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The origin of the brown frogs with 2n = 24 chromosomes

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Abstract. Late replication and C-banding analyses of somatic metaphase chromosomes were attempted on three species of brown frogs with 2n = 26 chromosomes (*Rana japonica*, *R. tsushimensis* and *R. temporaria*), and three with 2n = 24 chromosomes (*R. ornativentris*, *R. dybowskii* and *R. chensinensis*), which are distributed in the Palearctic region. The late replication banding patterns were highly conserved in these species. Four chromosome inversions were demonstrated in *R. ornativentris*, two in *R. dybowskii* and two in *R. tsushimensis*. From a detailed comparison of late replication and C-banding patterns between the 2n = 26 and the 2n = 24 species, it was found that an end-to-end fusion of two small chromosomes (nos 11 and 13) in an ancestral 2n = 26 species may have produced the medium-sized no. 6 chromosome of the 2n = 24 species.
Key words. Brown frogs; 2n = 24; 2n = 26; late replication band; C-band; an end-to-end fusion.

Brown frog species of the genus *Rana* are widely distributed in the Northern Hemisphere. Although *Rana* species usually have the 2n = 26 karyotype, brown frog species are characterized by two large groups with different chromosome numbers: 2n = 26 and 2n = 24 (refs 1–3). On the basis of electrophoretic analyses of enzymes and blood proteins in the 12 brown frog species distributed in the Palearctic region and North America[4], it was found that three species primarily deviated from ancestral frogs, and thereafter the remaining frogs were divided into two large groups of species: one with a diploid chromosome number of 26 and the other with 24. Each of these groups has expanded its distribution and differentiated into the

Table. Numbers of specimens and mitotic metaphases used for chromosomal analyses by conventional Giemsa staining (G), C-banding (C) and late replication banding (LR) in 11 populations of six brown frog species.

Species	Population (Country)	Sex	No. of frogs	Nos of metaphases photographed (analyzed) and specimens		
				G	C	LR
Rana japonica	Wakuya	female	22	15 4	135 (10) 18	135 (5) 10
	(Japan)	male	24	15 4	185 (23) 20	45 (10) 8
	Saeki	female	5	11 4	13 (4) 2	27 (8) 2
	(Japan)	male	9	37 9	27 (7) 3	41 (9) 4
	Hiroshima	female	4	2 1	32 (17) 2	3 1
	(Japan)	male	2	10 2	14 (2) 1	9 1
R. tsushimensis	Tsushima	female	3	32 3	44 (13) 3	54 (12) 3
	(Japan)	male	3	10 1	34 (7) 3	28 (3) 2
R. temporaria	St. Petersburg (Russia)	female	2	25 2	44 (20) 2	51 (23) 2
R. ornativentris	Hirosaki	female	8	12 1	68 (10) 8	73 (17) 8
	(Japan)	male	6	26 2	43 (8) 5	46 (4) 5
	Saeki	female	3	– –	10 3	11 (5) 3
	(Japan)	male	3	31 2	74 (18) 2	32 (13) 2
R. dybowskii	Tsushima	female	3	16 3	42 (12) 3	38 (15) 3
	(Japan)	male	4	20 3	55 (4) 4	55 (5) 4
R. chensinensis	Sapporo	female	2	28 2	31 (9) 2	35 (11) 2
	(Japan)	male	2	19 2	66 (17) 2	37 (9) 2
	Maritime	female	2	36 2	115 (16) 2	42 (18) 2
	territory (Russia)	male	2	22 2	21 (1) 1	16 (2) 2
(Hybrid)[a]	Beijing (China)[b]	male	3	26 3	80 (27) 2	32 (20) 3

[a]Hybrids of *Rana chensinensis* between the Maritime territory and Beijing populations were used for observation of the Beijing chromosomes.
[b]China, People's Republic of China

present individual species. Cytogenetic studies have not directly demonstrated the chromosomal homology conserved among brown frog species, or chromosomal rearrangements associated with speciation, especially the mechanisms of reduction in chromosome number from 26 to 24 or increase from 24 to 26 (refs 5–9), because it has not been possible to demonstrate G- and Q-bands in amphibian chromosomes as reproducibly as in other classes of vertebrates[10–17]. Recently, however, replication banding has been shown to induce multiple bands on amphibian metaphase chromosomes[18–25].

In the present study, we analyzed the chromosomes of six brown frog species distributed in the Palearctic region, by late replication and C-banding methods, in order to examine the chromosomal rearrangements associated with speciation, expecially the mechanisms of reduction in chromosome number from 26 to 24 or increase from 24 to 26. A detailed comparison of the banding patterns showed high conservation of the late replication banding patterns through these species and chromosomal mutations of inversions in several chromosomes. Furthermore, it was found that an end-to-end fusion of two small chromosome pairs causes the reduction in chromosome number from 26 to 24. This is the first report that verifies the mechanisms of chromosome rearrangements associated with the speciation of amphibians from the late replication banding patterns.

Materials and Methods

Collection station, numbers of specimens and metaphases observed in this study are shown in the table. Hirosaki, Aomori Prefecture and Wakuya, Miyagi Prefecture are situated in the northern region of Honshu, Japan, while Hiroshima and Saeki, Hiroshima Prefecture are situated in the south. Sapporo is in Hokkaido and Tsushima, Nagasaki Prefecture is in Kyushu. For the observation of *R. chensinensis* chromosomes from Beijing, male hybrids between the Maritime territory and Beijing populations, produced by artificial insemination and bred in our laboratory, were used.

Blood cells were cultured according to the method of Nishioka et al.[25]. Chromosome spreads were prepared

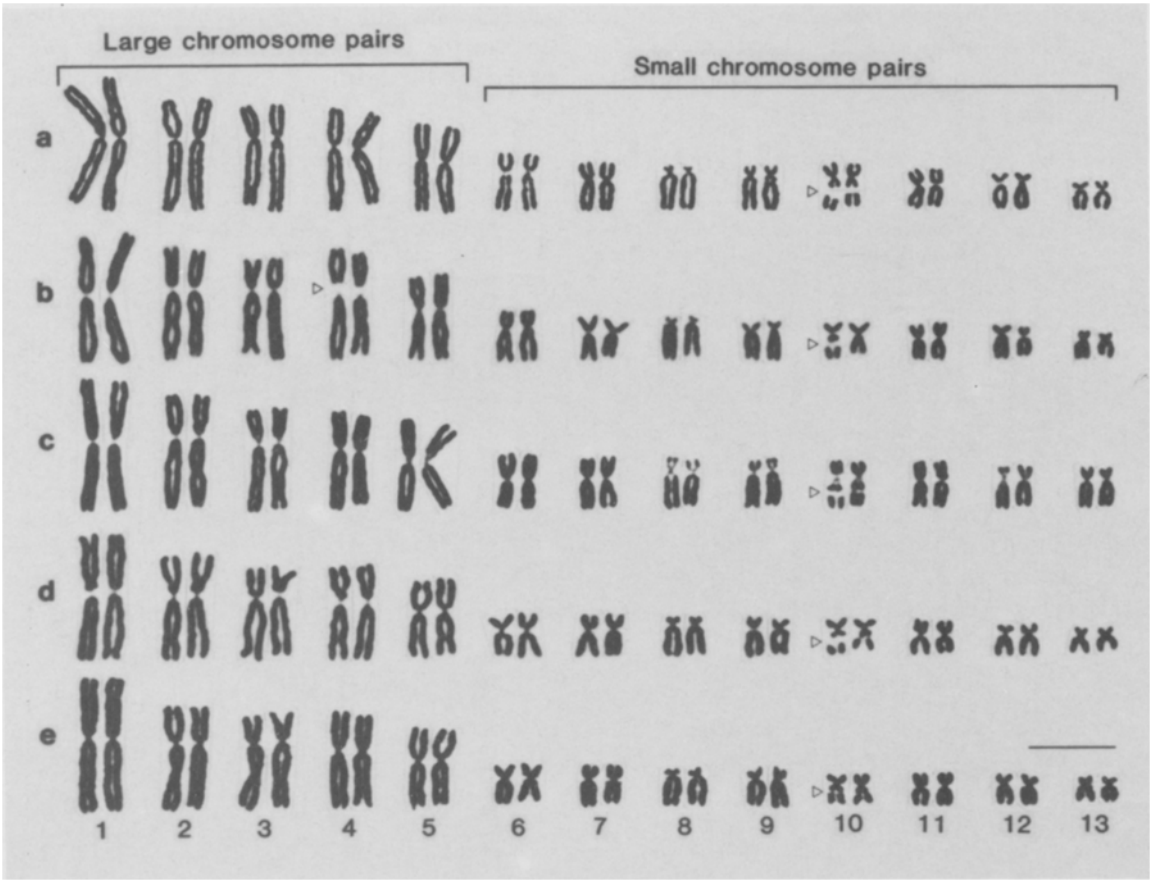

Figure 1. Karyotypes of the three brown frog species with 26 diploid chromosomes from five populations by conventional Giemsa staining: *a Rana japonica* male of the Hiroshima population, *b R. japonica* female of the Saeki population, *c R. japonica* male of the Wakuya population, *d R. tsushimensis* female, *e R. temporaria* female. Bar represents 10 μm. Large secondary constrictions are indicated by open triangles.

through the conventional air-drying method. Constitutive heterochromatin was stained with a modification of the technique of Sumner[26], described in detail by Nishioka et al.[25]. Late replication banding was achieved mainly by the 4Na-EDTA Giemsa staining method of Takayama et al.[27]. A small amount of 5-bromodeoxyuridine (BrdU) was added to the cultures, to give a final concentration of 10^{-4} M, six hours before the cells were harvested. Colchicine was also added, to a final concentration of 10 µg/ml, four hours before the harvest. Chromosome slides prepared by the ordinary air-drying method were heated overnight at 55 °C, then immersed in methanol for several seconds and incubated in 4Na-EDTA Giemsa solution (3% Giemsa solution in 2% 4Na-EDTA) for 3–5 min at 40 °C. The BrdU-incorporated regions of chromosomes were deeply stained with Giemsa. These regions corresponded to the regions replicated during roughly the last 4 h of the S phase, because the G_2 phase period was estimated at 2 h.

The distance of individual bands from the terminal of the short arm was measured on 15–23 metaphase figures by a digitizer (Olympus, Japan). The position of a band in the idiogram was shown by its mean distance as a percentage of the chromosome length. The relative chromosome length (chromosome length as a percentage of the total length of all haploid chromosomes) and the centromere position of a chromosome were measured in C-banded metaphase figures, and the means of

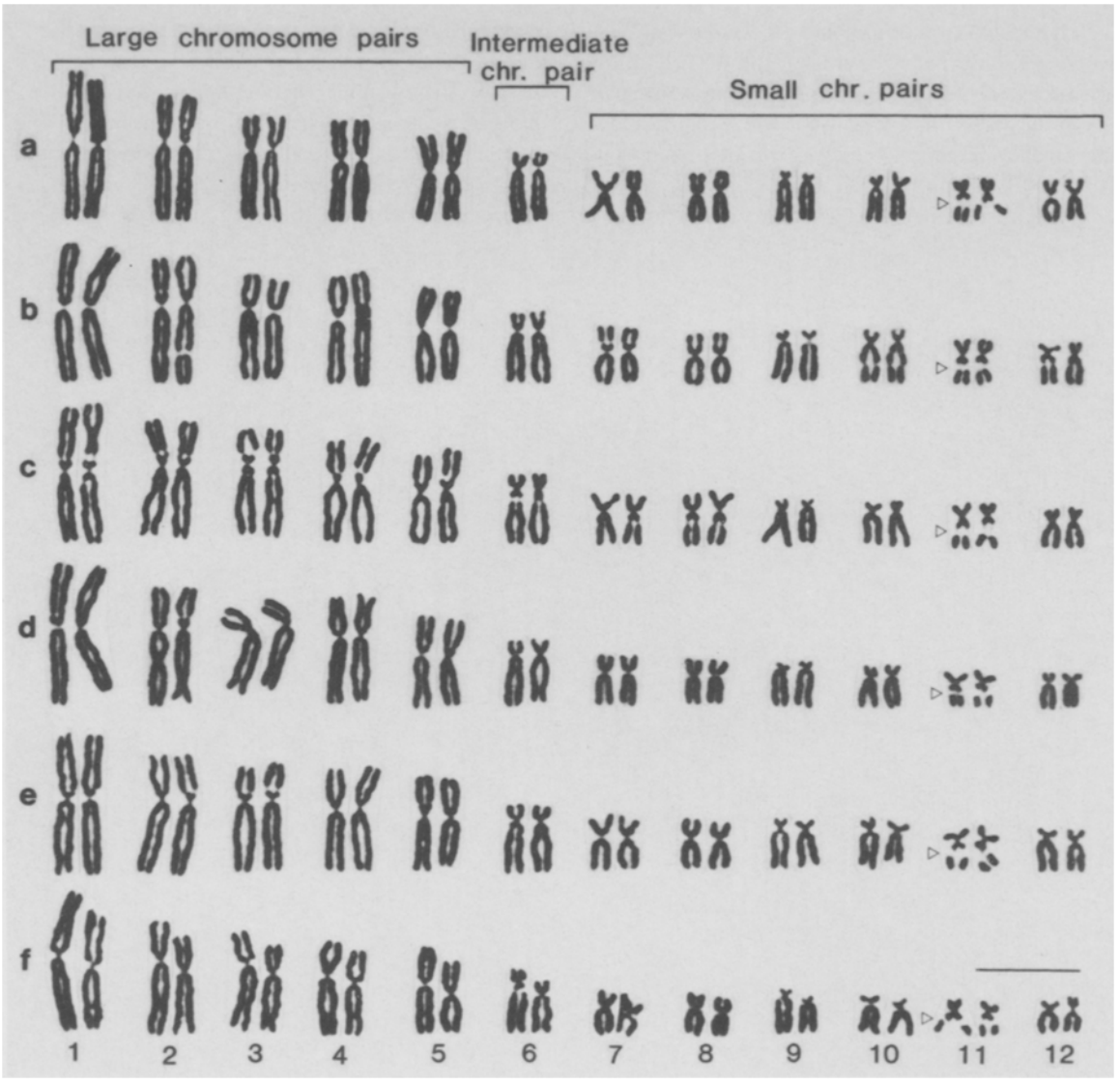

Figure 2. Karyotypes of the three brown frog species with 24 diploid chromosomes from six populations by conventional Giemsa staining: *a Rana ornativentris* male of the Saeki population, *b R. ornativentris* male of the Hirosaki population, *c R. dybowskii* male, *d R. chensinensis* female of the Sapporo population, Japan, *e R. chensinensis* female of the Maritime territory population, Russia, *f* Male hybrid of *R. chensinensis* between the Maritime territory (Russia) and Beijing (China) populations. Bar represents 10 µm. Large secondary constrictions are indicated by open triangles.

measurements of 11–33 metaphase figures were regarded as the values for the chromosome in the idiograms of C-banding patterns. However, in idiograms showing late replication banding patterns, the relative lengths of haploid chromosomes were unified in all six species, except in several chromosomes in which the relative lengths were adjusted in order to maximally match the banding patterns of other species. Twelve chromosome pairs of the 2n = 24 species were arranged to correspond to the chromosomes of the 2n = 26 species in a late replication banding pattern. No. 6 chromosomes of the 2n = 24 species were placed between the five large and six small chromosome pairs.

Results

Karyotypes. *Rana japonica* from three populations (Hiroshima, Saeki and Wakuya), *R. tsushimensis*, and *R. temporaria*, all had 26 chromosomes in diploid, comprising five large and eight small chromosome pairs (fig. 1). A large secondary constriction was located at the intermediate region of the long arm of chromosome no. 10. The Saeki population of *R. japonica* was remarkable because of its large secondary constriction in the proximal region of the short arm of chromosome no. 4, in addition to that of no. 10. In contrast, *R. ornativentris* from two populations (Saeki and Hirosaki), *R. dybowskii* and *R. chensinensis* from three populations (Sapporo, Maritime territory and Beijing) all had 24 chromosomes in diploid, consisting of five large and six small chromosome pairs plus one medium-sized pair (fig. 2). A large secondary constriction was located at the intermediate region of the long arm of chromosome no. 11. In addition, many small secondary constrictions (often wide ones) were observed in the proximal regions of the short arm, the long arm or both.

C-banding patterns. 1) The 2n = 26 species. Centromeric C-bands were deeply stained and detected in all 13 chromosome pairs of *R. japonica* and *R. temporaria*, but not in any chromosomes of *R. tsushimensis* (fig. 3). The centromeric bands in *R. japonica* from the Wakuya population were marked, and were the largest among these species, extending to the pericentric region of each chromosome. Distinct bands were also observed in the proximal regions of chromosomes nos 3 and 6 of *R. japonica*, nos 1–5 and 9 of *R. tsushimensis* and nos 1–5, 8 and 9 of *R. temporaria* (figs 3 and 5). Sexual heteromorphism of C-banding patterns was found in

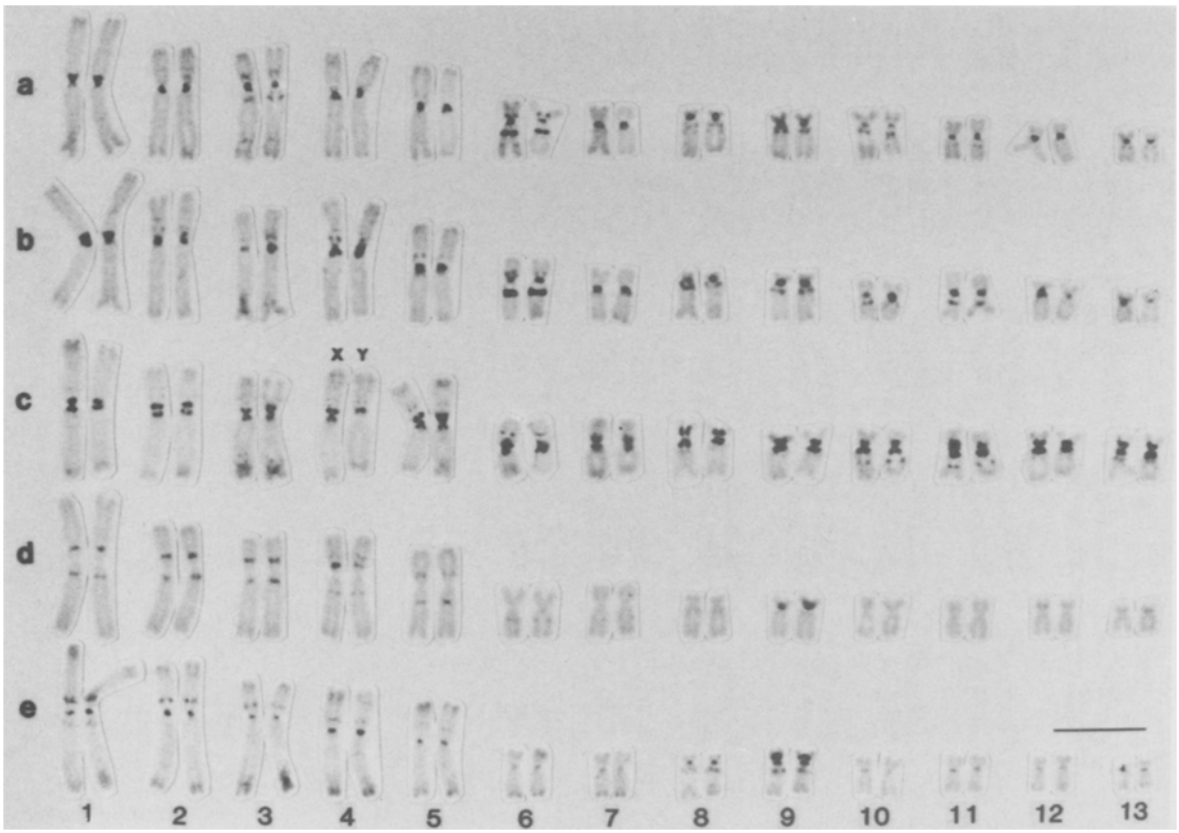

Figure 3. C-banded karyotypes of the three brown frog species with 26 diploid chromosomes from five populations: *a Rana japonica* male of the Hiroshima population, *b R. japonica* male of the Saeki population, *c R. japonica* male of the Wakuya population, *d R. tshushimensis* female, *e R. temporaria* female. Bar represents 10 μm.

chromosome pair 4 of *R. japonica* from the Wakuya population (in preparation).

2) The 2n = 24 species. Centromeric bands were observed, but stained weakly, in chromosomes nos 1–6 and 9–12 of the Sapporo population of *R. chensinensis* and in nos 4 and 5 of the Beijing population of *R. chensinensis*, but they were not seen in any of the chromosomes of *R. ornativentris* and *R. dybowskii* (figs 4 and 5). In contrast, C-bands in the proximal regions were stained clearly in many chromosome pairs of these three species. It is noteworthy that the medium-sized chromosome no. 6 had a gray C-band, which sometimes appeared as two separate gray bands, at the intermediate region of the long arm of the 2n = 24 species (figs 5 and 10). These gray bands were also observed at the terminal regions of other chromosomes.

3) Intra-population variation. Three chromosome pairs (nos 6, 9 and 10) of *R. ornativentris* showed intra-population variation in C-banding patterns (fig. 9). In *R. ornativentris* from Hirosaki, two types of chromosomes with different banding patterns were distinguished as follows: types A and O in chromosome no. 6, types 9 and 9^H in chromosome no. 9, and types 10 and 10^H in chromosome no. 10 (fig. 9). Because type 9^H appears at a low frequency (14.3%) in the population, and is not found in the Saeki population or the other species, the 9^H may be a secondary form produced by a paracentric inversion which moved one of the two bands in the long arm to a more proximal position in the same arm. *R. ornativentris* from Saeki had two types of chromosome no. 10: 10 and 10^S (fig. 9).

Late replication banding patterns. A total of 128–141 late replication bands in the haploid chromosome set

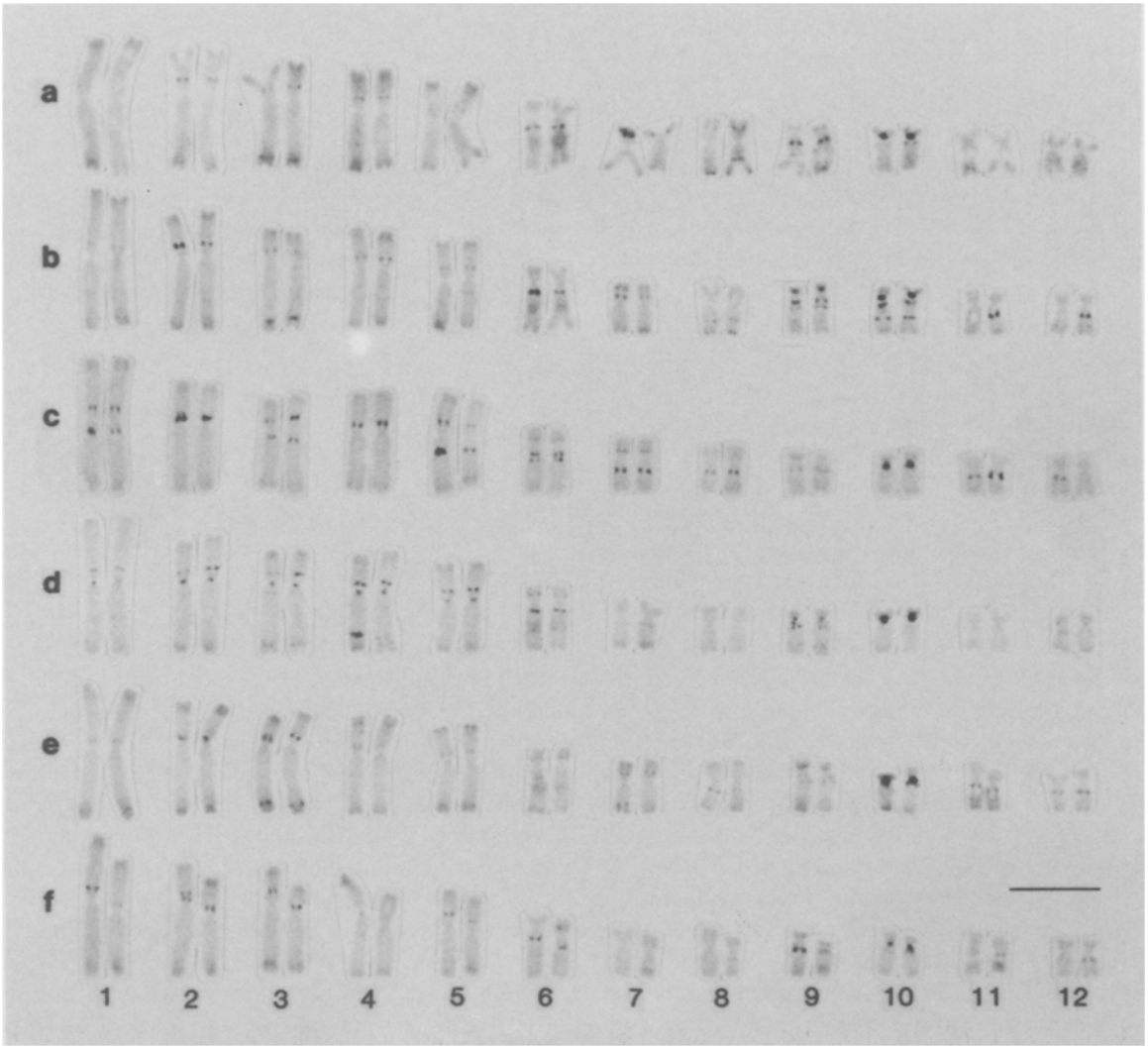

Figure 4. C-banded karyotypes of the three brown frog species with 24 diploid chromosomes from six populations: *a Rana ornativentris* male of the Saeki population, *b R. ornativentris* female of the Hirosaki population, *c R. dybowskii* female, *d R. chensinensis* male of the Sapporo population, Japan, *e R. chensinensis* female of the Maritime territory population, Russia, *f* Male hybrid of *R. chensinensis* between the Maritime territory (Russia) and the Beijing (China) populations. Bar represents 10 μm.

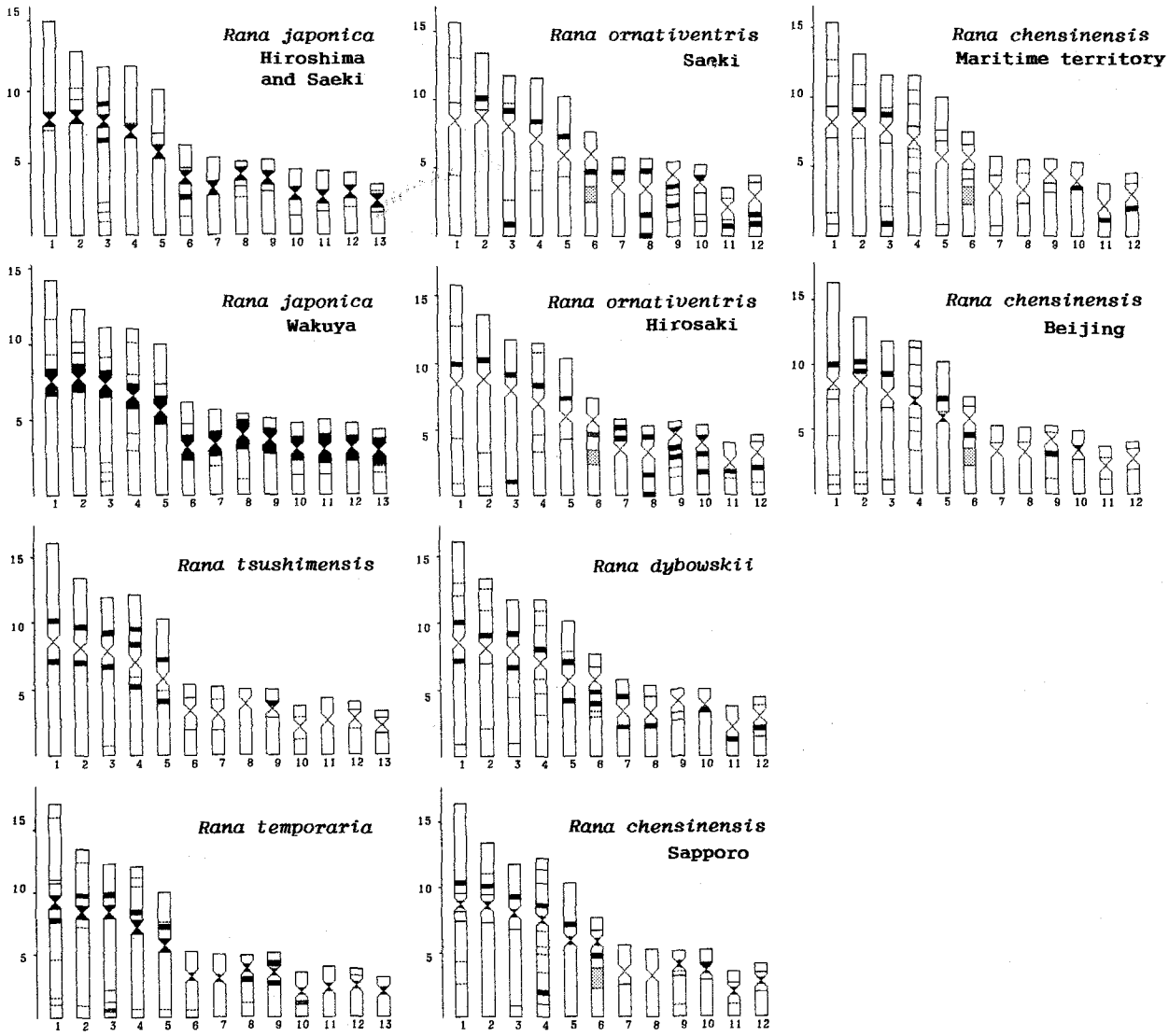

Figure 5. Idiogram of the C-banded karyotypes of the six brown frog species from 11 populations. Scales shown to the left of the no. 1 chromosomes represent the relative lengths of chromosomes.

was identified in the ten populations of the six brown frog species (figs 6–8). These bands appeared sequentially along the axis of individual chromosomes and showed characteristic patterns of each chromosome, making exact identification of all the chromosome pairs possible. No intra-individual differences could be found in the late replication banding patterns.

1) The $2n = 26$ species. The banding patterns of the $2n = 26$ species were very similar to each other (figs 6–8). However, species- and population-specific bands were also detected, as indicated by the arrowheads in figures 6 and 7. Comparison of these specific bands with the C-banding patterns revealed that they were all constitutively heterochromatic, that is, C-bands. In contrast, *R. tsushimensis* was remarkable because of the differences between the banding patterns of nos 2 and 8 and those of their counterparts in the other species. For no. 2, a pericentric inversion might have occurred, resulting in the movement of the band from the long arm nearest centromere to the proximal poisition of the short arm or *vice versa* (figs 6 and 8). More information on banding patterns of many *Rana* species is necessary to decide whether no. 2 of *R. tsushimensis* is a primary or secondary form. Chromsome no. 8 may be a secondary form produced by at least one paracentric inversion, because this banding pattern is restricted to *R. tsushimensis* among the brown frogs examined and other *Rana* species (unpublished).

2) The $2n = 24$ species. The banding patterns of $2n = 24$ species were also similar to each other (figs 6–8). The no. 12 chromosome of *R. ornativentris* from Saeki showed a different banding pattern from that of other species, but may be equivalent to the 12^{H} chromosome of the Hirosaki population described below (figs 7

Figure 6. Comparison of the late replication banding patterns in the six brown frog species from ten populations: Js, *R. japonica* of the Saeki population; Jw, *R. japonica* of the Wakuya population; Ts, *R. tsushimensis*; Te, *R. temporaria*; Os, *R. ornativentris* of the Saeki population; Oh, *R. ornativentris* of the Hiroskai population; Dy, *R. dybowskii*; Cs, *R. chensinensis* of the Sapporo, Japan; Cm, *R. chensinensis* of the Maritime territory, Russia; Cb, *R. chensinensis* of the Beijing, China. Chromosomes 1–5 in these six species are presented for comparison. Numbers on the left and right sides denote the chromosome numbers in the 2n = 26 and 2n = 24 species, respectively. Horizontal lines between the chromosomes connect the homologous bands. Arrowheads indicate the late replication bands that correspond to the the C-bands. Open triangles indicate the positions of the centromeres. Bar represents 10 μm.

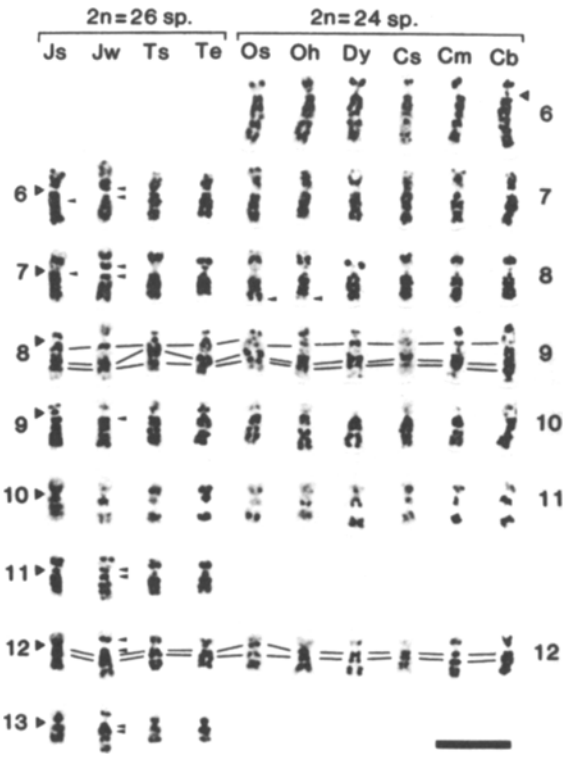

Figure 7. Comparison of the late replication banding patterns in the six brown frog species. Chromosomes 6–13 in the 2n = 26 species and 6–12 in the 2n = 24 species are presented for comparison. Abbreviations are the same as in figure 6. Bar represents 10 μm.

and 8). The intra-population variation of banding patterns was observed in nos 10 and 12 of *R. ornativentris* and nos 9 and 12 of *R. dybowskii* (fig. 9). The chromosomes 10^{H} and 12^{H} in *R. ornativentris* from Hirosaki, 10^{S} in *R. ornativentris* from Saeki and 9^{D} and 12^{D} in *R. dybowskii* may be secondary forms produced by a pericentric inversion because they are restricted to species or populations, and also appear at a low frequency ranging from 17.9%–41.7% (fig. 9).

3) Comparison of banding patterns between the 2n = 26 and the 2n = 24 species. Careful comparison of the banding patterns between three of the 2n = 26 species and three of the 2n = 24 species showed similarities in the following chromosome pairs: no. 1 of the 2n = 26 species and no. 1 of the 2n = 24 species; 2 and 2; 3 and 3; 4 and 4; 5 and 5; 6 and 7; 7 and 8; 8 and 9; 9 and 10; 10 and 11; 12 and 12 (fig. 8). In contrast, two chromosomes (nos 11 and 13) of the 2n = 26 species and one chromosome (no. 6) of the 2n = 24 species did not have counterparts. A close examination showed that no. 11 of the 2n = 26 species and about half of no. 6 of the 2n = 24 species, inclusive of the short arm, centromere and half the proximal portion of the long arm, shared similar banding patterns, and no. 13 of the 2n = 26 species and the remaining half of no. 6 of the 2n = 24 species, involving the distal half of the long arm, did so as well (fig. 10). Accordingly, the medium-sized chromosome no. 6 of the 2n = 24 species may have been primarily produced by an end-to-end fusion of two small chromosome pairs (nos 11 and 13) in an ancestral 2n = 26 species (fig. 10).

Discussion

Karyotypes of five European species of *Rana*, including *R. temporaria*, *R. graeca* and *R. arvalis*, were examined by Morescalchi[5] using a conventional staining method. *R. temporaria* and *R. graeca* have a diploid chromosome number of 26 and the karyotypes are both composed of five large and eight small chromosome pairs. However, the 2n = 24-karyotype of *R. arvalis* is composed of six large and six small chromosome pairs.

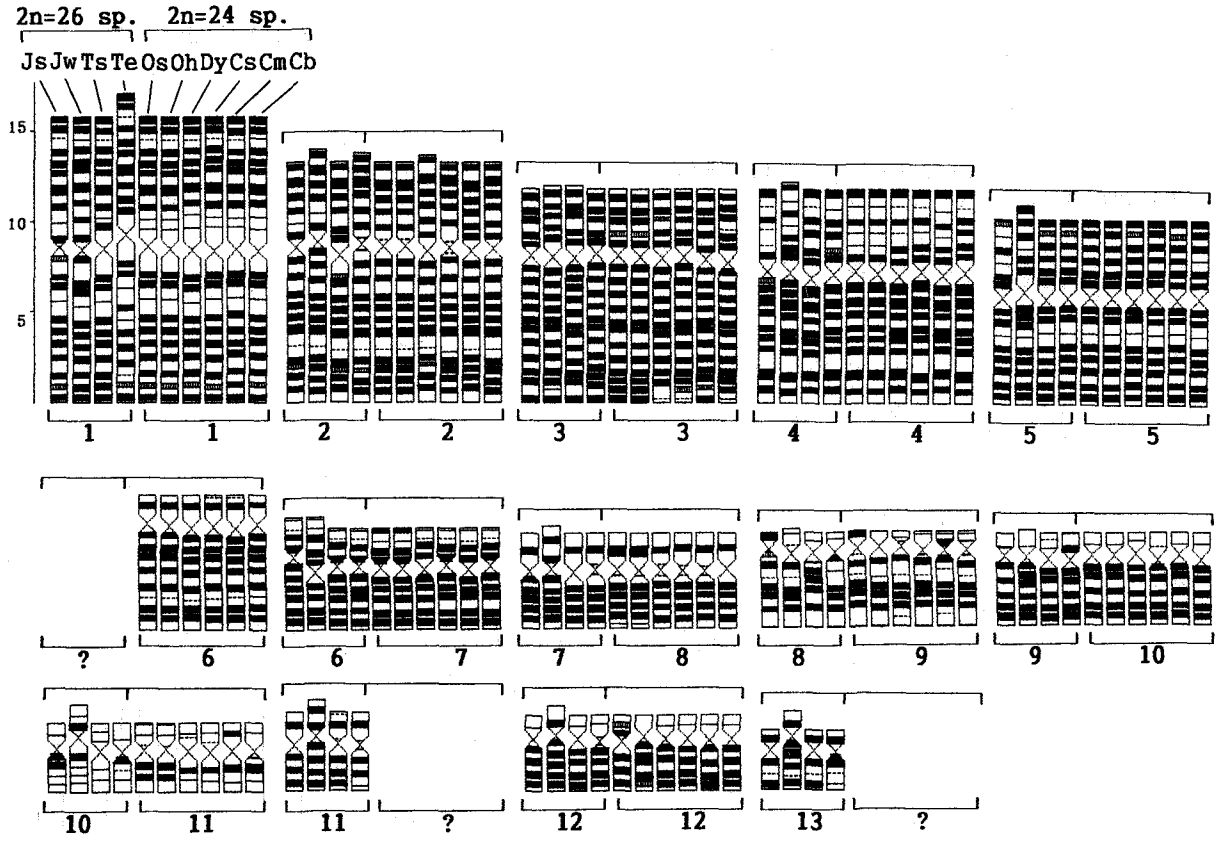

Figure 8. Idiogram of the late replication banding patterns in the chromosomes of the six brown frog species from ten populations. The arrangement order of chromosomes and abbreviations are the same as in figures 6 and 7. Under each chromosome set, chromosome numbers in the the 2n = 26 and 2n = 24 species are shown.

Comparative analysis of the length (relative to chromosome no. 1, 100%) and centromere position of each chromosome indicated that the large chromosome no. 6 of *R. arvalis* and the small chromosomes nos. 6 and 10 of the 2n = 26 species do not have counterparts. Therefore, it was concluded that chromosome no. 6 of *R. arvalis* was produced by the fusion of chromosomes nos 6 and 10 of a 2n = 26 species. However, since the total length of no. 6 and no. 10 from the 2n = 26 species is larger than the length of no. 6 of *R. arvalis*, the fusion was considered to be an unequal translocation with a loss of some chromosome material[1,5]. Green[8], who examined the karyotype and C-banding patterns of *R. dybowskii* from Korea, speculated that the chromosome number reduction from 2n = 26 to 24 in brown frogs is the result of a simple, two-stage process involving the initial production of small telocentric chromosomes by pericentric inversions and their subsequent fusion. This explanation has already been postulated by Morescalchi[28] as the mechanism of chromosome number reduction in African ranoid frogs. Each of these hypotheses would require a two-process chromosome rearrangement to produce chromosome no. 6 of the 2n = 24 species. On the other hand, Nishioka et al.[6] presumed that only a single process was involved in the production of chromosome no. 6. They examined the karyotypes of eight brown frog species distributed in the Palearctic region and North America, using conventional staining methods. Five of them (*Rana japonica*, *R. tsushimensis*, *R. amurensis coreana*, *R. temporaria* and *R. sylvatica*) have a diploid chromosome number of 26 and the remaining three (*R. ornativentris*, *R. dybowskii* and *R. chensinensis*) have 24. On the basis of a comparative karyotype analysis of these species, the union of chromosome no. 11 with chromosome no. 12 or 13 in an ancestral brown frog species with 26 chromosomes was considered to have produced the 24 diploid chromosome.

In the present study, as stated above, the late replication banding technique was applied to the somatic chromosomes of cultured lymphocytes from ten populations of six brown frog species distributed in the Palearctic region. The banding patterns of haploid chromosomes were highly conserved throughout these species. Careful comparison of the banding patterns between the 2n = 26 and 2n = 24 species showed that chromosome no. 6 of the 2n = 24 species was produced by an end-to-end fusion of two small chromosome pairs (nos 11 and 13) in ancestral 2n = 26 species (fig. 10). Additionally, the gray C-band found at the intermediate region of the

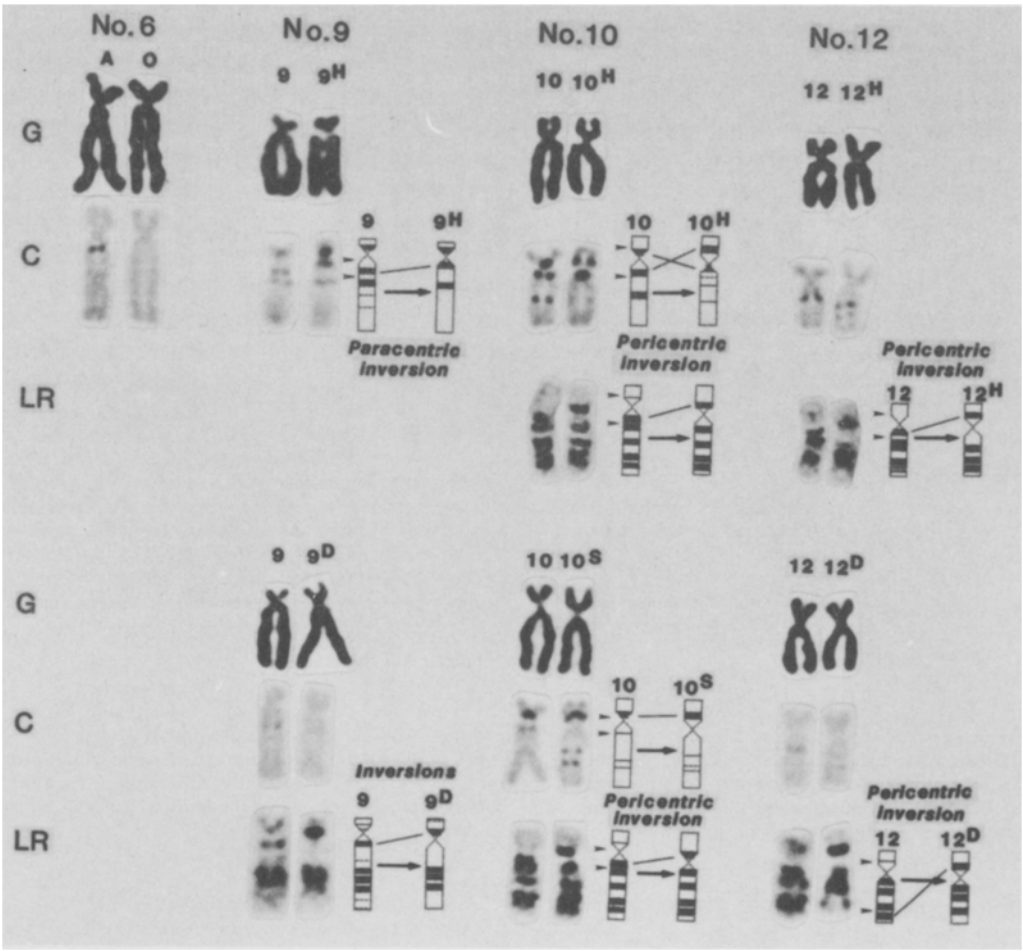

Figure 9. Chromosomes 6, 9, 10 and 12 of *R. ornativentris* and *R. dybowskii* showing intra-population variation in C-banding or late replication banding pattern. Configuration of each chromosome stained by conventional Giemsa staining (G), C-banding (C) and/or late replication banding (LR) is shown here for comparison. The presumed rearrangement necessary to produce each variant chromosome (indicated by H, S or D in the right superscript) is shown on the right side of C-banded or late replication banded chromosomes. Chromosome no. 6 and the 9^H, 10^H and 12^H variants are from the Hirosaki population of *Rana ornativentris*, the 10^S variant is from the Saeki population of the same species, and the 9^D and 12^D variants are from *R. dybowskii*. Arrowheads indicate the presumed breakpoints for inversions.

long arm of chromosome no. 6 from the 2n = 24 species presumably reflects the fusion of the distal gray, C-positive regions of chromosome nos 11 and 13 in ancestral 2n = 26 species (fig. 10). Thus, the production of chromosome no. 6 is probably the result of a single process end-to-end fusion between two chromosomes, and is not accompanied by any other chromosome rearrangements such as inversion and a loss of chromosome segments. Furthermore, the centromere of one (no. 13) of the two chromosomes that participated in the fusion may have been inactivated after their fusion (fig. 10).

A similar end-to-end fusion has occurred in Australian lizard[29]. *Gehyra purpurascens* (2n = 40) possesses a distinctive Z chromosome which is the largest in the diploid complement, revealing female heterogamety (ZW), and G-banding patterns of the Z coincide with those of chromosomes 7 and 8 in a related species, *G. nana*, which has 2n = 44 chromosomes. Therefore, it was concluded that the Z chromosome of *G. purpurascens* was derived from a tandem fusion between the telomeres of two acrocentric chromosomes 7 and 8 in a presumed ancestral species of *Gehyra* with 2n = 44. Additionally, the interstitial region of the long arm of the Z chromosome is intensely stained by N-banding (or gray-stained by C-banding) along with the telomeres of other chromosomes. It is noteworthy that the Australian lizard and brown frogs studied here share the same end-to-end fusion mechanism; that is, the two chromosomes are fused at each telomere of their 'long arms'.

Finally, as described above, chromosome no. 6 of the 2n = 24 brown frog species was probably derived from an end-to-end fusion of chromosomes nos 11 and 13 in an ancestral 2n = 26 species. On the contrary, nos 11 and 13 of the 2n = 26 species are unlikely to have derived from a fission of chromosome no. 6 of an ancestral 2n = 24 species, for the following two reasons: First, if a centric fission in chromosome no. 6 of an

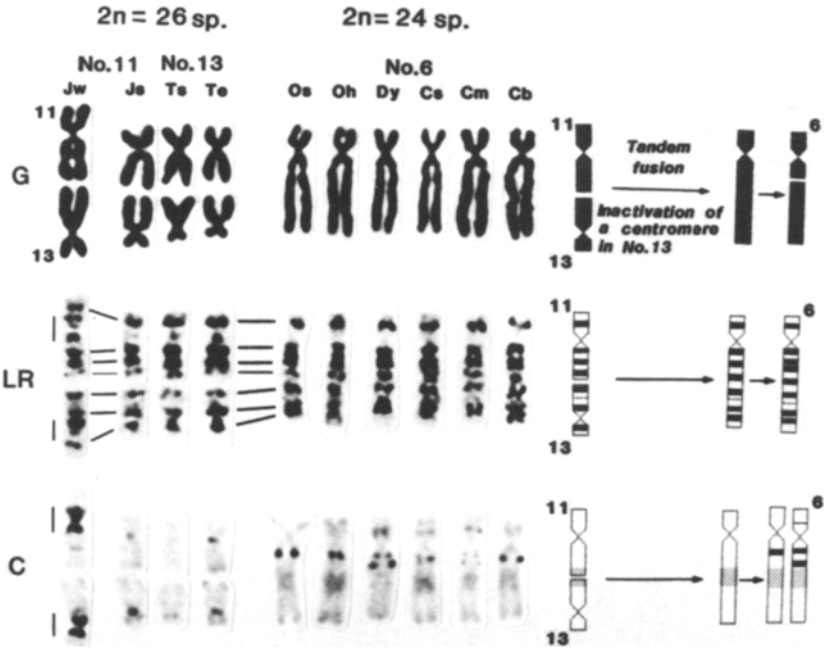

Figure 10. Comparison of chromosomes *11* and *13* of the *2n = 26 species with the no. 6* chromosomes of the 2n = 24 species by conventional Giemsa staining (G), late replicating banding (LR) and C-banding (C), and reconstruction of chromosome 6 of the 2n = 24 species from chromosomes 11 and 13 of a presumed ancestral 2n = 26 species. Horizontal lines connect the homologous late replication bands. Vertical bars represent the increased constitutive heterochromatin regions of the chromosomes of *R. japonica* from Wakuya. Abbreviations are the same as in figure 6.

ancestral 2n = 24 species could produce two small chromosomes, then they would be very different from chromosomes nos 11 and 13 of the 2n = 26 species, both in size and in their late replication banding pattern. Second, if chromosome no. 6 was broken at the intermediate region of the long arm and divided into two chromosomes, then the banding patterns of these two would certainly coincide with those of nos 11 and 13 from the 2n = 26 species, but one chromosome, derived from the distal half of the long arm of chromosome no. 6, could have no centromere. Consequently, the 2n = 24 brown frog species was probably derived from an ancestral 2n = 26 species.

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