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Karyotypes of *Rana tagoi* Okada with diploid number 28 in the Chausu Mountains of the Minamishinshu district of Nagano Prefecture, Japan (Anura: Ranidae)

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Abstract. Karyotypes of Tago's brown frog *Rana tagoi* from the Chausu mountains in Minamishinshu of Nagano Prefecture were examined by conventional Giemsa staining, C-banding and late replication (LR)-banding. Chromosome number was 2n = 28 in all cases. The 28 chromosomes consisted of four pairs (1–4) of large biarmed chromosomes, two pairs (5–6) of telocentric chromosomes and eight pairs (7–14) of small biarmed chromosomes. Chromosome pair 11 had a secondary constriction on the long arm. In females, the C-band on the long arm of chromosome pair 6 was detected in both homologs, but was absent from the arms of the homologs of chromosome pairs 5 and 9. In males, C-bands were found in the long arms of both homologs of chromosome pairs 5 and 6, were present only in one homolog of chromosome pair 5 for certain male specimens and found in only one homolog of chromosome pair 9. Specimens of *R. tagoi* (2n = 28) should thus have two pairs of telocentric chromosomes to provide the same number of chromosome arms, these originating quite likely from chromosome pair 1 in the 26-chromosome specimens by centric fission. Heteromorphic sex chromosomes of the XX-XY type in *R. tagoi* (2n = 28) in the Chausu mountains were identified. Karyotypes of tail-tip cells from a hybrid tadpole between female *R. tagoi* (2n = 26) from the Hinohara village in Tokyo and male *R. tagoi* (2n = 28) from the Chausu mountain population were examined by squash preparation. Chromosome number was 2n = 27 in all tadpoles. The 27 chromosomes consisted of one chromosome set of *R. tagoi* (2n = 28) and one of *R. tagoi* (2n = 26).

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Rana tagoi Okada (1928), Tago's brown frog, is a Japanese endemic species widely distributed in mountain areas of Honshu, Shikoku and Kyushu and also found on the Yaku and Oki islands. This frog may readily be found along small mountain streams and generally does not migrate any great distance. Specimens are quite similar to each other in morphology and ecology, but slight variations are apparent locally (Nishioka et al., 1987; Maeda and Matsui, 1989). *R. tagoi* lay ca. 30–100 very yolky eggs of 3.0–3.2 mm diameter in a small globular mass with light grayish-brown animal hemisphere. *R. tagoi* breeds from March to late June in slowly flowing underground water. The larvae are capable of metamorphosing even without feeding.

The authors obtained chromosomes of *R. tagoi* from 81 local populations in Japan and found the diploid number to be 26 (Fig. 1). Each karyotype consisted of 10 large and 16 small chromosomes (Fig. 2). Heteromorphic sex chromosomes of the XX/XY type in *R. tagoi* from five of 81 local populations and *R. sakuraii* from Bonbori river in Tokyo (Japan) were identified (Ryuzaki et al., 1999).

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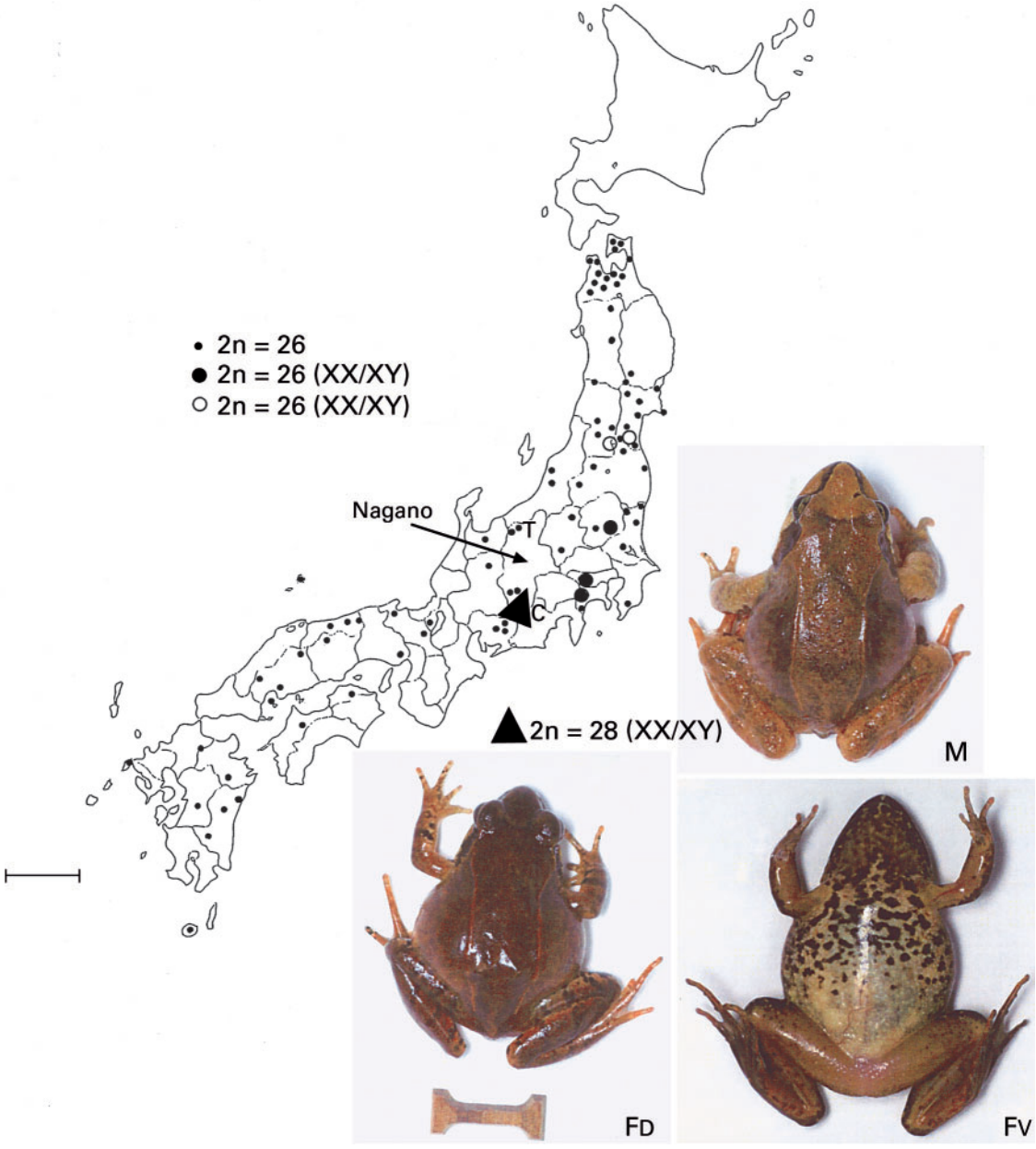

Fig. 1. Map of the islands of Japan. The small black circles indicate local populations of *R. tagoi* (2n = 26) and the large black circles *R. tagoi* (2n = 26 (X_2X_2/X_2Y_2)) and *R. sakuraii* (2n = 26 (X_1X_1/X_1Y_1)). The white large circles show *R. tagoi* (2n = 26 (X_1X_1/X_1Y_1)). Abbreviations: Nagano, Nagano Prefecture; T, Togakushi village and C, Chausu mountain range in the prefecture; M, male *R. tagoi* (2n = 28 (X_1X_1/X_1Y_2)); FD, female dorsal side and FV, female ventral side of *R. tagoi* (2n = 28 (X_1X_1/X_1Y_2)) (bar represents 1.7 cm). Bar of map represents 200 km. The triangle signifies provenance of *R. tagoi* with diploid number 2n = 28 (X_1X_1/X_1Y_2) from Chausu mountain range.

R. tagoi with diploid number 28 was recently found in the Chausu mountains in the Neba village district in Nagano (Japan). One hundred and eighty specimens were collected during the period from 2001 to 2003 from this mountain range for karyotype determination. *R. tagoi* (2n = 26) is considered not to inhabit the Neba village district since none could be found there. Hybrid tadpoles (2n = 27) were produced by female *R. tagoi* (2n = 26) from Hinohara Village in Tokyo and male *R. tagoi* (2n = 28) (Nishioka, 1972). These unusual characteristics were clearly demonstrated in the present study by karyological analysis, mating call sonogram, a crossing experiment and DNA sequences of the mitochondrial 16S and 12S rRNA genes for a certain local population of *R. tagoi* (2n = 28) in the Chausu mountains of Nagano Prefecture. As for *R. tagoi* with diploid number 28, a new species may possibly have developed over a long period of

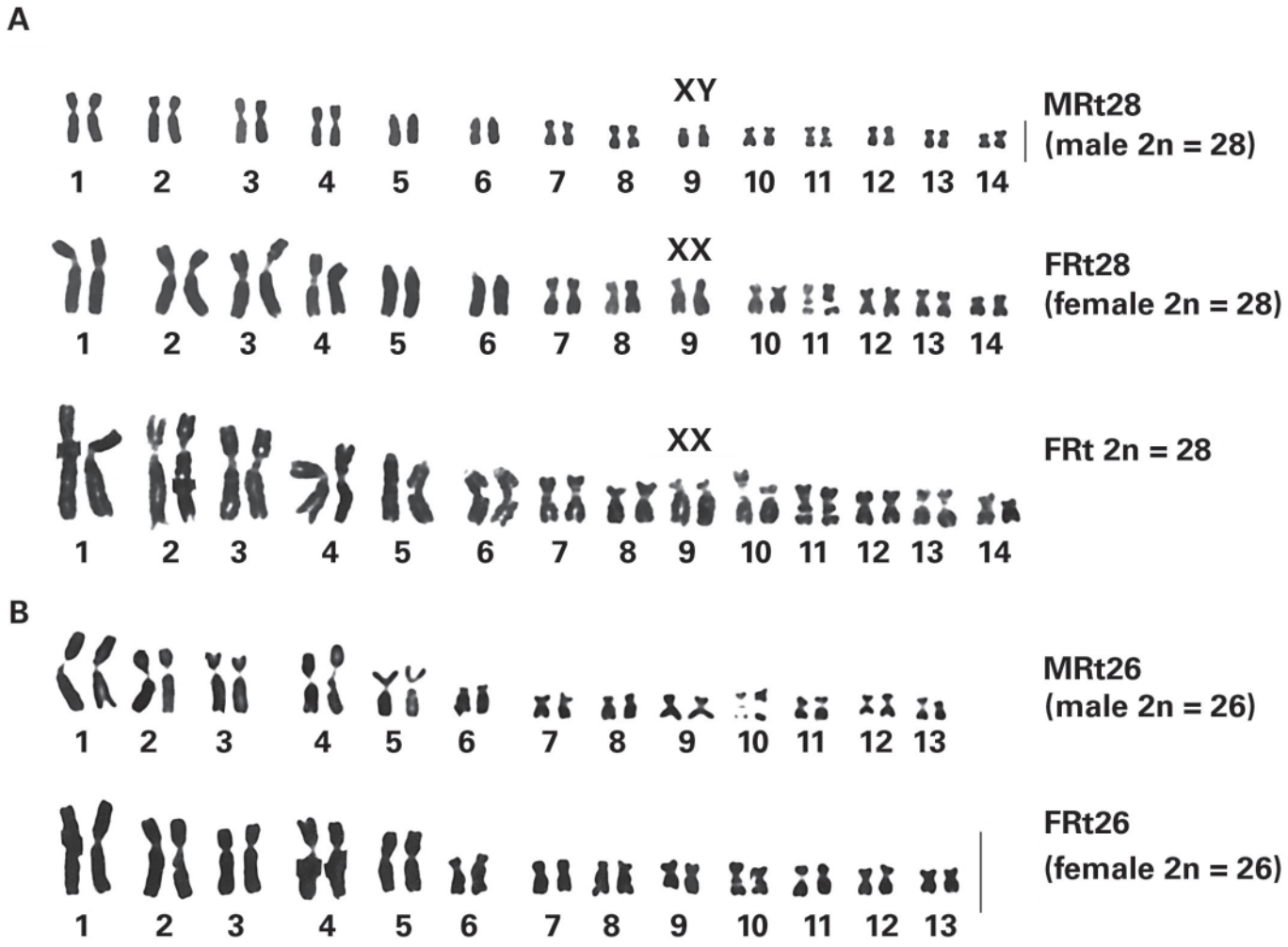

Fig. 2. Comparison of karyotypes of *R. tagoi* (2n = 28) (**A**) and *Rana tagoi* (2n = 26) (**B**) stained by conventional Giemsa staining. Chromosome pair 9 was identified as sex chromosomes. MRt28, *R. tagoi* male (2n = 28); FRt28, *R. tagoi* female (2n = 28). MRt26, *R. tagoi* male (2n = 26); FRt26, *R. tagoi* female (2n = 26). X, X chromosome; Y, Y chromosome. Bars represent 10 μm.

time during species differentiation in *R. tagoi* with diploid number 26. The present study was thus conducted to demonstrate that this frog species with diploid number 2n = 28 unambiguously differs from *R. tagoi* with 2n = 26 based on karyological findings.

Materials and methods

R. tagoi used in this study were mature males and females obtained in spring and autumn seasons from 2001 to 2003 at two sites in the Minamishinshu district: Chausu mountain range and Neba village and two sites in the Kitashinano district, Togakushi village and Hakuba village in Nagano Prefecture. The numbers of females and males from each of these sites are specified in Table 1.

Hybrid tadpoles were produced by artificial fertilization between a female *R. tagoi* (2n = 26) from Hinohara village in Tokyo and a male *R. tagoi* (2n = 28) from the Chausu mountains (Nishioka, 1972).

Karyotype determination was done by conventional Giemsa staining and C- and LR-banding for 40 frogs, i.e. 5 females and 5 males from the 4 sites. Karyotypes of *R. tagoi* were compared for relative length (RL) and centromere position (NVC) on each chromosome pair.

Conventional Giemsa staining

Metaphases were usually obtained following the procedure of Volpe and Gebhard (1968) for blood cell culturing with slight modification (Nishioka et al., 1987). The culture medium was prepared by mixing 4.8 ml RPMI 1640 (Gibco), 2.0 ml calf serum, 3.2 ml redistilled water and 0.3 ml PHA-M (Phytohemagglutinin, Difco). Penicillin and streptomycin were added at final concentrations of 100 IU/ml and 100 μg/ml, respectively. Venous blood (0.1–0.2 ml) was collected with a glass pipette containing 0.01–0.02 ml heparin solution (10 mg/ml RPMI 1640). To 2 ml culture fluid, 0.1–0.2 ml venous blood was added and the system was cultured for 3–5 days at 25°C. Chromosome preparations were done by conventional air drying. Hypotonic treatment was carried out in 0.075 M KCl solution and the cells were fixed in Carnoy's fluid (acetic acid:methanol = 1:3).

C-banding method (Giemsa staining after $Ba(OH)_2$ denaturation)

C-bands were visualized by the method of Sumner (1972) with slight modification of incubating chromosomes in 5% $Ba(OH)_2$ solution at 35°C for 5–10 min.

Table 1. Number of metaphases for chromosome analysis and banding patterns of chromosome pairs 5, 6 and 9 in *Rana tagoi* (2n = 28) and the respective chromosome pair in *Rana tagoi* (2n = 26)

Species and local population		Sex	No. of frogs[a]	No. of metaphases observed by the methods of			Bivalent chromosomes[b]			
				Giemsa staining	C-banding	LR-banding	no. 5	no. 6	no. 8	no. 9
Rana tagoi (2n = 28)	Chausu mountain range	Male	5 (80*)	4000*	33	29	t-t	t-t		smX-smY
		Female	5 (58*)	2900*	41	36	t-t	t-t		smX-smY
	Neba village	Male	5 (25*)	1250*	48	37	t-t	t-t		smX-smY
		Female	5 (17*)	850*	46	32	t-t	t-t		smX-smY
Rana tagoi (2n = 26)	Togakushi village	Male	5	250	44	23			sm-sm	
		Female	5	250	21	18			sm-sm	
	Hakuba village	Male	5	250	39	27			sm-sm	
		Female	5	250	35	30			sm-sm	

[a] *: Specimen numbers of *Rana tagoi* (2n = 28) from Chausu mountain range and Neba village were used only for analysis by Giemsa staining. Specimens of *R. tagoi* (2n = 26) were obtained from Togakushi and Hakuba villages in Nagano Prefecture. Numbers for analysis by C- and LR-banding are five males and females each of *R. tagoi* (2n = 28) and *R. tagoi* (2n = 26), respectively.
[b] t: telocentric chromosome; sm: submetacentric chromosome; smX: submetacentric X chromosome; smY: submetacentric Y chromosome.

Late replication (LR)-banding method

Late replication (LR) bands were produced by the method of Takayama et al. (1981) with slight modification. After cultivating of peripheral blood for 3–5 days, 5-bromodeoxyuridine (BrdU) was added to each culture at a final concentration of 10^{-4} M and then colchicine at 10 μg/ml 4 hours prior to harvesting. Chromosome preparations were done by conventional air drying. BrdU-labeled chromosome preparations were allowed to age for 1–2 days at room temperature and stained with 3% Giemsa solution at 40°C for 3–5 min. Giemsa solution was prepared in 2% EDTA-4Na aqueous solution (pH 11.5) (Nishioka et al., 1994).

Squash preparation

Squash preparations were done from tail-tips of tadpoles following essentially the method of Makino and Nishimura (1952): tadpoles were reared in 50 mg/l colchicine (Merck) solution for 15–18 hours at room temperature, tail-tips cut off, immersed in distilled water for 60–120 minutes and stained with 1% orcein (Chroma), dissolved in 45% acetic acid for 30–60 minutes on a glass slide and squashed under a cover glass after heat treatment for 20–30 seconds and mounted with PVLB (paraffin:vaseline:lanolin:Canadian balm = 2:1:1:1).

Results

Mitotic chromosomes of R. tagoi (2n = 28)

a) Conventional Giemsa staining. Chromosomes were examined for 50 metaphases from each specimen by conventional Giemsa staining. Enlarged photographs of 100 metaphase spreads were taken from males and females of *R. tagoi* and the best 10 were used for karyological examination. Chromosome number was 2n = 28 in all cases. The karyotypes were arranged in the order of chromosome size. Each of them contained four pairs (1–4) of large biarmed chromosomes, two pairs (5–6) of telocentric chromosomes and eight pairs (7–14) of small biarmed chromosomes. Chromosome 11 had a secondary constriction on the long arm (Fig. 2). For Tago's brown frog *R. tagoi* from the Chausu mountains, all 14 chromosome pairs in females were homomorphic, as evident from Figs. 2 and 3.

Chromosome pair 9 in males was composed of a larger submetacentric chromosome (l) and a smaller submetacentric chromosome (s). RL of l and s were 2.98 and 2.46, respectively. Chromosome types (NVC) of l and s were sm (32.05) and sm (27.35), respectively. Relative lengths of metaphase chromosomes (RL) were compared for RL_n l of males and females and RL_n s of males and females, where n is chromosome number (n = 10). RL was compared for (RL_n l – RL_n s) of males and (RL_n l – Rl_n s) of females. Chromosome length in *R. tagoi* males and females differed quite considerably for chromosome 9 ($P = 8.693 \times 10^{-9}$). Thus, l is the Y chromosome and s the X chromosome in the case of male chromosome pair 9. Female l and s are X chromosomes in *R. tagoi* (2n = 28).

b) C-banding. C-bands were examined for 41 metaphases from five females and 33 mitotic figures from five males. In twelve chromosome pairs, except for chromosome pairs 5 and 9, the bands showed no sex differences. C-bands of chromosome pair 5 in five females could be detected only at the centromere position. C-bands of chromosome pair 5 in four males were homomorphic, situated at interstitial position on the long arms and stained strongly. Bands of chromosome pair 5 in one male were heteromorphic, while those at the interstitial position of the long arm in only one specimen stained strongly. C-bands of chromosome pair 6 in females and males were homomorphic, situated at interstitial positions on the long arms and showed intense staining. Chromosome pair 9 bands were heteromorphic in males and those at the interstitial position on the long arm in only one chromosome 9 homolog exhibited intense staining. All C-bands of chromosome pair 9 in females were homomorphic (Figs. 3, 5).

c) LR-banding. LR-bands could be seen for 36 metaphases from five females and 29 metaphases from five males. LR-bands of *R. tagoi* from the Chausu mountain range were present on centromeres of fourteen pairs of chromosomes and on different positions of both chromosome arms of some of them. The bands were homomorphic in all cases without

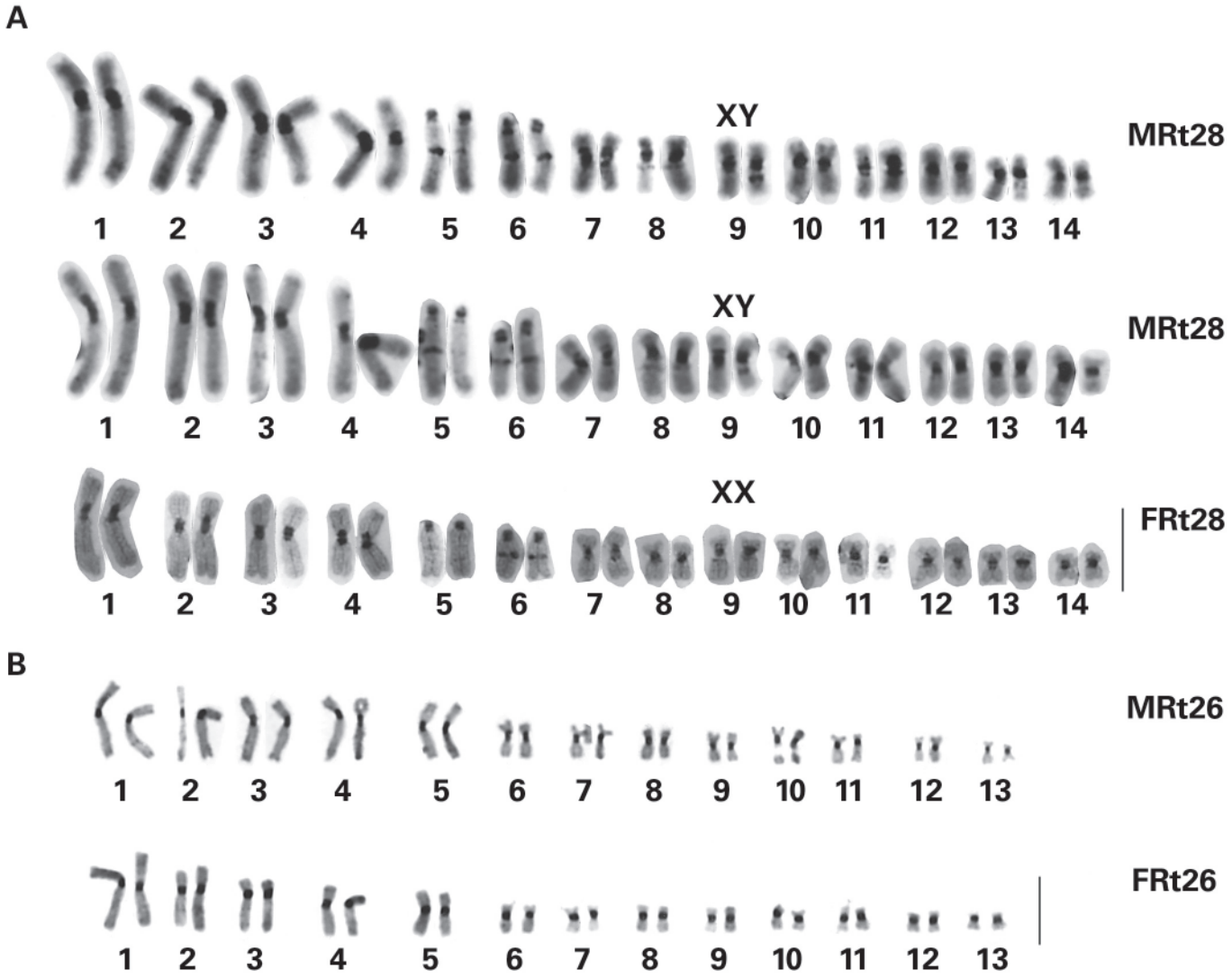

Fig. 3. Comparison of karyotypes of *Rana tagoi* (2n = 28) (**A**) and *R. tagoi* (2n = 26) (**B**) stained by C-banding. MRt28, *R. tagoi* male (2n = 28); FRt28, *R. tagoi* female (2n = 28). MRt26, *R. tagoi* male (2n = 26); FRt26, *R. tagoi* female (2n = 26). X, X chromosome; Y, Y chromosome. Bars represent 10 μm.

sex differences in all chromosome pairs of males and females. Band location, number and intensity of the homologs of each pair were basically the same. All chromosome pairs 5 consisted of telocentric chromosomes. One band was situated proximally, three between interstitial and terminal parts of the long arm and one at the terminal part of the short arm.

L (Y) and s (X) chromosomes of male chromosome pair 9 had the same bands. One band was situated proximally, one between interstitial and terminal parts of the long arm and one at the terminal part of the short arm (Fig. 4).

Mitotic chromosomes of R. tagoi (2n = 26)

a) Conventional Giemsa staining. Chromosomes were observed for 50 metaphases for each specimen by conventional Giemsa staining. Enlarged photographs of 100 metaphase spreads were taken from males and females of *R. tagoi* (2n = 26) and the best 6–7 were used for karyological examination. Chromosome number was 2n = 26 in all cases. The karyotypes were arranged in the order of chromosome size. The 26 chromosomes were comprised of five pairs (1–5) of large chromosomes and eight pairs (6–13) of small chromosomes. Chromosome 10 had a secondary constriction on the long arm. For Tago's brown frog *R. tagoi* from Togakusi village, all 13 chromosome pairs in females and males were homomorphic, as is also the case for *R. tagoi* on all the major islands of Japan except for Okutama in the Kanto region and Sitigasyuku in the Tohoku region (Figs. 1, 2). For *R. tagoi* from mountain regions in the Nishitama district in Tokyo, chromosome pair 8 was determined as sex chromosomes of the X_2X_2/X_2Y_1 type (Ryuzaki et al., 1999). For *R. sakuraii* which is sometimes sympatrically with *R. tagoi* from a mountain region in the

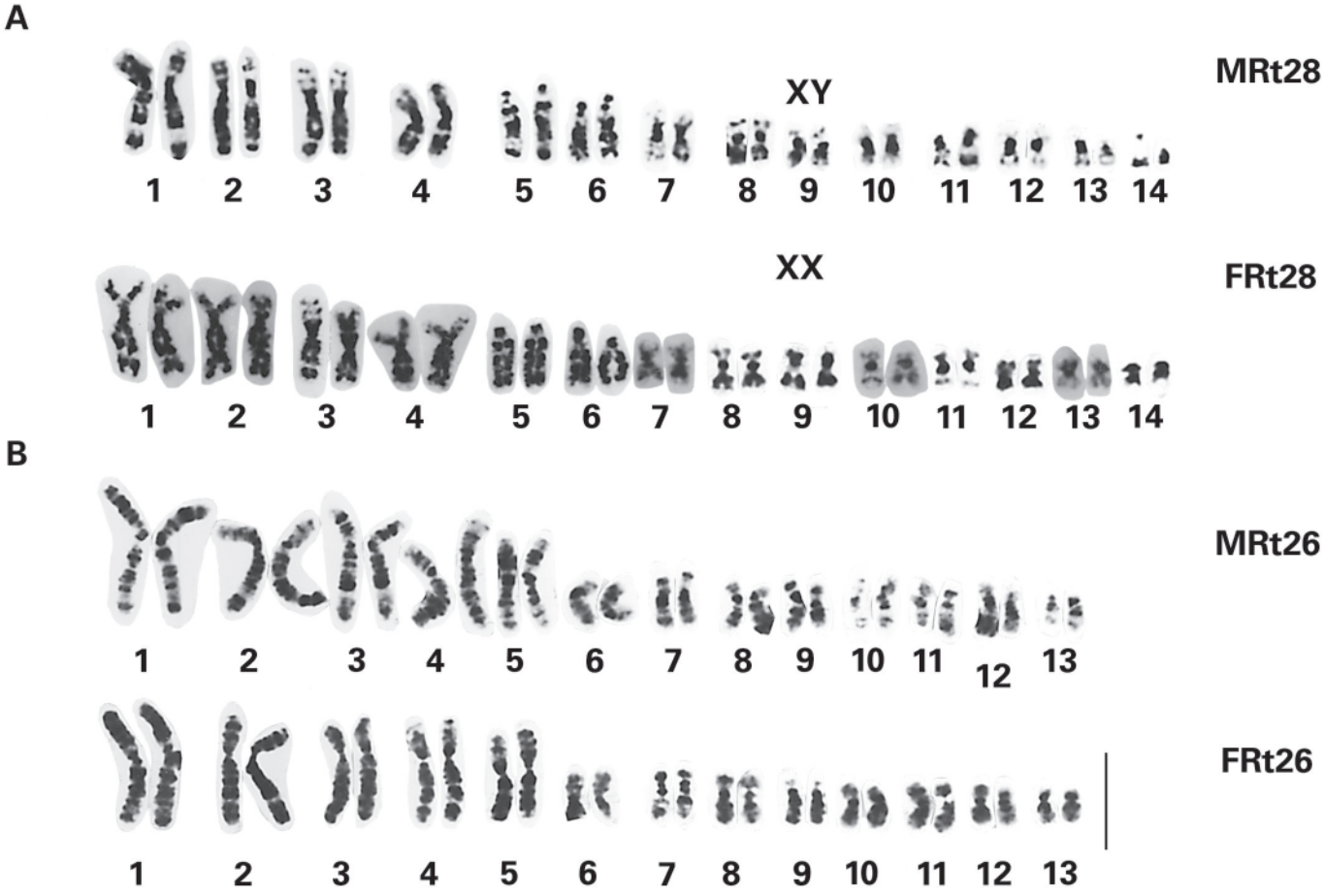

Fig. 4. Comparison of karyotypes of *R. tagoi* (2n = 28) (**A**) and *R. tagoi* (2n = 26) (**B**) stained by LR-banding. MRt28, *R. tagoi* male (2n = 28); FRt28, *R. tagoi* female (2n = 28). MRt26, *R. tagoi* male (2n = 26); FRt26, *R. tagoi* female (2n = 26). X, X chromosome; Y, Y chromosome. Bar represents 10 μm.

Niishitama district, chromosome pair 8 was shown to be the sex chromosome of the X_1X_1/X_1Y_1 type (Ryuzaki et al., 1999). These findings are shown by the large black circles in Fig. 1. For *R. tagoi* from a mountain region in the Sitigasyuku district in Miyagi Prefecture, chromosome pair 8 was identified as the sex chromosome of the X_1X_1/X_1Y_1 type as also noted in the case of *R. sakuraii*, as indicated by the large white circles in Fig. 1. Determination of the sex chromosome was carried out only for the Kanto and Tohoku regions.

b) C-banding. C-banding was studied for 50 metaphases from five females and the same number from five males. In all cases, bands could be seen only at the centromeres of the 13 chromosome pairs. In the karyotype, no chromosomes could be found showing sexual differences (Fig. 3).

c) LR-banding. LR-bands were observed for 30 metaphases from five females and then from five males. LR-bands of *R. tagoi* (2n = 26) from Togakushi village were present on centromeres of 13 pairs of chromosomes and at proximal, interstitial or terminal positions on long and short arms of some chromosomes. The bands were essentially homomorphic without sex differences in 13 chromosome pairs of males and females. Band portion, number and intensity of homologous chromosomes of each pair were basically the same (Fig. 4).

Comparison of mitotic chromosomes of R. tagoi (2n = 28) and R. tagoi (2n = 26)

Chromosome pairs 2, 3, 4 and 5 of *R. tagoi* (2n = 26) were essentially a complementary combination of chromosome pairs 1, 2, 3 and 4 of *R. tagoi* (2n = 28), according to the karyological data in Figs. 2 and 3. Chromosome pairs 6–13 of *R. tagoi* (2n = 26) were virtually complementary to chromosome pairs 7–14 of *R. tagoi* (2n = 28), respectively. For chromosome pair 1, no large metacentric chromosomes of *R. tagoi* (2n = 26) could be found in the karyotype of *R. tagoi* (2n = 28). For chromosome pairs 5 and 6 of *R. tagoi* (2n = 28), no corresponding telocentric chromosomes of *R. tagoi* (2n = 26) were found. Total relative length (TRL) of the longer chromosome (l-chromosome) in chromosome pairs 5 and 6 in female *R. tagoi* (2n = 28) and RL of the q arm in chromosome pair 1 in female *R. tagoi* (2n = 26) were 7.59 and 7.63 ($P = 0.8728$, according to the null hypothesis), respectively. TRL of the

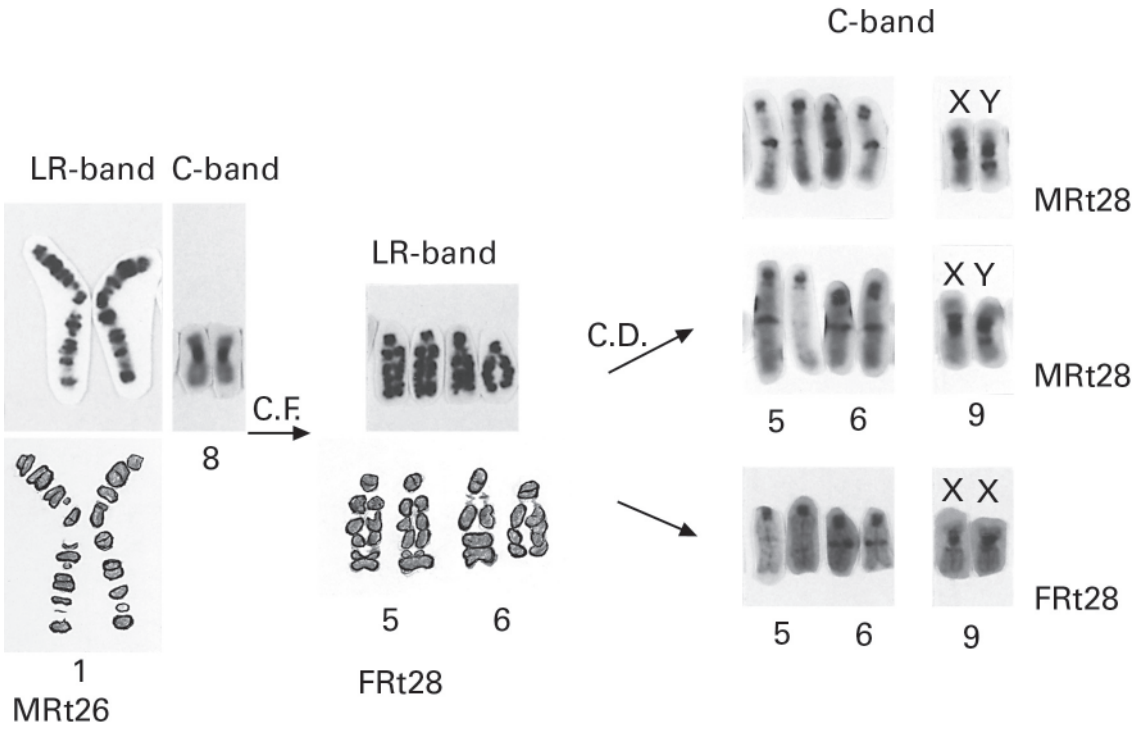

Fig. 5. Comparison of short and long arms of chromosome pair no. 1 of *R. tagoi* (2n = 26) and chromosome pairs 5 and 6 of *R. tagoi* (2n = 28) whose two pairs of telocentric chromosomes may originate from chromosome pair no.1 of *R. tagoi* (2n = 26) by centric fission (C. F.) as indicated by LR-banding. Based on karyological data, chromosome pair 9 of *R. tagoi* (2n = 28) may be expected to be chromosome pair 8 of *R. tagoi* (2n = 26). In *R. tagoi* (2n = 26), C-bands of chromosome pair 8 in males and females could be detected only at the centromere position (Fig. 3). Comparison of chromosome pairs 5, 6 and 9 of *R. tagoi* (2n = 28) with respect to the differentiation of the two pairs of telocentric chromosomes (C. D.) during chromosome evolution shown by C-banded chromosomes. MRt28, *R. tagoi* male (2n = 28); FRt28, *R. tagoi* female (2n = 28). MRt26, *R. tagoi* male (2n = 26); X, X chromosome; Y, Y chromosome.

shorter chromosome (s-chromosome) in chromosome pairs 5 and 6 in female *R. tagoi* (2n = 28) and RL of the p arm in chromosome pair 1 in female *R. tagoi* (2n = 26) were 7.37 and 7.46 (P = 0.6798), respectively. TRL of l-chromosomes in chromosome pairs 5 and 6 in male *R. tagoi* (2n = 28) and RL of the q arm in chromosome pair 1 in male *R. tagoi* (2n = 26) were 7.59 and 7.63 (P = 0.8728), respectively. According to the null hypothesis, TRL of l- and s-chromosomes in chromosome pairs 5 and 6 in male and female *R. tagoi* (2n = 28) are approximately the same as RL of l- and s-chromosomes in chromosome pair 1 in male and female *R. tagoi* (2n = 26), respectively.

The karyological findings in the present study may be considered equivalent to statistical data for karyotypes 2n = 26 and 2n = 28 of *R. tagoi* (Fig. 2). The main five LR-bands of chromosome pair 5 of *R. tagoi* (2n = 28) basically corresponded to the bands in the q arms of chromosome pair 1 of *R. tagoi* (2n = 26). The three major LR-bands of chromosome pair 6 of *R. tagoi* (2n = 28) were virtually the same as those of the p arms of chromosome pair 1 of *R. tagoi* (2n = 26) (Fig. 4). Chromosome pairs 5 and 6, the telocentric chromosomes of *R. tagoi* (2n = 28), likely originated from chromosome pair 1 of *R. tagoi* (2n = 26) via centric fission during the evolution of karyotypes (Fig. 5).

Hybrid frog (2n = 27) between female R. tagoi (2n = 26) from Hinohara village in Tokyo and male R. tagoi (2n = 28) from the Chausu mountains

Approximately 85–90% of the hybrid eggs developed into normally metamorphosed frogs. This percentage range was the same or somewhat less than for control mating. Karyotypes of tail-tip cells from hybrid tadpoles were examined using squash preparations.

Chromosome number was 2n = 27 in all tadpoles. Among the 27 chromosomes, there were only one chromosome 1 homolog and four (2–5) pairs of large chromosomes, only one homolog of each of the chromosome pairs 5 and 6 of telocentric chromosomes of *R. tagoi* (2n = 28) and eight pairs of small chromosomes corresponding to chromosome pairs 7–14 of *R. tagoi* (2n = 28), which is chromosome pairs 6–13 of *R. tagoi* (2n = 26) (Fig. 6).

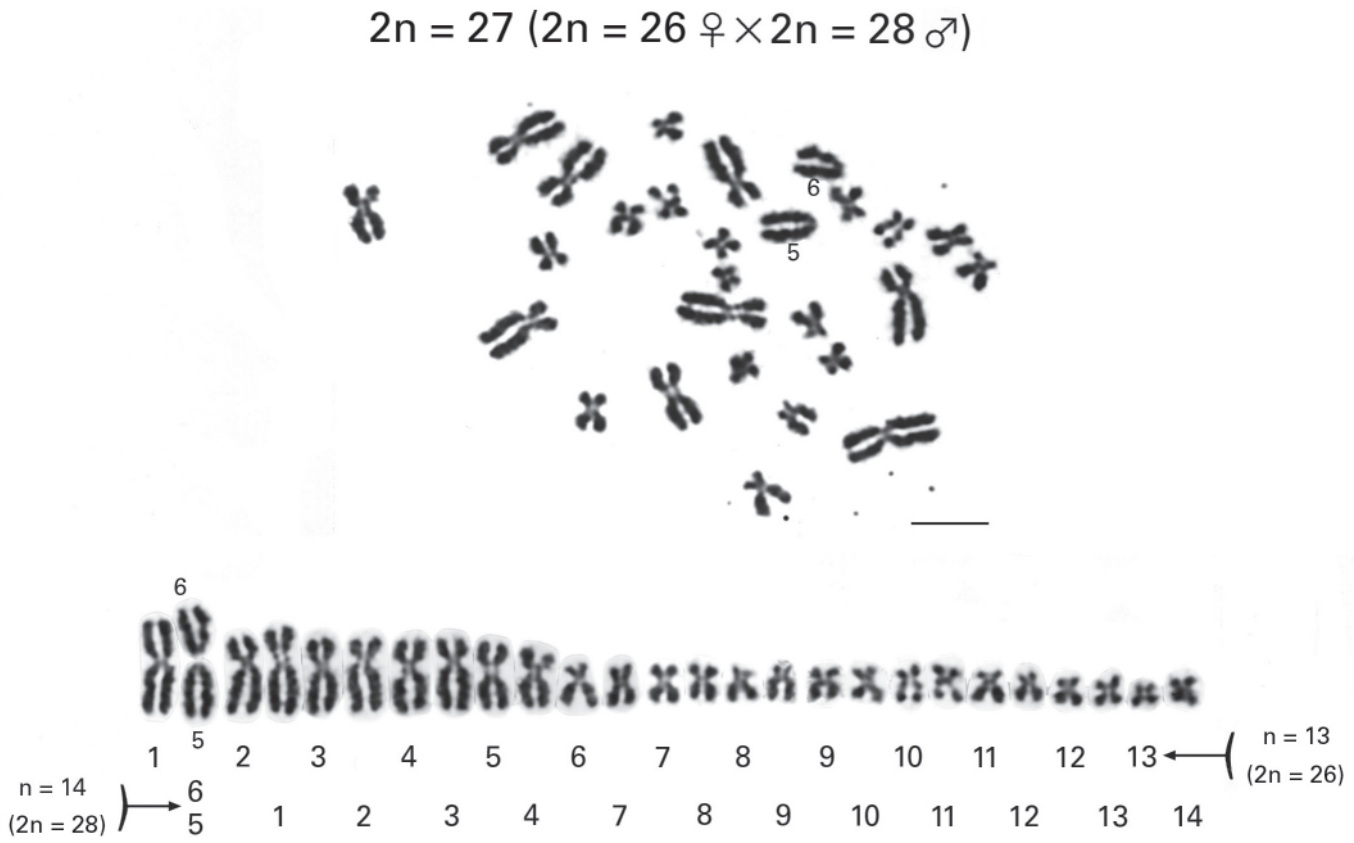

Fig 6. Karyotype of a hybrid frog (2n = 27) between female *R. tagoi* (2n = 26) and male *R. tagoi* (2n = 28) prepared by squash preparation. Female *R. tagoi* (2n = 26) originated from Hinohara village in Tokyo, male *R. tagoi* (2n = 28) originated from Chausu mountains. Chromosomes 5 and 6, the telocentric chromosomes of *R. tagoi* (2n = 28), likely originated from chromosome pair 1 of *R. tagoi* (2n = 26) via centric fission during evolution of karyotypes. Bar represents 10 μm.

Discussion

A recent list of chromosome numbers in anurans has been prepared for about 1,000 species. The list of Kuramoto shows that chromosome number variation occurs in most of the seven families of anuran amphibians classified with 33 genera (Kuramoto, 1990).

The authors collected all *R. tagoi* specimens with a diploid number of 28 in the Chausu mountains in the Minamishinshu district of Nagano Prefecture. Neba village at the most-southern end of the prefecture is situated in these mountains, at 1,000 to 1,415 m above sea level, and has an area of approximately 89 km^2 or nearly 10% of that of the mountain range (Fig. 1). One hundred and twenty-eight specimens were obtained in 2001 and 2003 for karyotype determination. The diploid number in all cases was 28. This *R. tagoi* (2n = 28) is apparently restricted to Neba village. No *R. tagoi* with diploid number of 26 could be found in the vicinity of this village but some were seen in Achi and Takagi villages and Oodaira in Iida city situated nearly 20 to 30 km from Neba. *R. tagoi* (2n = 28) has not been found in any of these places. Except for polyploid species (Bogart, 1980; Duellman and Trueb, 1986; Kuramoto, 1990), most genera differing in chromosome number are characterized by small clutch size and they lay large eggs, have parental investment and terrestrial and/or direct development.

Terrestrial breeding frogs from several families differ in karyotypic features and Bogart (1981) thus considers that chromosome variation may be predicted for species that produce small numbers of eggs and/or have terrestrial habitats. This in turn may promote inbreeding in small isolated demes with the possibility of fixing chromosome mutation. Lande (1979) has shown chromosome rearrangement not to give rise to significant phenotypic change. Any possible relation of karyotypic variation to deme size and life history may be confirmed by examining species differing in population size and mode of reproduction. The karyotypically most variable frog genus is *Eleutherodactylus* (Leptodactylidae) which includes species with diploid numbers from 18 to 36 (Bogart, 1970, 1973, 1981, 1991). *Eleutherodactylus* is the largest vertebrate genus with more than 400 known species to date (Frost, 1985) and new species continue to emerge. All species have been shown to be terrestrial breeders, with habitats confined to certain geographical areas (Schwartz and Thomas, 1975;

Schwartz and Henderson, 1985). Vences et al. (2000) did examinations of karyological data on *Nannophrys ceylonensis* (2n = 26), *N. marmorata* (2n = 26), *Indirana* sp. (2n = 30) and *I.* cf. *leptodactyla* (2n = 24). *N. ceylonensis* and *N. marmorata* possess 2n = 26 biarmed chromosomes. *Indirana* cf. *leptodactyla* has 2n = 24 biarmed chromosomes. *Indirana* sp. has 2n = 30 chromosomes; 16 are biarmed and 14 uniarmed (telocentric chromosomes). The 2n = 30 karyotype of *Indirana* sp. may represent a transitory stage in karyotype reduction by means of centric fission to produce telocentric chromosomes which subsequently undergo fusion (Vences et al., 2000). Centric fusion and fission are the most probable means for aneuploid numbers (heteroploidy) since karyotypes with greater numbers of chromosomes have been shown to be present in other individuals from the same population (Bogart, 1981; Jin et al., 2000).

Based on the findings by Schwartz and Thomas (1975), Schwartz and Henderson (1985) and Bogart (1991), *R. tagoi* would appear to have features in common with *Eleutherodactylus* and accordingly may exhibit small clutch size, lay large eggs and the larvae may be capable of metamorphosing without feeding. *R. tagoi* have also been shown to be restricted to certain geographical areas.

According to Bogart (1991), centric fusion and fission may likely account for changes in chromosome number in *Eleutherodactylus* and anomalous chromosomes have been found in few individuals. This demonstrates polymorphism within a population and these individuals should be considered translocation heterozygotes. Such individuals each possess a single, large metacentric chromosome produced via fusion of two telocentric chromosomes. *Eleutherodactylus cundalli* and *E. glaucoreius* normally possess 30 chromosomes but a specimen of each species was found in this study to have only 29 chromosomes. *E. heminota* and *E. bakeri* from Hispaniola showed chromosomal polymorphism involving presumed fusion of two telocentric chromosomes to produce 27 chromosomes instead of the usual 28. Two specimens of *E. glandulifer* had 31 chromosomes possibly due to fission in a 30-chromosome karyotype or fusion of two telocentric chromosomes in a 32-chromosome karyotype. All these changes represent independent events since chromosome numbers and telocentric chromosomes involved were different and the sampled populations were composed of species endemic to different islands. Fusion would thus be the most probable means for aneuploid number since karyotypes with higher numbers are present in other individuals from the same populations.

In much smaller genera that include species whose eggs develop in a terrestrial setting, such as *Arthroleptis* and *Cardioglossa* (Arthroleptidae), *Fritziana* (Hylidae), or *Leptopelis* (Hyperoliidae), certain terrestrial developmental and karyotypic variations observed in *Eleutherodactylus* have been shown to correlate (Bogart, 1991; Bogart and Tandy, 1981). Karyotypic examination of three species of African tree frogs of the genus *Leptopelis* has been conducted by Bogart and Tandy (1981). *Leptopelis bocagii* (2n = 22) has no telocentric chromosome, *L. vermiculatus* (2n = 24) has one pair of telocentric chromosomes and *L. parkeri* (2n = 30) has eight pairs of telocentric chromosomes. Karyotypic analyses of these and six other species of *Leptopelis* have also been done by these authors. Differences in chromosome number within the genus may possibly be explained by centric fusion or fission, but no 24-chromosome species would have two pairs of telocentric chromosomes to provide the same number of chromosome arms. *L. parkeri* has the same number of chromosome arms as *L. bocagii* but the chromosomes are much smaller.

Telocentric chromosomes were found in all four genera, *Colostethus, Dendrobates, Epipedobates* and *Minyobates* of the family Dendrobatidae, possibly indicating centric fusion and fission to be responsible for changes in chromosome number in this family. These processes may possibly accompany karyotypic differences in *Eleutherodactylus* but not likely so in the case of dendrobatid frogs (Bogart, 1991). In the 24-chromosome *Colostethus*, some species have only metacentric and submetacentric chromosomes, whereas *Colostethus subpunctatus* has five pairs of telocentric chromosomes. In *Dendrobates*, if number reduction from 20 to 18 chromosomes results from fusion, the 20-chromosome species should possess two pairs of telocentric chromosomes. It is evident that determination of the number of chromosome arms is of little value for understanding karyotype evolution in the family Dendrobatidae. It is also evident that dendrobatid chromosomes have undergone extensive restructuring via translocation and inversion.

Chromosome number polymorphism is not the only variation found in *Eleutherodactylus* karyotypes. The karyotype of *E. nortoni* manifests considerable variation in position and extent of constitutive heterochromatin as indicated by C-banding. Centromeric, telocentric and interstitial bands are present and also, entire arms of some chromosomes show intense staining. Chromosome 3 of *E. nortoni* is heteromorphic for secondary constriction (King, 1980). Only one male was available for chromosome analysis and thus determining whether the heteromorphic pair is related to a similar XY/XX sex chromosome was not possible here. Heteromorphism was found in *Centrolenella antisthenesi* by Schmid et al. (1989) and in species of *Gastrotheca* (Schmid et al., 1988).

Schmid et al. (2002) did an examination of homomorphic XY sex chromosomes and a derived Y-autosome translocation in *Eleutherodactylus riveroi* present along with homomorphic XY sex chromosomes and a derived Y-autosome translocation in *Eleutherodactylus maussi*.

R. tagoi with diploid number 28 should have two pairs of telocentric chromosomes to ensure that the number of chromosome arms is the same. Differences in chromosome number in *R. tagoi* may have come about via centric fission. The two pairs of telocentric chromosomes may have originated from chromosome pair 1 by centric fission. Telocentric chromosomes may have undergone modification or development and can be differentiated by C-banding now. These findings on the Japanese frog are reported here for the first time. For *R. tagoi* (2n = 28 (X_1X_1/X_1Y_2)), chromosome pair 9 was examined as sex chromosomes of the X_1X_1/X_1Y_2 type in all specimens. Up to the present, differentiation of sex chromosomes has been observed only for the Kanto and Tohoku regions. For *R. tagoi* (2n = 26 (X_2X_2/X_2Y_1)) and *R. sakuraii* (2n = 26 (X_1X_1/X_1Y_1)) from a mountain region in the Nishi-

tama district in Tokyo, chromosome pair 8 was determined as XX/XY type sex chromosomes in all specimens (Ryuzaki et al., 1999). In a chromosome study on *Eleutherodactylus*, anomalous chromosomes were seen in a few individuals within populations (Bogart, 1991). But the present study clearly indicates *R. tagoi* (2n = 28) to be abundantly present only in Neba village and the surrounding Chausu mountains. The mating call sonogram of *R. tagoi* (2n = 28) has been shown to clearly differ from that of *R. tagoi* (2n = 26) (Ryuzaki, manuscript in preparation). Reciprocal crosses between *R. tagoi* (2n = 26) from Hinohara village in Tokyo and *R. tagoi* (2n = 28) from the Chausu mountains were carried out by artificial fertilization. Approximately 85–90% of the eggs developed into normally metamorphosed frogs. This percentage is essentially the same or only slightly less than for control mating. Chromosome number was 2n = 27 in all examined tadpoles. Cytogenetic analysis of hybrid frogs is presently underway. As for *R. tagoi* with diploid number 28, it can be concluded from karyological data, mating call sonogram, a crossing experiment and DNA sequences of the mitochondrial 16S and 12S rRNA genes (Ryuzaki, manuscript in preparation) that a new species may possibly have developed during species differentiation in *R. tagoi* with diploid number 26 over a long period of time. These frogs with diploid numbers 28 and 26 are shown by the present study to differ uniquely and precisely because of this, they are sibling species, as is also the case for *R. nigromaculata* and *R. brevipoda* (Nishioka, 1972). Consequently, a new name designation is necessary so as to differentiate the two frog species. Whether the peculiar differentiation of this karyotype is restricted only to the Chausu mountains and whether other karyotypes may be present in this frog are points that should be clarified and studies for this purpose are presently being conducted.

The present study presents the karyological features of the karyotypes of *R. tagoi* (2n = 28) found in the Chausu mountains in the Minamishinshu district of Nagano Prefecture in Japan and gives reason to designate a new species.

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