	5													1
(I)II, No. 4	118	115	3													1
(I)II, No. 5	28	26	2													1
(I)II, No. 6	37	32	4	1												1
(I)II, No. 7	40	40														1
(I)II, No. 8	46	36	10													1
(I)II, No. 9	43	40	3													1
(I)II, No. 10	121	120	1													1
(I)II, No. 11	116	107	9													1
(I)II, No. 12	153	153														1
(I)II, No. 13	77	73	3	1												1
(I)II, No. 14	130	122	6							1	1					1
(I)II, No. 15	131	127	3		1											1
(H)HI, No. 1	213	5	77	56	31	22	10	5	2	1		1	2		1	4
(H)HI, No. 2	177	1	3	8	10	8	4	8	9	14	24	23	35	20	10	4
(H)HI, No. 3	145	4	14	35	29	20	13	8	12	4	4	1	1			4
(H)HI, No. 4	131	2	31	35	15	14	7	11	8	4	3		1			4
(H)HI, No. 5	176	4	29	34	50	28	20	6	4	1						4
(H)HI, No. 6	213				1			2	1	16	32	39	39	59	24	5
(H)HI, No. 7	140	5	66	37	21	3		2		3	1		2			4
(H)HI, No. 8	143		1	1				1	2	4	12	24	31	46	21	5
(H)HI, No. 9	94			1			1	10	17	20	23	13	5	1	3	4
(H)HI, No. 10	104	26	48	22	4	1	2			1						3
(H)HI, No. 11	134	2	3	3		1	2	9	21	16	32	15	13	13	4	4
(H)HI, No. 12	200	35	81	54	21	7	1			1						3
(H)HI, No. 13	277	11	77	84	51	30	14	3	3	1			2		1	4
(H)HI, No. 14	135	2	57	49	21	5		1								4
(H)HI, No. 15	214	19	75	69	29	19	2			1						3
(H)HI, No. 16	135		9	20	30	37	21	13	3	2						4
(H)HI, No. 17	132	3	34	46	28	13	6	1	1							3
(H)HI, No. 18	138	3	13	24	42	30	16	4	6							3
(I)IH, No. 1	120	5	32	43	29	9	2									3
(I)IH, No. 2	66		14	21	16	11	2	1			1					4
(I)IH, No. 3	172	8	26	44	48	30	10	2	4							3
(I)IH, No. 4	175	2	73	54	26	7	8	3				2				3
(I)IH, No. 5	188	1	67	69	34	14		1		2						3
(I)IH, No. 6	125	1	37	39	28	14	2				2			2		4
(I)IH, No. 7	129	48	47	24	10											2
(I)IH, No. 8	129	38	52	27	8	1	3									2
(I)IH, No. 9	263	23	135	70	26	7	2									2
(I)IH, No. 10	194	17	81	58	22	11	3	1		1						3
(I)IH, No. 11	192	46	78	41	21	5	1									2
(I)IH, No. 12	128	26	36	42	18	1	2	1	2							2

(H)HH: Hiroshima population. (I)II Ichinoseki population.
(H)HI: Hybrid between Hiroshima femald and Ichinoseki male.
(I)IH: Hybrid between Ichinoseki female and hiroshima male.

hybrids were compared. It was found that ring-shaped bivalents were overwhelmingly more numerous in both the large and small chromosomes of the parental populations, whereas ring-shaped bivalents decreased and rod-shaped bivalents increased in both the large and small chromosomes of reciprocal hybrids (table 3).

A total of 14982 (93.9%) and 16665 (92.9%) bivalents were ring-shaped in the Hiroshima and Ichinoseki pop-

Table 3. Numbers of ring- and rod-shaped bivalents in the Hiroshima and Ichinoseki populations of *Rana japonica* and their reciprocal hybrids.

Type of frog	No. of bivalents	Large chromosomes		Small chromosomes		Total		Mean no. of bivalents per cell
		Ring (%)	Rod (%)	Ring (%)	Rod (%)	Ring (%)	Rod (%)	
(H)HH No. 1–10	15952	5418 (88.5)	703 (11.5)	9564 (97.3)	267 (2.7)	14982 (93.9)	970 (6.1)	12.97
(I)II No. 1–15	17929	5920 (86.0)	960 (14.0)	10745 (97.2)	304 (2.8)	16665 (92.9)	1264 (7.1)	12.95
(H)HI No. 1–18	25124	2600 (32.9)	5296 (67.1)	10881 (63.2)	6347 (36.8)	13481 (53.7)	11643 (46.3)	8.66
(I)IH No. 1–12	21047	2785 (39.8)	4208 (60.2)	10086 (71.8)	3968 (28.2)	12871 (61.2)	8176 (38.8)	11.19

(H)HH: Hiroshima population. (I)II: Ichinoseki population.
(H)HI: Hybrid between Hiroshima female and Ichinoseki male.
(I)IH: Hybrid between Ichinoseki female and Hiroshima male.

ulations, respectively, whereas the other 970 (6.1%) and 1264 (7.1%) bivalents were rod-shaped. In the hybrids between Hiroshima females and Ichinoseki males, a total of 13481 (53.7%) were ring-shaped, whereas the other 11643 (46.3%) was rod-shaped. In the hybrids between Ichinoseki females and Hiroshima males, a total of 12871 (61.2%) was ring-shaped and the other 8176 (38.8%) were rod-shaped. The mean numbers of bivalents per spermatocyte in the Hiroshima and Ichinoseki populations were 12.97 and 12.95, respectively, whereas the comparable figures were 8.66 and 11.19 for the reciprocal hybrids (table 3).

Discussion

Extensive studies have examined the behavior of meiotic chromosomes in spermatogenesis of F_1 hybrids between species, subspecies or races of amphibians in which chromosome structures were expected to differ[1–5,13]. Such studies found that the meiotic chromosomes of these hybrids showed a drastic reduction in chiasma frequency, chiasmata being restricted to chromosome ends, and an increase in univalent chromosomes. The degree of meiotic aberration seems to depend on the taxonomic levels at which hybridization was observed. Callan and Spurway[1] reported that the intersubspecific hybrids between three European newt subspecies, *Triturus c. carnifex*, *T. c. cristatus* and *T. c. karelinii* ($2n = 24$) had 0.9–4.3, 2.44 on averge, univalents per spermatocyte. White[2] reported that the number of univalents per spermatocyte in an interspecific hybrid between *T. marmoratus* and *T. cristatus* ($2n = 24$) was 13.92. Spurway and Callan[3] observed that the mean univalent frequency per spermatocyte was 11.3 ± 0.2 in the interspecific hybrids between *T. vulgaris* and *T. helveticus* ($2n = 24$). Okumoto[5] recorded that the mean numbers of univalents per spermatocyte were 13.52 and 14.12 in the interspecific reciprocal hybrids between two Japanese pond frog species, *Rana nigromaculata* and *R. brevipoda* ($2n = 26$). The present study showed that the numbers of univalents per spermatocyte for the interpopulational reciprocal hybrids between the Hiroshima and Ichinoseki populations of *Rana japonica* were 8.68 and 3.62. These values are almost the same as, or somewhat larger than, those of the intersubspecific hybrids[1] stated above (in which the number of univalents per spermatocyte were 0.9–4.3, 2.44 on average), but are smaller than those of other interspecific hybrids[2,3,5] (in which almost half of all chromosomes were univalents). It thus seems probable that meiotic aberrations may get worse when putative taxonomic distance is increased (from populational level to the level of species).

With regard to chiasma frequency, Callan and Spurway[1] observed that chiasma frequencies per spermatocyte were 30.7–42.2, 35.4 on average in three subspecies, *T. c. carnifex*, *T. c. cristatus* and *T. c. karelinii*, whereas comparable figures for the hybrids were 15.1–21.9, 18.85 on average. White[2] reported that the numbers of chiasmata per spermatocyte in *T. c. cristatus* and *T. c. carnifex* were 35.2 and 31.0, respectively, whereas the comparable figure, 5.84, was very small in the hybrids. Spurway and Callan[3] reported that the mean chiasmata per spermatocyte in *T. vulgaris* and *T. helveticus* were 23.3 ± 1.1 and 23.25 ± 1.05, respectively, whereas the comparable figure was 7.6 ± 0.3 for the hybrid between these two species. Corresponding to these observations in urodeles, the present study showed that ring-shaped bivalents (93.4%) were far more numerous than rod-shaped ones (6.6%) in both parental Hiroshima and Ichinoseki populations, whereas ring- and rod-shaped bivalents represented 57.1% and 42.9%, respectively, in the reciprocal hybrids. Okumoto[5] observed a similar phenomenon in the interspecific hybrids between two Japanese pond frog species, *Rana nigromaculata* and *R. brevipoda*. The increase of rod-shaped

bivalents in hybrids of anurans is thought to correspond to the decrease in chiasma frequency.

According to Sumida[6], the Hiroshima and Ichinoseki populations are reproductively isolated from each other by incomplete male hybrid sterility. The reciprocal hybrids showed a remarkable preponderance of males. Male reciprocal hybrids were completely or partially sterile. The present study shows that abnormalities of spermatogenesis in reciprocal hybrids depend on the failure of pairing of the hybrids' homologous chromosomes, the formation of univalents and the subsequent degeneration of the spermatids. Wide variation was seen in the extent to which degeneration occurred. It seems likely that the degree of abnormality in spermatogenesis corresponded to the extent of univalent formation. Compared with male hybrids, female reciprocal hybrids showed almost complete fertility[6]. This suggests that pairing was much more nearly complete in oogenesis and that no degeneration of eggs comparable to spermatid degeneration occurred.

Sumida and Nishioka[14] studied intraspecific differentiation of *Rana japonica*, which is widely distributed in Japan, not only by experimental crossing but also by analyses of chromosomes and isozymes. They reported distinct differentiation between the eastern and western groups, which include the Hiroshima and Ichinoseki populations, respectively. It is noteworthy that the differentiation of *R. japonica* into eastern and western groups was accompanied by incomplete hybrid sterility resulted from abnormal spermatogenesis. The author considered the degree of this differentiation to be an incipient speciation of *R. japonica*.

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