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Sex Chromosome Differentiation in the Japanese Brown Frog, *Rana japonica* I. Sex-related Heteromorphism of the Distribution Pattern of Constitutive Heterochromatin in Chromosome No. 4 of the Wakuya Population

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ABSTRACT—To clarify the sex-related differences of chromosomes in the Japanese brown frog, *Rana japonica*, its chromosomes were analyzed by C- and late replication banding techniques. This species was characterized by ♂XY/♀XX sex chromosomes. There were three types (A, O and B) of chromosome No. 4 with respect to C-banding pattern. The type A had a C-band at the basal portion of the long arm, the type O had no such band, and the type B had double bands at the corresponding region. Almost all (97.6%) of the females examined showed the AA type and 2.4% the AO type, while 85.7% of the males examined showed the AO type, 10.7% the AA type, 1.2% the OO type and 2.4% the AB type. Histological examination of the testes of these AA, OO and AB males and the sex ratio of their offspring with AA females revealed that the OO and AB males were genetic XY males, while the AA males were composed of spontaneously sex-reversed XX females and genetic XY males. These results suggest that the genetic XY males of AA and the presumably genetic XX female of AO were produced by a recombination between the C-band in chromosome No. 4 and the site of a male-determining gene on the same chromosome. The OO male was probably produced from a mating of an XY male of AO with an XX female of AO.

INTRODUCTION

Sex chromosomes in amphibians vary widely among species [16, 18, 19] and populations [3, 7, 12, 20] with respect to a degree of differentiation. This feature presumably reflects the primitive differentiation of the sex chromosomes in amphibian species. Accordingly, cytogenetic and genetic examination of the amphibian sex chromosomes is important in comprehending why and how the chromosomes including sex determining genes have differentiated into the so-called distinguishable sex chromosomes by changing their external appearance.

The Japanese brown frog, *Rana japonica*, is widely distributed in Honshu except for Aomori Prefecture, Shikoku and Kyushu. Its sex-determining mechanism has been well examined by breeding experiments and sex-reversal [6, 10, 11]. However, sex chromosomes of this species have not been identified by conventional staining [8, 9, 13, 21, 22] and banding methods [4]. In recent years, C- and replication banding techniques have been used in amphibian species to demonstrate minor innerstructural changes taking place in the Y or W chromosome, which is apparently identical with the X or Z chromosome [2, 15, 17]. Thus, the present study attempts to clarify the sex-related differences of chromosomes in *Rana japonica* using these banding techniques.

MATERIALS AND METHODS

Animals

Specimens of *Rana japonica* Günther used in this study were collected from the paddyfields in Wakuya-machi, Toda-gun, Miyagi Prefecture situated in the northeastern area of Honshu, Japan.

Chromosome preparation and banding techniques

Mitotic metaphases were obtained by the *in vitro* blood cell culture [12]. A frog was first anesthetized with ether. After incision of the skin (about 1.5 cm in length) at the rear of the tympanum, the branchial vein and/or the muscular-cutaneus vein was cut with iridectomy scissors and watchmaker's forceps, and the blood was collected with a glass pipette containing 0.01~0.02 ml of heparin solution (10 mg/ml RPMI1640). The injury to the skin was healed without any proper treatment. 0.1~0.2 ml of blood was cultured in 2 ml of medium composed of 60% RPMI1640, 20% calf serum, 20% redistilled deionized water and 3% phytohemagglutinin (M) with penicillin and streptomysin at final concentrations of 100 Iu/ml and 100 μg/ml, respectively, at 25°C for 3–5 days. After the hypotonic treatment with 0.075M KCl solution, the cells were fixed in Carnoy's fluid (acetic acid : methanol = 1 : 3). Chromosome spreads were then prepared using the conventional air-drying method.

C-banding was performed according to the method of Sumner [23] with slight modifications. Chromosome preparations aged one day were treated with 0.2 N HCl for 40 min, and they were incubated in 5% $Ba(OH)_2$ solution at 35°C.

Late replication bands were produced mainly by the method of Takayama *et al.* [24]. The period of G_2 phase in the lymphocyte cell-cycle of this species was estimated at 2 hours (data not shown). Therefore, in order to visualize the chromosomal regions replicated during about four hours in late S phase, after 3–5 days of growth of non-synchronized peripheral lymphocytes *in vitro*, 5-bromodeoxyuridine was added to the cultures 6 h before the cell harvest (final concentration 10^{-4} M). Colchicine was added 4 h before the harvest to make the final concentration 10 μg/ml. The BrdU-chromosome preparations were kept for 1–2 days at room temperature, and then stained with 3% Giemsa solution diluted in 2% 4Na-EDTA aqueous solution for 3–5 minutes at 40°C.

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Cross experiments and histological preparation

Cross experiments were performed in February. Ovulation was accelerated by injecting suspension of bullfrog pituitaries into the abdominal cavity. For histological observation, gonads fixed in Navashin's fluid were sectioned (10–12 μm in thickness) and stained with Delafield's haematoxylin and eosin.

RESULTS

Mitotic chromosomes

a. Conventional Giemsa staining

Rana japonica collected from Wakuya had, without exception, 26 chromosomes in diploid, comprising five large and eight small chromosome pairs, and chromosome No. 10 had the large, remarkable secondary constriction at the middle portion of the long arm, as reported previously in this species [8, 9, 13, 21, 22]. One homologue of chromosome pair 4 was slightly shorter than the other in the males, while in the females each was similar in size (Fig. 1).

b. C-banding

Chromosomes of this species were characterized by dark and well defined C-bands at the basal portions of both arms (Fig. 2). Each of the five large pairs could be identified by the length, shape and C-banding pattern (Fig. 3). Nos. 1, 2, 4 and 5 were metacentric, and No. 3 was submetacentric. Because No. 1 was largest and No. 5 was smallest, they were discernible from three other chromosome pairs. Though it was similar in size and shape to No. 5, the following type O of No. 4 was clearly distinguished from No. 5 by the unique

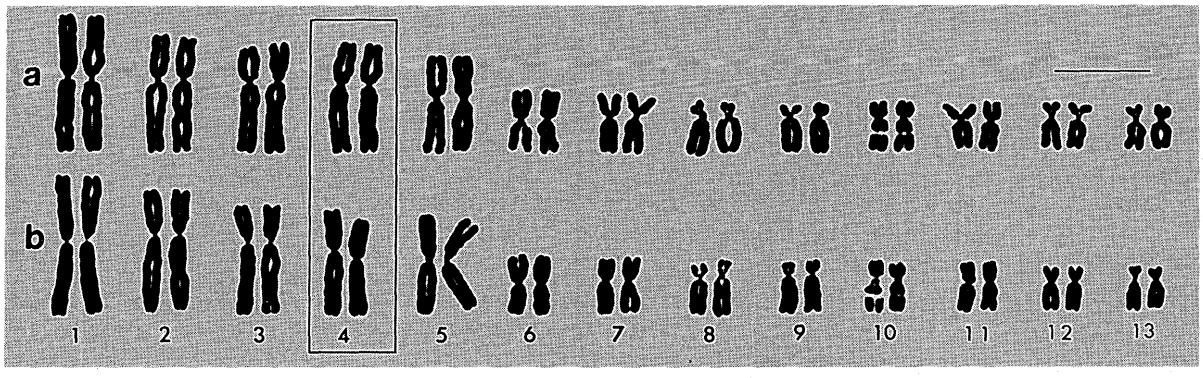


FIG. 1. The karyotypes of *Rana japonica* from the Wakuya population by conventional Giemsa staining: (a) female and (b) male. No. 4 chromosomes are boxed. Bar, 10 μm.

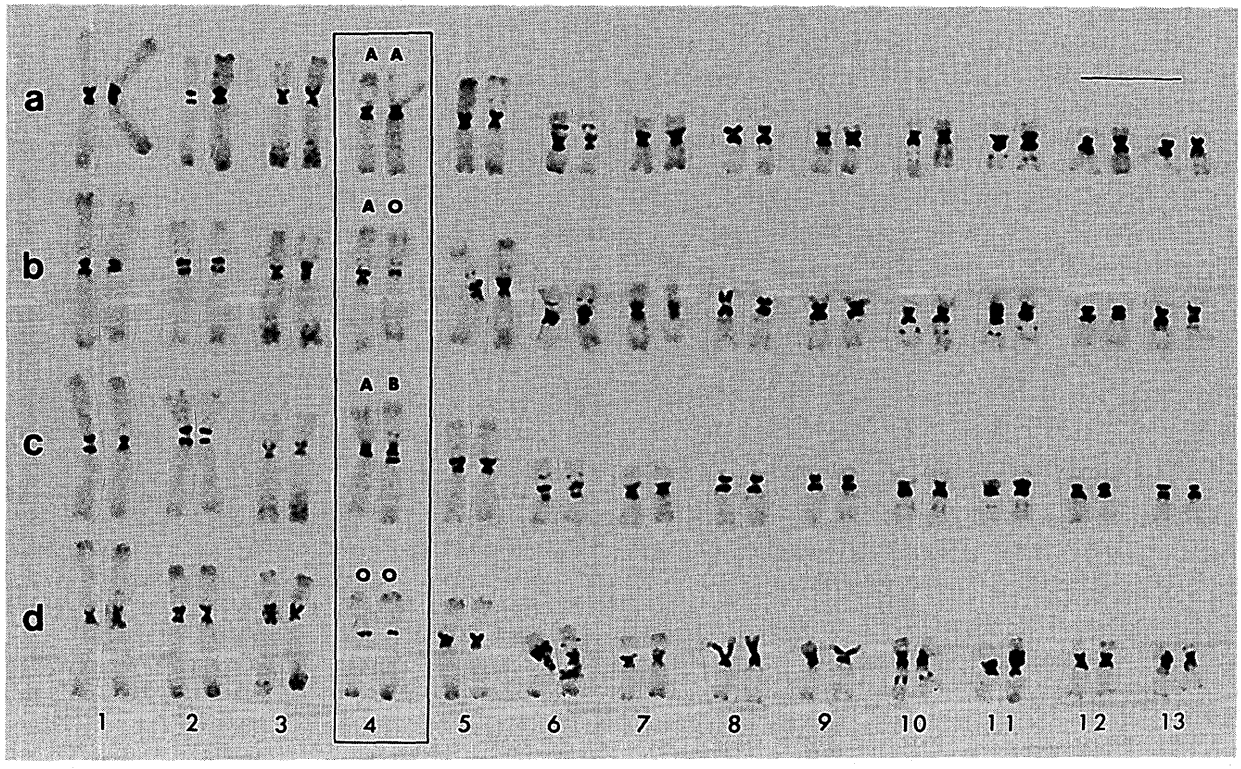


FIG. 2. C-banded karyotypes of *Rana japonica* from the Wakuya population: (a) female of AA type (AA1), (b) male of AO type (AO1), (c) male of AB type (AB1) and (d) male of OO type (OO1). No. 4 chromosomes are boxed. Bar, 10 μm.

C-banding pattern (Figs. 2 and 3). No. 3 was easily identified by its shape and three subterminal weak bands of the long arm, which were pointed out by arrowheads in Fig. 3. Nos. 2 and 4 were similar in shape, although No. 2 was somewhat larger than No. 4. Moreover, No. 2 had three weak C-bands: two sequential bands in the intermediate region of the short arm, and one interstitial band of the long arm. These two bands of the short arm were often observed, while one of the long arm only rarely. In contrast, No. 4 had four weak bands: one subterminal and one proximal bands of the short arm and two interstitial bands of the long arm. Bands of the short arm were frequent, while those of the long arm rare. Therefore, Nos. 2 and 4 could be distinguished from each other by these four frequently observable weak bands of the short arms, as pointed out by arrowheads in Fig. 3. In addition, these two chromosomes could also be distinguished on the basis of dark and well defined pericentromeric bands. For No. 2, two pericentromeric bands on short and long arms got separated from each other, whereas for No. 4 they often appeared joined together due to their close location (Figs. 2–4).

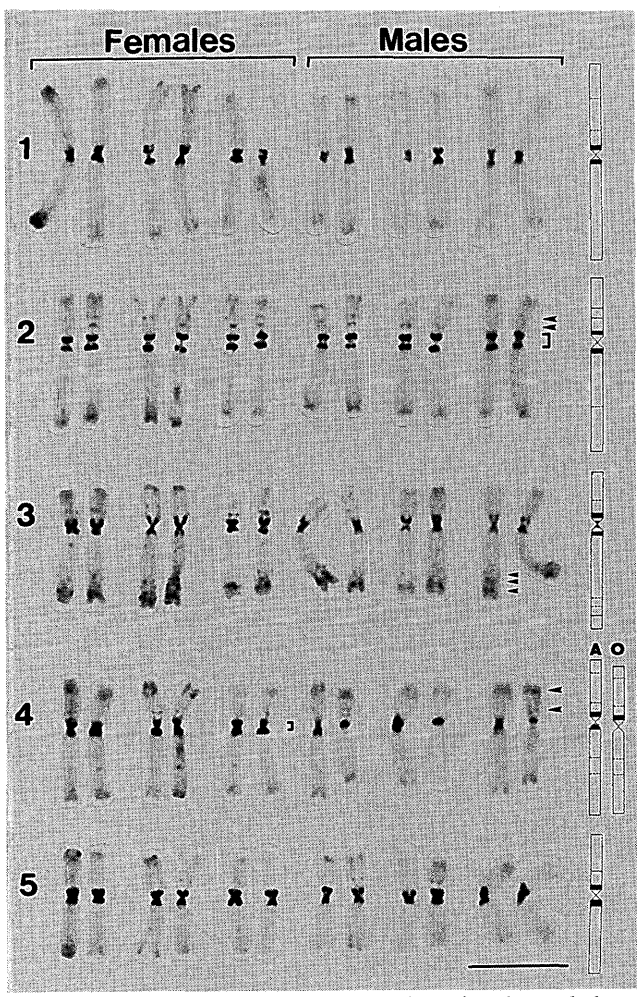


FIG. 3. Partial C-banded karyotypes of three females and three males. Chromosomes 1~5 are presented. Chromosome number is indicated on the left side. Idiogram showing C-banding patterns of chromosomes 1~5 is arranged on the right side. The arrowheads point to C-bands that characterize chromosomes 2, 3 and 4. Distance between the two pericentromeric bands on chromosomes 2 and 4 is shown by a short vertical bar. Bar, 10 μm.

When C-banded karyotypes of 125 specimens were examined, No. 4 chromosome was divided into three types (A, O and B) with respect to C-banding pattern (Fig. 2). Type A chromosome had a band at the basal portion of the long arm like other chromosomes, type O had no such band, and type B had double bands at the corresponding region. Type O chromosome was slightly shorter than type A at a ratio of 0.931 ± 0.011 (standard error), calculated from 30 metaphases of 11 males. And, type B was slightly longer than type A at a ratio of 1.099 ± 0.021, calculated from eight metaphases of two males. As shown in Table 1, of the 41 females examined, 40 were homomorphic (AA), and the remaining one was heteromorphic (AO). In contrast, of the 84 males examined, 72 were heteromorphic (AO), nine, homomorphic (AA), one, homomorphic (OO), and two, heteromorphic (AB). These observations show that the C-band located at the basal portion of the long arm of chromosome No. 4 is sex-linked. There were no definite banding differences between the A chromosomes of AA females and AA males, and also between the AO chromosomes of the AO males and AO female (Fig. 4). The C-bands in other chromosomes showed no such sex-related heteromorphism.

c. Late replication banding

The 140–141 late replicated bands were identified in the haploid chromosome set (Fig. 5). These bands showed characteristic pattern of each chromosome, making exact identification of all the chromosomes possible (Fig. 6). For chromosome No. 4, seven bands were equally confirmed in the short arms of the type A and O chromosomes, while nine and eight bands were visualized in the long arms of the type A and type O chromosomes, respectively (Fig. 7). The first band from the basal region of the long arm of the type A chromosome, which corresponded to the C-band at the basal portion of the long arm, was not detected in the type O

TABLE 1. Number of mitotic metaphases used for chromosome analyses and the banding pattern of chromosome No. 4 in the Wakuya population of *Rana japonica*

Sex	No. of analyzed frogs	No. of mitotic metaphases observed (photographed)			C-band type of chromosome No. 4
		Giemsa staining	C-banding	LR-banding	
Female	40	14 (14)	643 (165)	116 (116)	AA
	1	–	11 (11)	–	AO
Total	41	14 (14)	654 (176)	116 (116)	
Male	9	–	160 (46)	2 (2)	AA
	72	17 (17)	1352 (167)	29 (29)	AO
	2	–	40 (18)	–	AB
	1	–	20 (10)	–	OO
Total	84	17 (17)	1572 (241)	31 (31)	

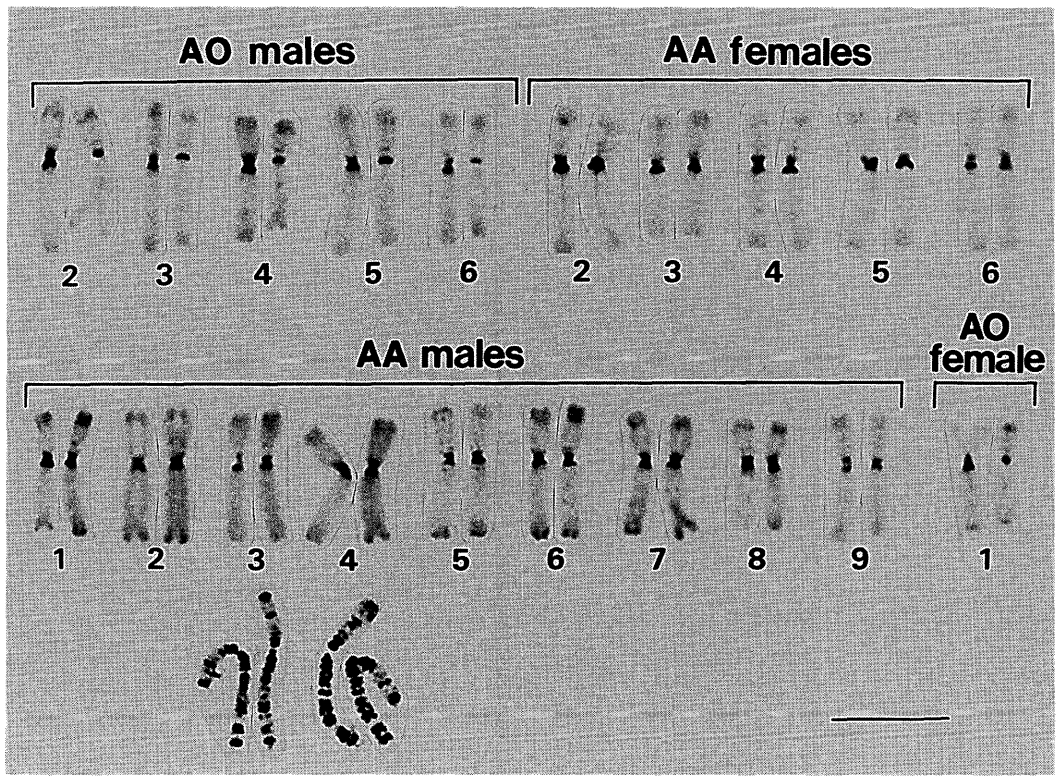


FIG. 4. C-banding patterns of the No. 4 chromosomes from five AO males (AO2~6), five AA females (AA2~6), nine AA males (AA1~9) and one AO female (AO1). Individual numbers are shown under the C-banded chromosome pairs. Late replication banding patterns of the No. 4 chromosomes from AA3 and AA4 males are shown under their C-banded chromosome pairs. Bar, 10 μm.

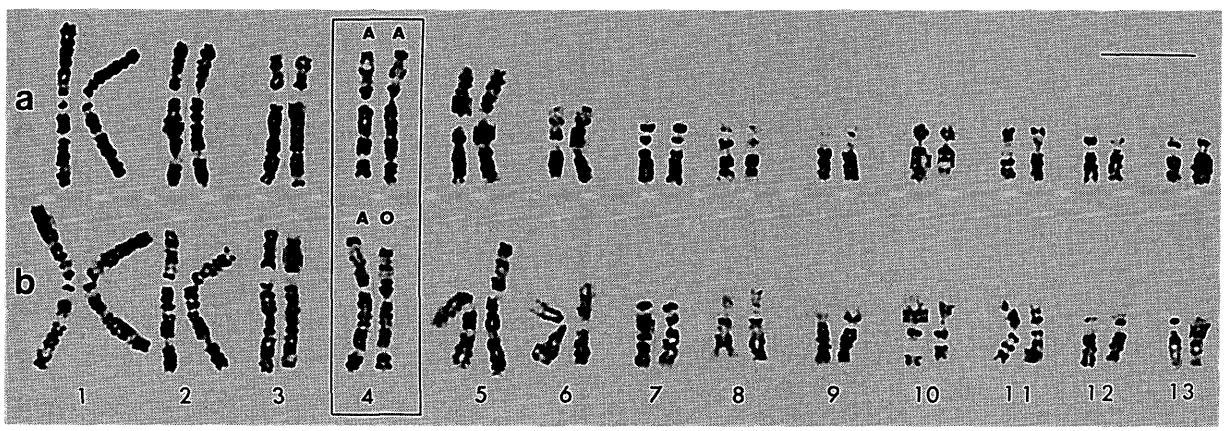
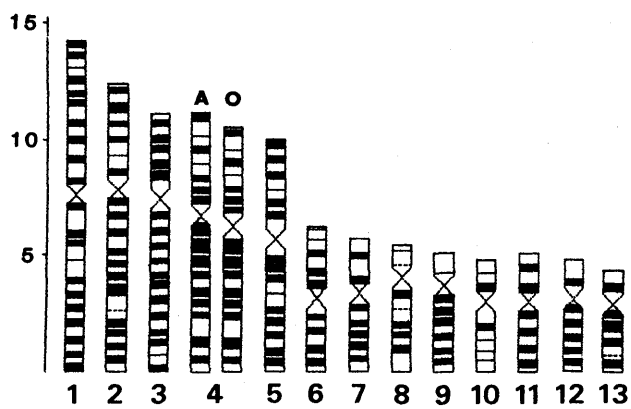


FIG. 5. Late replication banded karyotypes of *Rana japonica* from the Wakuya population: (a) female of AA type (AA1) and (b) male of AO type (AO15). No. 4 chromosomes are boxed. Bar, 10 μm.



chromosome. The patterns of the remaining bands of the type A chromosome were identical with those of the type O chromosome. Between the two A chromosomes of the AA males, there were no differences of banding patterns (Fig. 4). In the remaining chromosomes there were no differences in the banding patterns between females and males.

FIG. 6. Idiogram showing late replication banding pattern of haploid chromosome set in *Rana japonica*. Scale on the left side shows relative chromosome length.

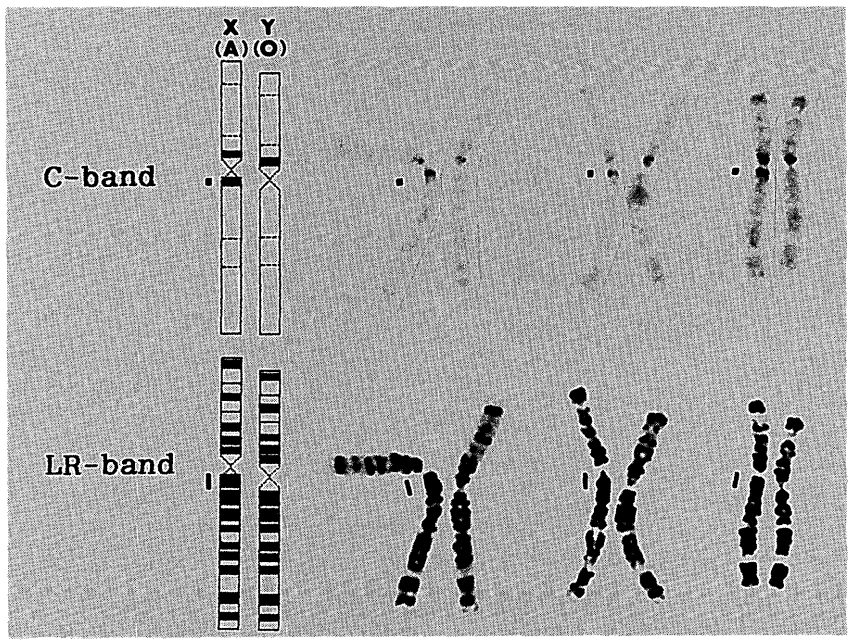


FIG. 7. Selected No. 4 chromosomes in a male of AO type (AO15) after C-banding and LR-banding. C-banding was performed after destaining of the LR-banded chromosome preparation. Almost all regions along the A and O chromosomes are homologous except the basal portions of the long arms, which are indicated by dots or bars.

Meiotic chromosomes

The pairing of the chromosomes at first meiotic metaphase was examined in the AO males. Identification of chromosome pair No. 4 was difficult, even in the C-banded metaphases, owing to the considerable spiraling and condensation of each bivalent (Fig. 8). Therefore, the number of large rod-shaped bivalents included in a complement was counted. The results are shown in Table 2. The number of complements containing one large rod-shaped bivalent varied considerably between males examined (17.9~66.7%). Consequently, during meioses of the AO males of *R. japonica*, it seems that each homologue of chromosome pair 4 does not always pair in an end to end fashion, thus differing from the sex-bivalents of mammals and also those of other frogs having highly evolved sex chromosomes.

Examination of the genetic sex of AA, AB and OO males

By C-banding analyses of the mitotic chromosomes, a

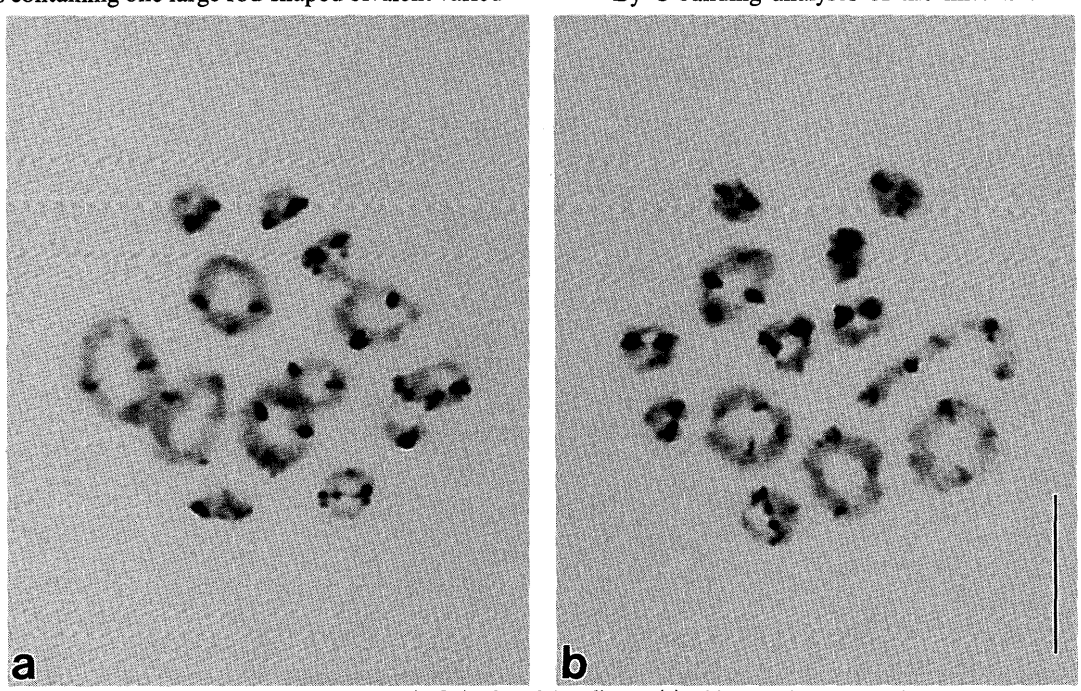


FIG. 8. First meiotic metaphase plates of the AO male (AO5) after C-banding. (a) This complement consists of five large and eight small ring-shaped bivalents. (b) Four large and eight small ring-shaped bivalents and one large rod-shaped bivalent. Bar, 10 μm.

TABLE 2. Pairing of the chromosomes at first meiotic metaphases of ten AO males

Individual No.	No.of meioses	No. of meiotic complements containing 0–5 large rod-shaped bivalents						No. of meioses including univalents
		0	1	2	3	4	5	
AO5	207	**167**	37 (17.9%)	0	0	0	0	3
AO6	209	**152**	53 (25.4%)	3	0	0	0	1
AO7	212	**132**	71 (33.5%)	8	0	0	0	1
AO8	210	7	**140** (66.7%)	47	11	0	0	5
AO9	214	22	**116** (54.2%)	64	7	0	0	5
AO10	210	12	**113** (53.8%)	67	13	0	0	5
AO11	210	85	**106** (50.5%)	16	2	0	0	1
AO12	200	4	**89** (44.5%)	85	17	2	0	3
AO13	218	1	56 (25.7%)	**146**	10	0	0	5
AO14	210	5	89 (42.4%)	**92**	22	2	0	0
Total	2100	587	870 (41.4%)	528	82	4	0	29

small number of males of AA, AB and OO and a female of AO that do not fit the ♂AO/♀AA system were found. Two cases can be supposed for the explanation of this fact. One is due to sex-reversal and the other to a recombination between a C-band at the basal portion of the long arm of chromosome No. 4 and a site of a male-determining gene located on the same chromosome. If sex-reversal could occur, AA males should be sex-reversed females and an AO female a sex-reversed male. And, an OO would be a genetic YY male produced from mating of a genetic XY male of AO with a sex-reversed XY male of AO. If this is true, some structural abnormality characteristic of transforming gonads might be observed in their testes. Therefore, the histological structure of testes was carefully examined.

a. Structure of testis

All the AA, AB and OO males examined had typical testes with normal structure except the two AA males (AA1, AA7), each of which had an auxocyte within the seminiferous tubule (Fig. 9a). Thus, these two males (AA1, AA7) seem to be spontaneously sex-reversed females.

In order to confirm the genetic sex of the AA males (AA1~5), the AB male (AB1) and the OO male (OO1), they were crossed with AA females, and the sex-ratio of the offspring was examined. Although the AO female laid a number of eggs, they did not begin to cleave at all. Therefore, the sex-ratio of the offspring between the AO female and an AO male could not be examined.

b. Sex of the offspring

Four AO males were mated with six AA females as controls, and five AA males, one OO and one AB males were mated with the six AA females. The percentage of normally cleaved eggs was low (21.0~35.2%) in the matings of the

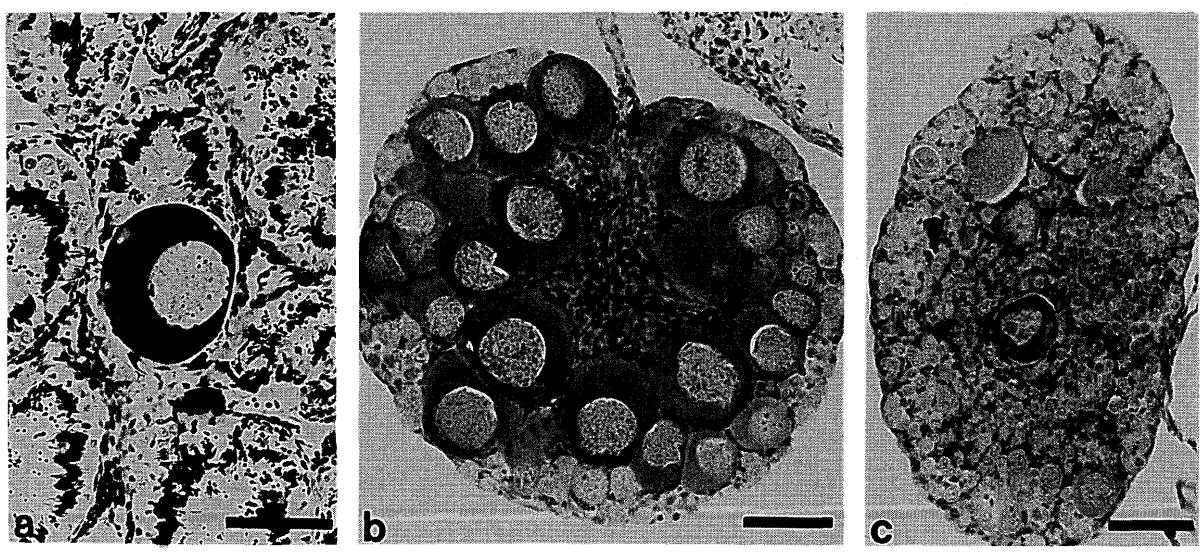


FIG. 9. Cross sections of gonads in an adult male (a) and 1~1.5-month-old frogs after metamorphosis (b and c). (a) Testis of AA7 male with a growing auxocype in the seminiferous tubule. (b) Gonad of a hermaphrodite, transforming from ovary into testis. (c) Gonad of a hermaphrodite having small groups of oocytes in testis. Bars, 100 μm.

AA2, AA5 and AB1 males, while high (72.8~99.6%) in other matings (data not shown). The sex of young frogs within 1.5 months after metamorphosis and adults of six months or one year old obtained from these matings was examined.

The results are shown in Table 3. In control matings, out of a total of 1061 frogs, 526 were females and 535 (50.4%) were males. The sex-ratio was just 1:1 (Table 3). 13 young frogs were hermaphrodites, the gonads of which were testes as a whole, where nearly all the gonia were surrounded with rete cells and there were no ovarian cavities, although there were small groups of oocytes (Fig. 9c).

In experimental matings using the AA males, two types of sex ratio were found. The offspring of the AA2 and AA4 males were females and males at a ratio of about 1:1, which is one type. Nine hermaphrodites at young frog stage were similar to those in the controls, having testes with some oocytes. On the other hand, almost all of the offspring from the AA1, 3 and 5 males were females, which is the other type. Twenty-one hermaphrodites at the young frog stage contained 15 hermaphrodites that had testes with some oocytes similar in histological structure to those of the controls, and six that had gonads at the beginning of sex-reversal, where a multiplication of rete cells was found in the medullary parts of the gonads and in the cortical parts there were abundant oogonia and young oocytes (Fig. 9b). Sex-ratio of offspring from the AB male and the OO male was about 1:1 (Table 3).

These sex-ratios reveal that the three AA males (AA1, 3 and 5) are spontaneously sex-reversed XX females, while the two AA males (AA2 and 4) as well as the AB and OO males are genetic XY males (Table 4).

Inheritance of the C-band in chromosome No. 4

Segregation of the three kinds of C-bands (O, A and B) in chromosome No. 4 was examined in the offspring from the above matings (Table 5). In the control matings of the AO males with the AA females, all the females examined showed the AA type and all the males examined the AO type. In the experimental mating of the AB male with the AA female, all of the females showed the AA type and all of the males the AB type. From these results, the O chromosome of the AO males and the B chromosome of the AB male could be considered Y chromosomes and inheritable to the next generation. In the experimental matings of the AA males with the AA females, all the frogs examined were of the AA type. Likewise, in the experimental mating of the OO male with the AA female, all the frogs examined were of the AO type. These results show that no addition or deletion of the C-band at the basal portion of the long arm of chromosome No. 4 occurred in the course of gametogenesis, or during the development and growth of these males.

TABLE 3. Sex of offspring in the matings of five AA, one AB and one OO males with AA females

Series	Parents		No. of meta-morphosed frogs (%)	1~1.5-month-old frogs whose sex was examined					Six months- or one-year old frogs examined			Sex of all frogs examined		
	Female	Male		Total	♀	♂	♀E	♀C	Total	♀	♂	Total	♀	♂ (%)
Cont.	AA1	AO1	189	117	52	65			48	29	19	165	81	84 (50.9)
	AA2	AO2	196 (87.9)	114	56	57		1	48	24	24	162	80	82 (50.6)
	AA3	AO3	207 (87.0)	111	48	61		2	73	37	36	184	85	99 (53.8)
	AA4	AO3	195 (91.1)	181	87	87		7				181	87	94 (51.9)
	AA5	AO4	190 (79.2)	111	52	56		3	67	44	23	178	96	82 (46.1)
	AA6	AO4	206 (90.4)	191	97	94						191	97	94 (49.2)
	Total		994 (87.0)	825	392	420		13	236	134	102	1061	526	535 (50.4)
Exp.	AA1	AA1	153	81	60	5	4	12	55	42	13	136	102	**34 (25.0)**
	AA2	AA2	90 (21.5)	47	25	22			43	15	28	90	40	50 (55.6)
	AA2	AA3	194 (59.1)	99	93	1	2	3	68	55	13	167	148	**19 (11.4)**
	AA3	AA4	211 (69.9)	104	62	41		1	80	36	44	184	98	86 (46.7)
	AA4	AA4	196 (85.2)	192	89	95		8				192	89	103 (53.6)
	AA5	AA5	126 (32.1)	61	61	0			55	55	0	116	116	**0 (0.0)**
	AA6	AA5	106 (31.8)	99	99	0						99	99	**0 (0.0)**
	3 males (AA1, 3 and 5)			340	313	6	6	15	178	152	26	518	465	53 (10.2)
	2 males (AA2 and 4)			343	176	158		9	123	51	72	466	227	239 (51.3)
	AA2	AB1	90 (15.6)	52	29	23			35	17	18	87	46	41 (47.1)
	AA5	OO1	300 (82.9)	197	99	96		2	73	34	39	270	133	137 (50.7)
	AA6	OO1	210 (87.1)	184	103	81						184	103	81 (44.0)
	Total		510 (84.6)	381	202	177		2	73	34	39	454	236	218 (48.0)

♀E, hermaphrodite whose gonad was at the beginning of sex-reversal from ovary into testis
♀C, hermaphrodite whose gonad was testis as a whole with some oocytes

TABLE 4. Testes and the male offspring of nine AA males, two AB males and one OO male of *Rana japonica* collected from the Wakuya population

Individual No.	Growing auxocytes	Male offspring (%)	Presumed sex chromosome constitution
AA1	+	25.0	XX
AA2	−	55.6	XY
AA3	−	11.4	XX
AA4	−	50.3	XY
AA5	−	0.0	XX
AA6	−	NE	?
AA7	+	NE	XX
AA8	−	NE	?
AA9	−	NE	?
AB1	−	47.1	XY
AB2	NE	NE	?
OO1	−	48.0	XY

NE, not examined
+, detected −, not detected

TABLE 5. Segregation of C-bands on chromosome No. 4 in the offspring from matings of AO, AA, AB and OO males with AA females

Series	Parents		Sex	No. of analyzed frogs	C-band type of chromosome No. 4		
	Female	Male			AA	AO	AB
Cont.	AA2	AO2	Female	14	14	0	0
			Male	14	0	14	0
	AA5	AO4	Female	10	10	0	0
			Male	12	0	12	0
	Total		Female	24	24	0	0
			Male	26	0	26	0
Exp.	AA1	AA1	Female	1	1	0	0
			Male	2	2	0	0
	AA2	AA2	Female	1	1	0	0
			Male	1	1	0	0
	AA2	AA3	Female	2	2	0	0
			Male	1	1	0	0
	AA3	AA4	Female	1	1	0	0
			Male	2	2	0	0
	Total		Female	5	5	0	0
			Male	6	6	0	0
	AA2	AB1	Female	17	17	0	0
			Male	18	0	0	18
	AA5	OO1	Female	3	0	3	0
			Male	2	0	2	0

DISCUSSION

Male heterogamety in the sex-determining mechanism of Rana japonica

Moriwaki [10] obtained 16 parthenogenetic diploids (seven males and nine females) of *Rana japonica* by cold treatment of unfertilized eggs after pricking them with a glass needle. When one of these males was mated with a normal female caught in the field, the progeny were all females [11]. Kawamura and Yokota [6] performed sex-reversal of genetic females of *R. japonica* into phenotypic males by an injection of testosterone propionate at the tadpole stage. All the injected larvae became mature males. When 12 of these males were mated with normal females, the progeny from four of these males were females and males with the ratio 1:1, while almost all of the progeny from the remaining eight males were females. These two experiments clearly show that the males which produced only female progeny in the matings with normal females are sex-reversed XX females, and consequently, in *R. japonica* the male is the heterogametic sex (♂XY/♀XX system). The present C-banding analysis of the chromosomes with the Wakuya population of *R. japonica* has demonstrated that chromosome pair 4 was heteromorphic (AO) in the males and homomorphic (AA) in the females for a C-band at the basal portion of the long arm. When the AO males were mated with AA females, all the male offspring were of the AO type and all the female offspring were of the AA type. Thus, it is clear that the C-band located at the basal portion of the long arm of chromosome No. 4 is sex-linked, and chromosome No. 4 is a sex chromosome itself.

Lower amount of constitutive heterochromatin on the Y chromosome than on the X chromosome

In general, much more constitutive heterochromatin has been accumulated in the Y or W chromosomes than in the X or Z chromosomes of mammals, birds and some lower vertebrates. In contrast, the amounts of constitutive heterochromatin distributed in the Y chromosomes (O) were less than in the X chromosomes (A) in *Rana japonica*. This is one of the unique features for the sex chromosomes of *R. japonica* elucidated in this study. Sex chromosomes similar to those of *R. japonica* have so far been found in the marsupial frog, *Gastrotheca walkeri* [18]. The C-banded karyotype exhibits a large amount of constitutive heterochromatin in the pericentromeric region of each of the chromosomes including X, while the Y chromosome contains an extremely low amount of constitutive heterochromatin at the centromeric region. Such Y chromosomes have been reported also in Japanese Gold fish and Chinese Funa [14] and the Asian black turtle, *Siebenrockiella crassicollis* [1]. In *Rana japonica*, amplification of the heterochromatin occurred in the pericentromeric regions of all the chromosomes, but only the basal portion of the long arm of the Y chromosome escaped from the occurrence possibly by the sex-specific innerstructural change, which may be one of the initial steps

on the differentiation of the Y chromosomes.

Various morphs of the X and Y chromosomes within a species

The other unique feature for the sex chromosomes of *R. japonica* confirmed in this study is the presence of the AA, OO and AB males and the AO female within the ♂AO/♀AA system. Four of the nine AA males were found to be spontaneously sex-reversed females, because almost all of the progeny from these three males when mated with the AA females were females, and one AA male included a growing auxocyte within the seminiferous tubule of the testis (Table 4). Other two AA males were found to be genetic XY males, because the progeny of these two males when mated with the AA females were females and males in the ratio 1:1. The OO male was also found to be a genetic XY male, not a YY male, judging by the 1:1 sex ratio of the progeny. This OO male may have been produced from a mating of an AO male of XY with an AO female of XX in the field. The AO female may not be a sex-reversed XY male, but an XX genetic female, because sex-reversal of genetic XY male into phenotypic female is firmly resisted in *Rana* species [5]. The heteromorphic AB male was also found to be a genetic XY male on the basis of the sex ratio of 1:1 in the progeny, and the B chromosome was found to be a Y chromosome from chromosome analysis of the progeny, where all males were of the AB type and all females were of the AA type.

Thus, in the Wakuya population two morphs (A and O) of the X chromosomes and three morphs (O, A and B) of the Y chromosomes coexist. The south American frog, *Gastrotheca psuestes*, is characterized by ♂XY/♀XX sex chromosome system, and it was shown that in 11 males the XY chromosomes are still homomorphic, but in 15 males the Y chromosome displays a prominent telomeric C-band in the long arm, which is absent in the X [19]. The North American salamander, *Aneides ferreus*, is characterized by ♀ZW/♂ZZ sex chromosomes in the Vancouver island populations, while in the northern California populations all males are homomorphic (telocentrics/telocentrics), and the females are either homomorphic (T/T) or heteromorphic (T/metacentrics) [7, 20]. In the two species referred to above, the frequency of specimens with the morph of the Y or W chromosome which is identical to the X or Z in appearance is relatively high, 42.3% in *Gastrotheca pseustes* and 26.9% in *Aneides ferreus*. By contrast, for *Rana japonica* the genetic XY males of AA rarely occurred at the frequency of 2.4% (2/84)~6.0% (5/84); if the uncertain three AA males are all sex-reversed XX females, the number of the genetic XY males of AA would be two in total, and if those are all genetic XY males, it would be five. Furthermore, a genetic XX female of AO occurred at the similar frequency of 2.4% (1/41). Thus, it is reasonable to infer that in *R. japonica* the XY males of the AA type and the XX female of the AO type were produced through a recombination between the site of a C-band at the basal portion of the long arm of chromosome No. 4 and the site of a male-determining gene on the same chromosome during male meiosis. Since the late replication banding patterns in the euchromatic regions of the A and O chromosomes were almost identical with each other and both AO and autosomal bivalents of the ring-like appearance were formed in male meiosis, the A and O chromosomes may be genetically homologous, and crossing over should occur at the euchromatic regions of XY (AO) as often as autosomes.

Consequently, chromosome No. 4 of *R. japonica* is a sex chromosome without doubt. However, sex-linkage of the C-band on chromosome No. 4 is incomplete. Therefore, the heteromorphic situation of the sex chromosomes will possibly not be held in this population, if the number of recombinants and sex-reversed females increases in future generations. Such unstable heteromorphism of the sex chromosomes may reflect the primitive state of sex chromosome differentiation.

Iizuka [4] has performed C- and Ag-banding analyses on the chromosomes of *R. japonica* with the Hitachi-ota population from Ibaraki Prefecture, but failed to identify the sex chromosomes. Therefore, in order to know whether such a peculiar differentiation of the sex chromosomes is a restricted system only to the Wakuya population under present study, or whether other different types of sex chromosome exist in this species, it is necessary to do an extensive chromosomal analysis of various geographic populations.

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