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Reproductive Isolating Mechanisms and Molecular Phylogenetic Relationships among Palearctic and Oriental Brown Frogs

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ABSTRACT—Crossing experiments were made among various brown frog species and populations collected from Japan, China, Russia and Taiwan. The main purpose of these experiments was to confirm the existence of reproductive isolating mechanisms among *Rana pirica* from Japan, *R. chensinensis* from China and *R. chensinensis* from Russia, and between these three taxa and the other brown frogs distributed in the Palearctic and Oriental regions. It was found that there was no or a slight gametic isolation among the three taxa. While there was a nearly equal number of male and female offspring in the control groups, the hybrid frogs were all males, and completely sterile upon attaining sexual maturity. Thus, each of the Japanese *R. pirica* and the Russian *R. chensinensis* is a valid species, distinct from the Chinese *R. chensinensis*. The phylogenetic tree based on nucleotide sequence data from the mitochondrial 12S and 16S rRNA genes of the Palearctic and Oriental brown frogs showed that the three taxa are included in a cluster together with the other species with 2n=24 chromosomes. The present crossing experiments and molecular data support the hypothesis that each of them is a separate but closely related species.

Key words: reproductive isolation, hybrids, molecular phylogeny, mitochondrial genes, brown frogs

INTRODUCTION

The brown frog distributed in Hokkaido, Japan was identified with *Rana temporaria temporaria* Linne by Okada (1931), and later with *R. t. chensinensis* David by Balcells (1956). Kawamura and Kobayashi (1960) reported that in a cross between a Japanese *R. temporaria* female from Hokkaido and a *R. temporaria* male from Germany, a small number of normally cleaved eggs became normally swimming tadpoles, but none completed metamorphosis. Kawamura and Nishioka (1962) confirmed this result by repeating the same kind of crosses and made clear that in the reciprocal combination no fertilization occurs. These results suggested that the Japanese form treated as *R. temporaria* is reproductively isolated from the European *R. temporaria* by means of gametic isolation and hybrid inviability. On the basis of the results of crossing experiments and differences in karyotypes, Kawamura (1962) placed the Hokkaido population, which had thus far been called *R. t. chensinensis,* in a valid species and provisionally named this population *R. chensinensis*, originally described from China, pending verification of isolating mechanisms between the Japanese form and continental *R. chensinensis* by crossing experiments.

Matsui (1991) described the Japanese *R. chensinensis* (sensu Kawamura, 1962) as a new species, *R. pirica,* based on the available morphological information on the nominate population of *R. chensinensis* (Hu *et al.*, 1966). Nonetheless, it remains to be clarified whether reproductive isolating mechanisms exist between Japanese *R. pirica* and the continental *R. chensinensis.*

Kawamura *et al*. (1981) made a total of 97 kinds of crossing experiments using 14 brown frog species distributed in Japan, Korea, Formosa, Europe and North America. They found that all these species were completely isolated from one another by gametic isolation, hybrid inviability, hybrid sterility or combination of two or three of these reproductive isolating mechanisms. However, the Chinese and Russian populations of *R. chensisnensis* used in the present study were not treated by them. Nishioka *et al*. (1992) elucidated the genetic differences among 30 populations of 12 brown frog species distributed in the Palearctic and Oriental regions by allozyme analysis. They showed that *R. pirica* from Hokkaido and *R. chensinensis* from China and Russia are far separated from each other by large genetic distances.

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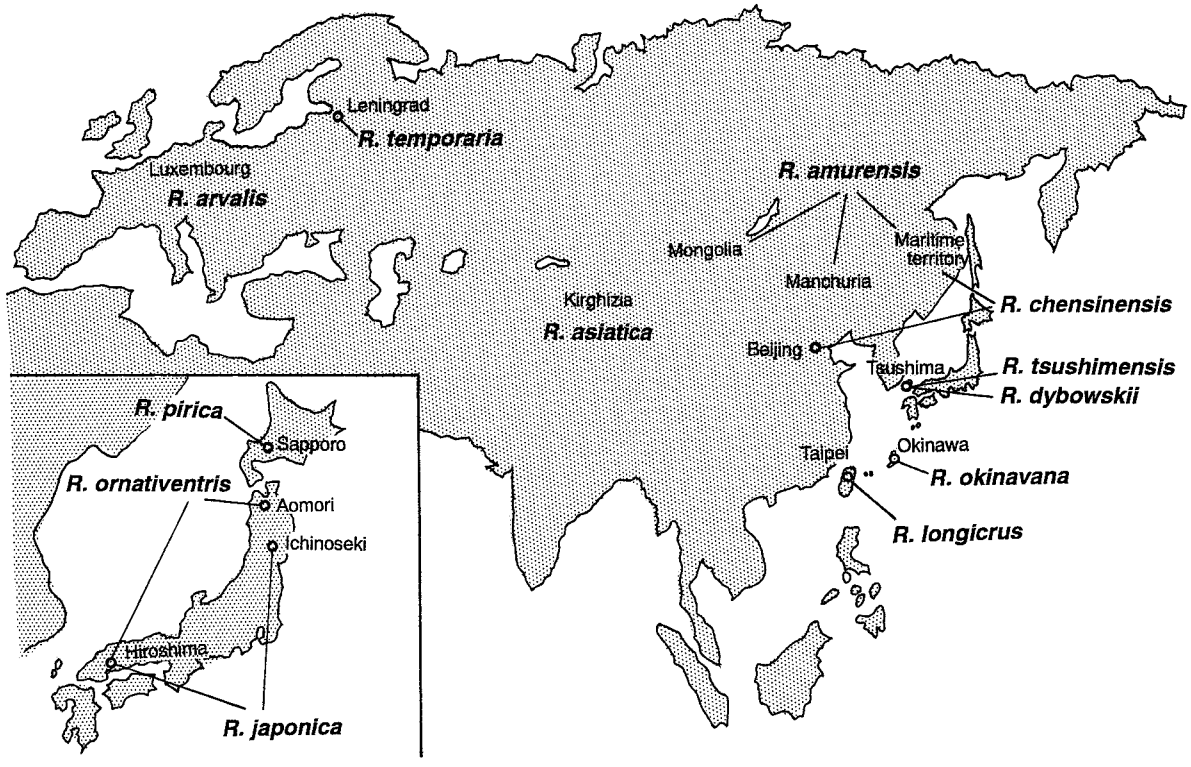

Fig. 1. Map showing localities of Palearctic and Oriental brown frogs used in the present study.

Table 1. Specimens of the Palearctic and Oriental brown frogs used in the present study.

	Species	Collecting station		Chromosome No. (2n)	No. of frogs			Abbreviation or haplotype	Accession No.	
		Country	Locality		Total	Female	Male		12S rRNA	16S rRNA
ingroup	*Rana chensinensis*	Russia	Maritime territory	24	16	10	6	*chen.* R	AB058852	AB058870
	Rana chensinensis	China	Beijing	24	17	3	14	*chen.* C	AB058853	AB058871
	Rana pirica	Japan	Hokkaido, Sapporo	24	25	12	13	*piri.* J	AB058854	AB058872
	Rana dybowskii	Japan	Tsushima	24	14	9	5	*dyb.* J	AB058855	AB058873
	Rana ornativentris	Japan	Hiroshima	24	8	4	4	*ornat.* J^H	AB058856	AB058874
	Rana ornativentris	Japan	Aomori	24	2	0	2	*ornat.* J^A	AB058857	AB058875
	Rana japonica	Japan	Hiroshima	26	14	6	8	*jap.* J^H	AB058858	AB058876
	*Rana japonica**	Japan	Iwate, Ichinoseki	26	1	1	0	*jap.* J^I	AB058859	AB058877
	Rana tsushimensis	Japan	Tsushima	26	22	10	12	*tsu.* J	AB058860	AB058878
	Rana okinavana	Japan	Okinawa	26	1	0	1	*oki.* J	AB058861	AB058879
	Rana longicrus	Taiwan	Taipei	26	8	5	3	*long.* T	AB058863	AB058881
	Rana temporaria	Russia	Leningrad	26	6	2	4	*temp.* R	AB058864	AB058882
	*Rana arvalis**	Luxembourg	Luxembourg	24	1	1	0	*arv.* L	AB058865	AB058883
	Rana asiatica	Russia	Kirghizia	26	3	3	0	*asia.* R	AB058866	AB058884
	*Rana amurensis**	Mongolia	North Mongolia	26	1	1	0	*amu.* M	AB058867	AB058885
	*Rana amurensis**	Russia	Maritime territory	26	1	1	0	*amu.* R	AB058868	AB058886
	*Rana amurensis**	China	Manchuria	26	1	1	0	*amu.* C	AB058869	AB058887
outgroup	*Rana latouchii**	Taiwan	Taipei	26	1	0	1	*lat.* T	AB058862	AB058880
	Total				142	69	73			

Asterisks show species or populations used only for DNA analysis.

In the present study, extending the work by Kawamura *et al*. (1985), crossing experiments were performed among *R. pirica* and Chinese and Russian *R. chensinensis* and between these three taxa, and the other brown frogs distributed in the Palearctic and Oriental regions in order to elucidate the existence of reproductive isolating mechanisms among them. The present study also investigated molecular phylogenetic relationships among 18 populations of 13 Palearctic and Oriental frog species using nucleotide sequences of the 12S and 16S rRNA genes of mtDNA.

MATERIALS AND METHODS

A total of 142 frogs of 18 populations belonging to 13 species distributed in the Palearctic and Oriental regions were used in the present study (Table 1, Fig. 1). Only one frog represented each population or species for DNA analysis. *Rana latouchii* was used as an outgroup. *Rana chensinensis* from Russia and China and *R. pirica* from Hokkaido were similar in external morphology, but they can be distinguished from one another by some external characters such as snout and dorsolaterl fold (Fig. 2). Crossing experiments were carried out by artificial insemination. The population-level sampling of one species in the crossing experiments was limited in only one or two from wide distribution area. Ovulation was induced by injecting bullfrog pituitaries into the body cavity. Tadpoles were fed on boiled spinach, and metamorphosed frogs were fed on crickets. Histological observation of testes of mature males was made after fixation in Navashin's fluid, sectioning at 10 μm, and staining with Heidenhain's iron hematoxylin (Kawamura *et al*., 1981). The testes were observed on the basis of abnormality in inner structure according to Kawamura and Nishioka (1972) and Sumida (1981, 1996).

DNA extraction

Total genomic DNAs for PCR were extracted from clipped toes using standard protocols of chemical digestion (0.1M Tris-HCl pH8.0, 0.2M EDTA, 1% SDS, 20 mg/ml proteinase K) followed by phenol/chloroform extraction (Sumida *et al*., in preparation). Air-dried DNA pellets were eluted in TE. The frog samples used were frozen and stored at the Institute for Amphibian Biology, Hiroshima University.

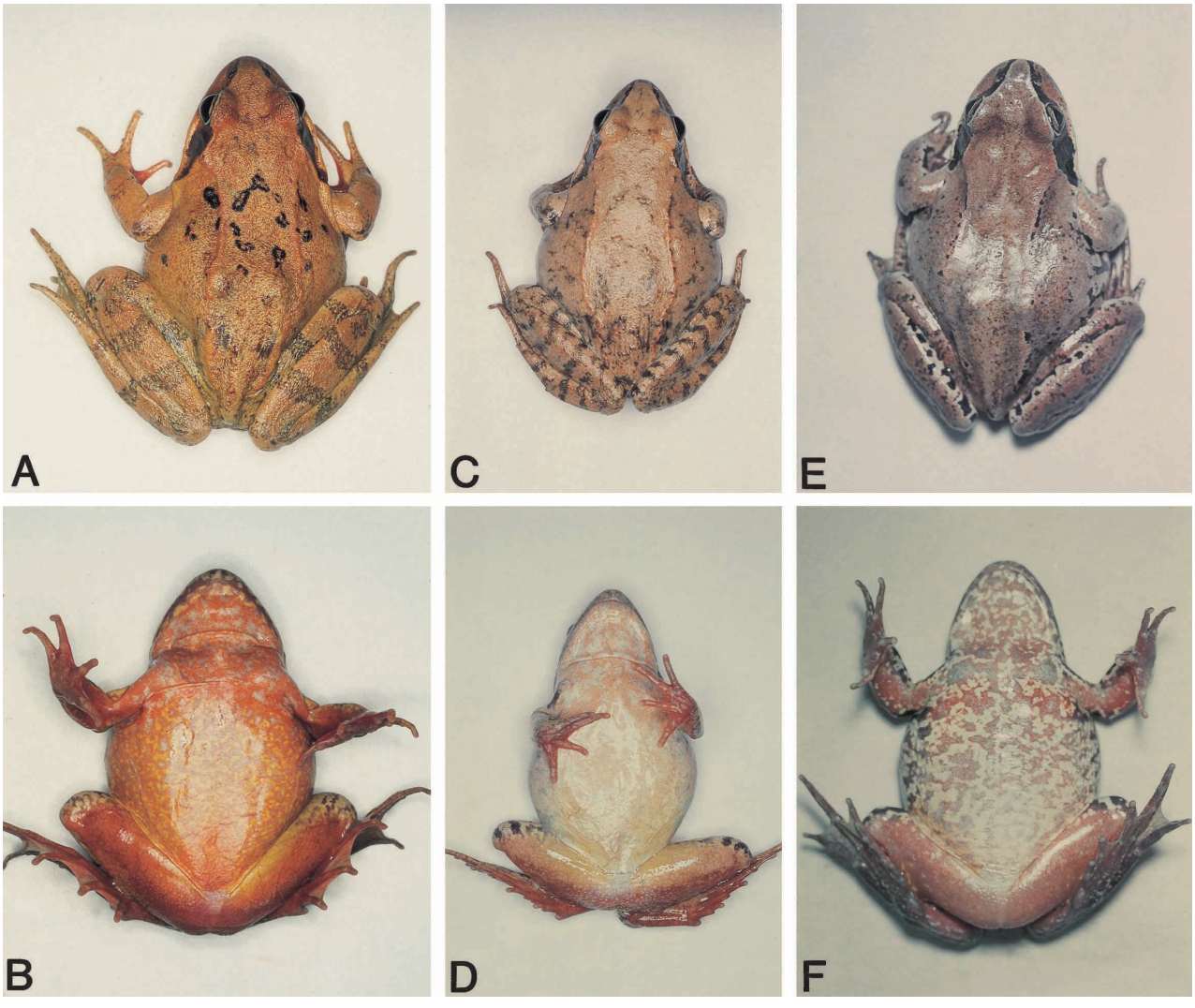

Fig. 2. Dorsal and ventral views of *Rana chensinensis* from Russia and China, and *Rana pirica* from Hokkaido (the previous name *R. chensinensis*). These three taxa are morphologically distinguished from one another. (A, B) Female *Rana chensinensis* from Russia. × 0.87. (C, D) Female *Rana chensinensis* from China. × 0.87. (E, F) Female *Rana pirica* from Hokkaido. × 0.80.

PCR primers

PCR primers (F51 and R51) were designed by Sumida *et al*. (2002) to cover a 546-bp segment of the 16S rRNA gene corresponding to sites 3976–4549 in the *X. laevis* sequence reported by Roe *et al*. (1985). The primer sequences were F51 (5'-CCC GCC TGT TTA CCA AAA ACA T-3') and R51 (5'-GGT CTG AAC TCA GAT CAC GTA-3') (Sumida *et al*., 2002). The primers (FS01 and R16) designed by Sumida *et al*. (1998, 2000a,b, 2001) were used for amplification and sequencing of a 411-bp segment of the 12S rRNA gene corresponding to sites 2816–3225 in the *R. catesbeiana* sequence (Yoneyama, 1987). The primer sequences were FS01 (5'-AAC GCT AAG ATG AAC CCT AAA AAG TTC T-3') and R16 (5'-ATA GTG GGG TAT CTA ATC CCA GTT TGT TTT-3') (Sumida *et al*., 1998, 2000a,b).

PCR and direct sequencing

PCR mixtures were prepared with the TaKaRa Taq™ Kit as recommended by the manufacturer (TaKaRa) in a final volume of 50 µl. The 12S and 16S rRNA genes were amplified by 30 cycles, each cycle consisting of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C. The PCR products were purified by MicroSpin™ S-300 HR Columns (Pharmacia Biotech) and used for sequencing. Purified DNA was sequenced by the DyeDeoxy™ Terminator Cycle Sequencing method using a 373A DNA Sequencing System Ver. 1.2 (ABI). Nucleotide sequences were analyzed using DNASIS (Ver. 3.2, Hitachi Software Engineering). Alignments for DNA sequences were determined based on maximum nucleotide similarity using CLUSTALW (Thompson *et al*., 1994). Nucleotide sequences were deposited in DDBJ, EMBL, and GenBank nucleotide sequence databases under the Accession Nos. AB058852 ~ AB058887 (Table 1).

Phylogenetic analysis

Phylogenetic relationships were estimated by the neighbor-joining (NJ) method (Saitou and Nei, 1987) on the basis of sequence divergences calculated by Kimura's two parameter method (Kimura, 1980), using the program included in Ver. 3.5c of PHYLIP (Felsenstein, J. Univ. of Washington, Seattle, 1993, unpubl.). Confidences in topology were assessed by 1,000 bootstrap replications (Felsenstein, 1985). The nucleotide sequences of *X. laevis* (Roe *et al*., 1985) were used as an outgroup to construct the phylogenetic tree.

RESULTS

Developmental capacity of hybrids

Tables 2 and 3 show the results of a total of 57 kinds of crossing experiments among 12 populations of 10 species distributed in the Palearctic and Oriental regions. In all of the control matings except for the Chinese *R. chensinensis*, the eggs developed normally and completed metamorphosis; 81.6~98.6% of the total number of eggs cleaved nor-

Table 2. Developmental capacity and sex of brown frog hybrids and the controls. I.

Parents		Total no. of eggs	No. of normal cleavages (%)	No. of normally hatched tadpoles (%)	No. of normally metamorphosed frogs (%)	Sex of frogs examined			Testes	
Female (No. of frogs)	Male (No. of frogs)					Total	♀ (%)	♂ (%)	No. of males observed	Inner structure
***chen.* R (10)**	***chen.* R (6)**	**841**	**800(95.1)**	**657(78.1)**	**514(61.1)**	**436**	**215(49.3)**	**221(50.7)**	**9**	**Normal**
chen. R (2)	*chen.* C (2)	193	182(94.3)	134(69.4)	99(51.3)	96	0	96(100)	5	Abnormal
chen. R (10)	*piri.* J (3)	434	424(97.7)	197(45.4)	145(33.4)	135	0	135(100)	5	Abnormal
chen. R (10)	*dyb.* J (2)	443	419(94.6)	189(42.7)	38(8.6)	25	0	25(100)	5	Abnormal
chen. R (8)	*ornat.* J^H (1)	258	207(80.2)	154(59.7)	131(50.8)	116	0	116(100)	5	Abnormal
chen. R (8)	*ornat.* J^A (1)	255	185(72.5)	159(62.4)	140(54.9)	119	0	119(100)	5	Abnormal
chen. R (8)	*temp.* R (2)	401	40(10.0)	19(4.7)	1(0.2)					
chen. R (8)	*jap.* J^H (2)	250	129(51.6)	78(31.2)	0					
chen. R (8)	*tsu.* J (2)	610	520(85.2)	415(68.0)	0					
chen. R (8)	*oki.* J (1)	296	31(10.5)	0	0					
***piri.* J (12)**	***piri.* J (11)**	**1448**	**1300(89.8)**	**666(46.0)**	**520(35.9)**	**433**	**202(46.7)**	**231(53.3)**	**11**	**Normal**
piri. J (5)	*chen.* R (3)	397	331(83.4)	227(57.2)	73(18.4)	68	0	68(100)	5	Abnormal
piri. J (9)	*chen.* C (5)	1370	488(35.6)	111(8.1)	35(2.6)	34	0	34(100)	7	Abnormal
piri. J (2)	*temp.* R (2)	74	50(67.6)	13(17.6)	0					
***chen.* C (3)**	***chen.* C (2)**	**234**	**129(55.1)**	**22(9.4)**	**16(6.8)**	**16**	**6(37.5)**	**10(62.5)**	**7**	**Normal**
chen. C (3)	*piri.* J (2)	190	108(56.8)	22(11.6)	20(10.5)	20	0	20(100)	5	Abnormal
chen. C (3)	*ornat.* J^H (1)	110	61(55.5)	16(14.5)	8(7.3)	8	0	8(100)	5	Abnormal
chen. C (3)	*dyb.* J (2)	156	81(51.9)	8(5.1)	6(3.8)	6	0	6(100)	5	Abnormal
chen. C (3)	*jap.* J^H (2)	126	85(67.5)	18(14.3)	3(2.4)	3	0	3(100)	5	Abnormal
chen. C (3)	*tsu.* J (2)	137	90(65.7)	18(13.1)	0					
chen. C (3)	*long.* T (1)	114	41(36.0)	0	0					

Bold represents control mating.

mally, 46.0~97.2% became normally hatched tadpoles, and 35.9~90.7% metamorphosed normally (Tables 2 and 3). The eggs used in the Chinese *R. chensinensis* matings were considered to be in an unfavorable condition, but 6.8% of the total number of eggs completed metamorphosis (Table 2). On the other hand, various degrees of abnormalities were found in the offspring produced by the interspecific crosses. No eggs cleaved, normally or abnormally, in any of the following crosses: the eight crosses using female *R. temporaria*, the crosses between female *R. ornativentris* from Hiroshima and male *R. chensinensis* from Russia and China, and the crosses between female *R. japonica* from

Table 3. Developmental capacity and sex of brown frog hybrids and the controls. II.

Parents		Total no. of eggs	No. of normal cleavages (%)	No. of normally hatched tadpoles (%)	No. of normally metamorphosed frogs (%)	Sex of frogs examined			Testes	
Female (No. of frogs)	Male (No. of frogs)					Total	♀ (%)	♂ (%)	No. of males observed	Inner structure
***ornat.* J^H(2)**	***ornat.* J^H(3)**	**525**	**511(97.3)**	**478(91.0)**	**384(73.1)**	**226**	**101(44.7)**	**125(55.3)**	**5**	**Normal**
ornat. J^H (2)	*chen.* R (2)	269	0	0	0					
ornat. J^H (2)	*chen.* C (2)	185	0	0	0					
ornat. J^H (2)	*temp.* R (2)	398	324(81.4)	5(1.3)	0					
***dyb.* J (9)**	***dyb.* J (4)**	**1925**	**1750(90.9)**	**1253(65.1)**	**1079(56.1)**	**521**	**266(51.1)**	**255(48.9)**	**5**	**Normal**
dyb. J (1)	*chen.* R (2)	194	73(37.6)	70(36.1)	34(17.5)	32	0	32(100)	5	Abnormal
dyb. J (9)	*chen.* C (5)	3123	295(9.4)	210(6.7)	131(4.2)	121	0	121(100)	5	Abnormal
dyb. J (1)	*temp.* R (2)	143	138(96.5)	50(35.0)	12(8.4)	11	0	11(100)	5	Abnormal
***jap.* J^H (4)**	***jap.* J^H (6)**	**987**	**973(98.6)**	**959(97.2)**	**895(90.7)**	**782**	**386(49.4)**	**396(50.6)**	**5**	**Normal**
jap. J^H (2)	*chen.* R (2)	221	0	0	0					
jap. J^H (4)	*chen.* C (2)	741	414(55.9)	300(40.5)	257(34.7)	243	0	243(100)	5	Abnormal
jap. J^H (2)	*temp.* R (2)	271	134(49.4)	126(46.5)	100(36.9)	100	0	100(100)	5	Abnormal
***tsu.* J (10)**	***tsu.* J (10)**	**1231**	**1182(96.0)**	**1074(87.2)**	**869(70.4)**					
tsu. J (3)	*chen.* R (2)	207	68(32.9)	31(15.0)	0					
tsu. J (10)	*chen.* C (6)	1064	761(71.5)	697(65.5)	1(0.1)					
tsu. J (3)	*temp.* R (2)	247	227(91.9)	30(12.1)	0					
***long.* T (5)**	***long.* T (2)**	**496**	**460(92.7)**	**422(85.1)**	**389(78.4)**	**355**	**196(55.2)**	**159(44.8)**	**5**	**Normal**
long. T (5)	*chen.* C (2)	573	444(77.5)	419(73.1)	0					
***temp.* R (2)**	***temp.* R (2)**	**136**	**111(81.6)**	**93(68.4)**	**83(61.0)**	**77**	**43(55.8)**	**34(44.2)**	**5**	**Normal**
temp. R (2)	*chen.* R (2)	129	0	0	0					
temp. R (2)	*chen.* C (1)	206	0	0	0					
temp. R (2)	*piri.* J (2)	144	0	0	0					
temp. R (2)	*dyb.* J (1)	145	0	0	0					
temp. R (2)	*ornat.* J^H (1)	196	0	0	0					
temp. R (2)	*ornat.* J^A (1)	155	0	0	0					
temp. R (2)	*jap.* J^H (1)	139	0	0	0					
temp. R (2)	*tsu.* J (1)	147	0	0	0					
asia. R (3)	*chen.* R (2)	266	246(92.5)	0	0					
asia. R (3)	*chen.* C (1)	225	194(86.2)	0	0					
asia. R (3)	*piri.* J (2)	235	212(90.2)	0	0					
asia. R (3)	*temp.* R (2)	346	302(87.3)	18(5.2)	0					
asia. R (3)	*dyb.* J (1)	247	231(93.5)	0	0					
asia. R (3)	*ornat.* J^H (1)	286	267(93.4)	0	0					
asia. R (3)	*ornat.* J^A (1)	272	251(92.3)	0	0					
asia. R (3)	*jap.* J^H (1)	341	273(80.1)	0	0					
asia. R (3)	*tsu.* J (1)	272	214(78.7)	0	0					

Bold represents control mating.

Hiroshima and male *R. chensinensis* from Russia (Tables 2 and 3). No eggs developed into normally hatched tadpoles in any of the following crosses: the 8 interspecific crosses using female *R. asiatica,* the crosses between female *R. chensinensis* from Russia and male *R. okinavana,* and the crosses between female *R. chensinensis* from China and male *R. longicrus* (Tables 2 and 3). No tadpoles metamorphosed normally in the interspecific crosses between female *R. chensinensis* from Russia and males of *R. japonica* from Hiroshima and *R. tsushimensis,* between female *R. pirica* and male *R. temporaria,* between female *R. chensinensis* from China and male *R. tsushimensis*, between female *R. ornativentris* from Hiroshima and male *R. temporaria*, between female *R. tsushimensis* and males of *R. chensinensis* from Russia and *R. temporaria*, and between female *R. asiatica* and male *R. temporaia* (Tables 2 and 3).

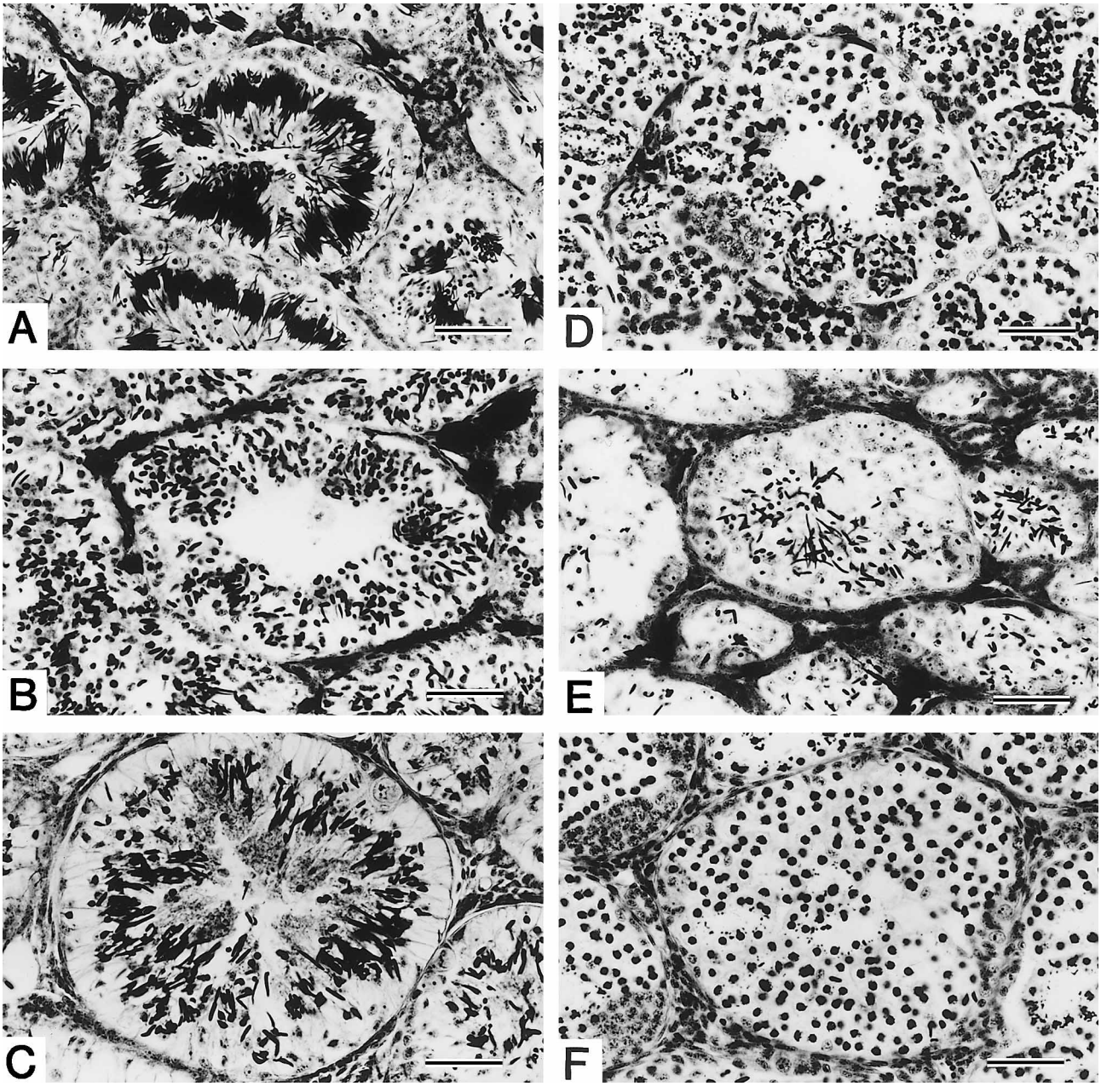

Fig. 3. Cross-sections of the testes of mature male hybrids between female *Rana chensinensis* from Russia and males of three species and the control males. Scale bars equal 50 μm. (A) Control *R. chensinensis* from Russia, *chen.* R ♀No.1 × *chen.* R ♂ No.1. (B) Hybrid between female *R. chensinensis* from Russia and male *R. chensinensis* from China, *chen.* R ♀ No.1 × *chen.* C ♂ No. 1. (C) Hybrid between female *R. chensinensis* from Russia and male *R. chensinensis* from China, *chen.* R ♀ No.1 × *chen.* C ♂ No. 2. (D) Hybrid between female *R. chensinensis* from Russia and male *R. pirica* from Sapporo, *chen.* R ♀ No.1 × *piri.* J ♂ No. 1. (E) Hybrid between female *R. chensinensis* from Russia and male *R. pirica* from Sapporo, *chen.* R ♀ No.1 × *piri.* J ♂ No. 2. (F) Hybrid between female *R. chensinensis* from Russia and male *R. ornativentris* from Hiroshima, *chen.* R ♀ No.1 × *ornat.* J^{H} ♂ No. 1.

The tadpoles metamorphosed normally in only 16 out of the 48 interspecific crosses.

Sexes of the hybrids

The metamorphosed frogs from all of the control matings included both males and females, and the percentages of females ranged from 37.5 to 55.8%. On the other hand, all the hybrid frogs produced from interspecific crosses were males (Tables 2 and 3).

Testes of mature hybrids

The inner structures of testes of mature males derived from 8 control matings and 16 interspecific crosses were examined by histological observation (Tables 2 and 3). The testes of males derived from the control matings were completely normal in inner structure: the seminiferous tubules were filled with compact bundles of normal spermatozoa (Figs. 3A and 4A, D). On the other hand, the testes of all male hybrids produced from interspecific crosses were

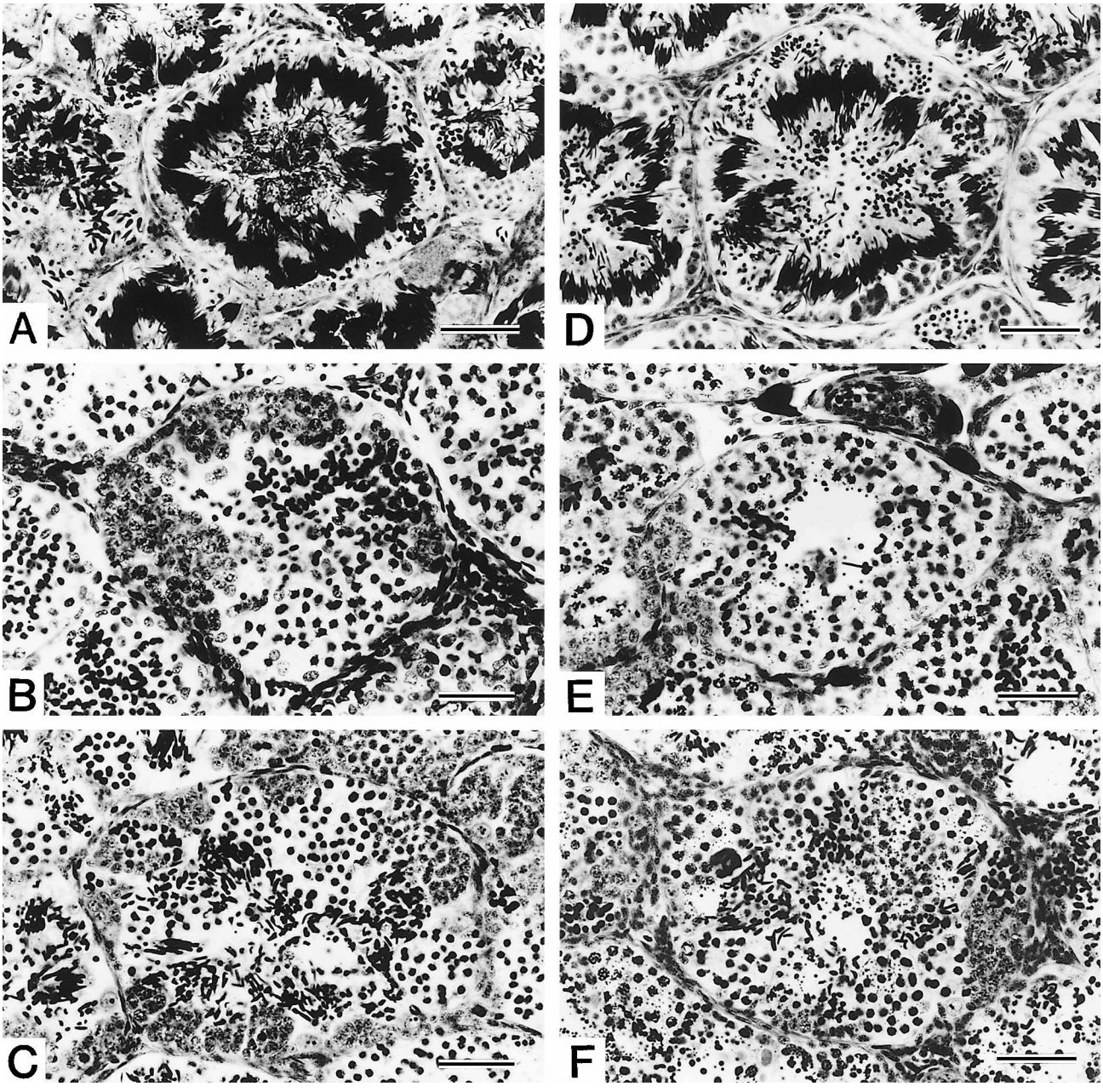

Fig. 4. Cross-sections of the testes of mature male hybrids between female *Rana pirica* and males of *R. chensinensis* from Russia and China, between female *R. chensinensis* from China and male *R. pirica*, and between female *R. japonica* from Hiroshima and male *R. chensinensis* from China and the control males. Scale bars equal 50 μm. (A) Control *R. pirica*, *piri.* J ♀ No.1 × *piri.* J ♂ No.1. (B) Hybrid between female *R. pirica* and male *R. chensinensis* from Russia, *piri.* J ♀ No.1 × *chen.* R ♂ No. 1. (C) Hybrid between female *R. pirica* and male *R. chensinensis* from China, *piri.* J ♀ No.1 × *chen.*C ♂ No. 1. (D) Control *R. chensinensis* from China, *chen.* C ♀ No.1 × *chen.* C ♂ No. 1. (E) Hybrid between female *R. chensinensis* from China and male *R. pirica*, *chen.* C ♀ No.1 × *piri.* J ♂ No. 1. (F) Hybrid between female *R. japonica* from Hiroshima and male *R. chensinensis* from China, *jap.* J^H ♀ No.1 × *chen.* C ♂ No. 1.

abnormal in inner structure: the seminiferous tubules contained no normal spermatozoa, and there were various quantities of large abnormal spermatozoa and pycnotic nuclei (Figs. 3B-F and 4B, C, E, F). When mature male hybrids were mated with the control females, all male hybrids were completely sterile in reproductive capacity.

Molecular phylogeny

Nucleotide sequences were determined in a 411-bp segment of the 12S rRNA gene and 546-bp segment of the 16S rRNA gene in 18 frogs from 18 populations of 13 species distributed in the Palearctic and Oriental regions. Eighteen haplotypes were observed in these frogs (Table 1), and each population or species had a specific haplotype. The sequenced 411-bp segment of the 12S rRNA gene provided 116 variable sites including eight gaps between species, and the sequenced 546-bp segment of the 16S rRNA gene provided 121 variable sites including 13 gaps.

Table 4 shows the nucleotide sequence divergences and the numbers of nucleotide substitutions in all pairs of 18 haplotypes of the 12S and 16S rRNA genes in the Palearctic and Oriental frogs. The nucleotide sequence divergences of the 12S and 16S rRNA genes were 0.53%, 1.50% and 5.90% between two haplotypes of *R. ornativentris, R. japonica* and *R. chensinensis,* respectively, and 0.11%~0.32% among three haplotypes of *R. amurensis* (Table 4). There were 5, 17 and 54 nucleotide substitutions between two haplotypes of *R. ornativentris, R. japonica* and *R. chensinensis,* respectively, and 1~3 among three haplotypes of *R. amurensis* including gaps (Table 4). The nucleotide sequence divergences were 2.69%~6.61% ($\bar{x}$=4.15%), and there were 26~62 nucleotide substitutions including gaps among seven haplotypes of 2n=24 brown frog species (Table 4). The nucleotide sequence divergences were 2.80%~8.31% ($\bar{x}$=6.21%), and there were 29~80 nucleotide substitutions including gaps among 10 haplotypes of 2n=26 brown frog species (Table 4). The nucleotide sequence divergences were 3.56%~7.77% ($\bar{x}$=6.24%), and there were 33~73 nucleotide substitutions including gaps between seven haplotypes of 2n=24 brown frog species and 10 haplotypes of 2n=26 brown frog species (Table 4). The nucleotide sequence divergences were 13.96%~15.22% ($\bar{x}$=14.68%), and there were 122~133 nucleotide substitutions including gaps between outgroup *R. latouchii* and ingroup 12 brown frog species (Table 4). The nucleotide sequence divergences were 29.79%~32.27% ($\bar{x}$=30.71%), and there were 278~293 nucleotide substitutions including gaps between outgroup *X. laevis* and ingroup 12 brown frog species (Table 4). The nucleotide sequence divergences were 29.02%, and there were 262 nucleotide substitutions including gaps between outgroup *R. latouchii* and *X. laevis* (Table 4).

When the nucleotide sequence divergences in the 12S rRNA gene were plotted against those in the 16S rRNA gene in all pairwise comparisons of 19 mtDNA haplotypes

Table 4. Percent divergences estimated by Kimura's two-parameter method (above diagonal) and the numbers of transition sites (Ts), transversion sites (Tv) and gaps (G) (Ts / Tv / G) (below diagonal) among haplotypes of nucleotide sequences of the mitochondrial 12S and 16S rRNA genes of the brown frogs distributed in the Palearctic and Oriental regions and *R. latouchii* (*lat.* T) and *X. laevis* (*xeno.*) as outgroups.

Haplotype	*chen.*R	*chen.*C	*piri.*J	*dyb.*J	*ornat.*J^{H}	*ornat.*J^{A}	*jap.*J^{H}	*jap.*J^{I}	*tsu.*J	*oki.*J
*chen.*R	–	5.90	3.89	5.11	4.79	4.90	7.09	6.73	7.37	7.69
*chen.*C	50/3/1	–	3.46	3.79	3.36	3.02	6.65	6.30	7.39	6.78
*piri.*J	32/3/0	23/7/1	–	2.69	2.80	2.69	6.19	5.05	7.04	6.08
*dyb.*J	43/4/2	29/5/3	19/6/2	–	3.69	3.57	5.96	5.38	6.92	6.30
*ornat.*J^{H}	40/4/2	24/7/1	20/6/2	27/5/4	–	0.53	6.43	5.96	7.63	7.14
*ornat.*J^{A}	42/3/2	22/6/1	19/5/2	27/6/4	4/1/0	–	6.31	5.85	7.39	7.02
*jap.*J^{H}	53/10/5	46/10/6	41/16/5	38/15/7	44/12/7	44/11/7	–	1.50	6.35	6.89
*jap.*J^{I}	54/6/6	47/8/7	36/10/6	38/11/8	44/11/6	44/10/6	14/0/3	–	5.88	6.42
*tsu.*J	49/18/4	46/19/5	43/22/4	45/18/8	46/20/6	42/20/6	42/16/5	42/12/8	–	5.49
*oki.*J	58/14/2	48/16/3	42/16/2	42/16/4	48/17/4	48/16/4	49/13/3	47/11/4	40/6/4	–
*long.*T	57/8/5	47/11/6	39/12/5	41/11/7	46/11/5	46/11/5	27/3/6	24/2/3	45/17/7	47/11/5
*temp.*R	53/4/2	37/6/3	35/8/2	35/7/4	35/7/4	35/6/4	40/6/5	44/4/4	47/12/6	47/12/2
*arv.*L	54/6/2	38/10/3	33/9/2	38/9/4	32/10/4	32/9/4	41/8/5	37/9/4	45/15/6	41/13/2
*asia.*R	51/5/2	44/8/3	33/10/2	43/10/4	37/9/4	35/8/4	41/8/5	37/5/6	43/18/4	48/14/2
*amu.*M	59/9/3	48/11/4	49/9/3	51/11/5	49/10/3	47/9/3	51/13/6	50/12/5	56/16/7	53/13/3
*amu.*R	61/9/3	49/11/4	50/9/3	52/11/5	48/10/3	46/9/3	50/13/6	49/12/5	56/16/7	54/13/3
*amu.*C	61/9/3	50/11/4	51/9/3	53/11/5	49/10/3	47/9/3	51/13/6	50/12/5	57/16/7	55/13/3
*lat.*T	81/46/8	77/47/9	72/48/8	76/44/8	75/48/8	78/47/8	76/40/13	71/42/12	76/44/10	70/44/8
xeno.	114/103/65	114/108/62	116/98/63	117/109/57	119/109/59	120/108/59	117/111/64	118/104/67	113/108/63	121/109/63

Abbreviations of haplotypes are given in Table 1.

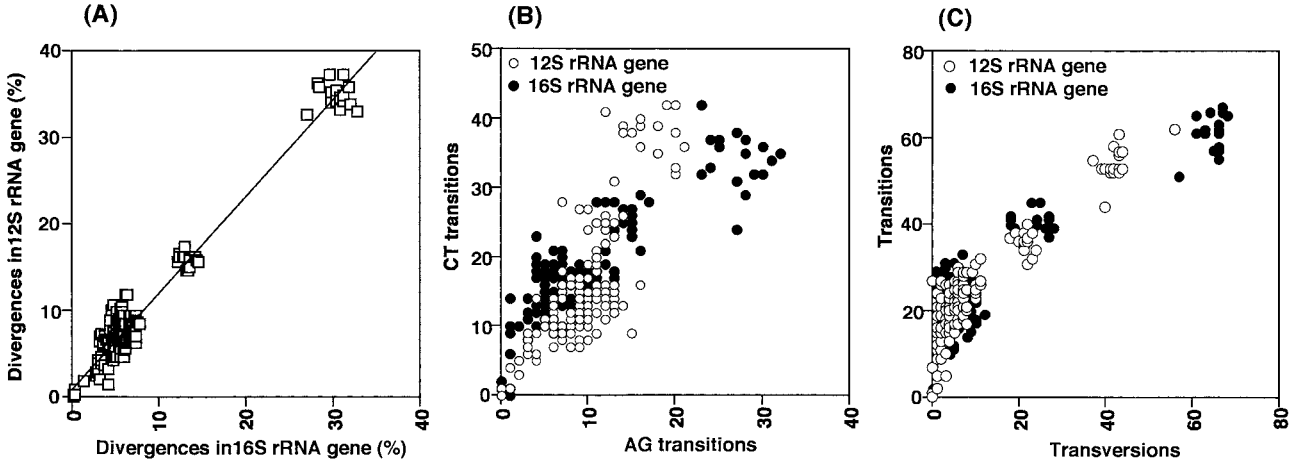

Fig. 5. (A) Dependence of percent sequence divergences of the 12S rRNA gene on the 16S rRNA gene. (B) Dependence of C⇔T (CT) transitions on A⇔G (AG) transitions of the 12S and 16S rRNA genes. (C) Dependence of transitions on transversions of the 12S and 16S rRNA genes.

of the ingroup and outgroup, the rates of sequence divergences for the 12S rRNA gene were approximately 1.2 times greater than those for the 16S rRNA gene in all pairwise comparisons of 19 haplotypes (Fig. 5A). The comparisons of the mtDNA haplotypes from the ingroup and outgroup in the 12S and 16S rRNA genes showed more C ⇔ T than A⇔G transitions (C⇔T / A⇔G ratios = 1.66) (Fig. 5B). The frequencies of transitions in the 12S and 16S rRNA genes appeared to plateau in pairwise comparisons between the ingroup Palearctic and Oriental brown frog species and outgroup *R. latouchii* / *X. laevis*, where multiple substitutions occurred at the same site (Fig. 5C).

The NJ tree based on the nucleotide sequence divergences of the 12S and 16S rRNA genes showed that the outgroup *R. latouchii* was clearly separated from the brown frog species (Fig. 6). The ingroup brown frog species, the monophyly of which was strongly supported in 100% of 1,000 bootstrap iterations, were largely divided into six

long.T	*temp*.R	*arv*.L	*asia*.R	*amu*.M	*amu*.R	*amu*.C	*lat*.T	*xeno*.
7.19	6.25	6.61	6.26	7.54	7.65	7.77	15.21	30.28
6.52	4.80	5.38	5.93	6.64	6.75	6.87	14.73	31.05
5.61	4.69	4.58	4.80	6.39	6.50	6.62	14.20	29.91
5.83	4.69	5.14	5.93	6.86	6.97	7.09	14.01	31.28
6.18	4.70	4.71	5.04	6.63	6.52	6.63	15.11	30.74
6.06	4.47	4.59	4.70	6.29	6.17	6.29	15.22	30.88
3.57	5.03	5.61	5.49	7.58	7.46	7.58	14.62	30.33
2.80	5.24	5.04	4.81	6.87	6.75	6.87	14.34	30.61
6.91	6.54	6.67	6.21	8.19	8.19	8.31	15.03	31.76
6.54	6.29	5.96	6.65	7.34	7.46	7.57	13.96	32.27
–	5.13	5.48	5.03	5.61	5.27	5.38	14.84	29.79
41/6/3	–	3.56	4.55	5.81	5.93	6.04	15.05	30.53
39/11/3	28/5/0	–	4.68	6.27	6.38	6.50	14.29	30.31
38/7/4	40/2/2	38/4/2	–	6.16	6.05	6.16	14.93	31.05
40/12/4	46/7/3	50/7/3	47/9/3	–	0.32	0.21	14.64	30.42
37/12/4	47/7/3	51/7/3	46/9/3	3/0/0	–	0.11	14.64	30.42
38/12/4	48/7/3	52/7/3	47/9/3	2/0/0	1/0/0	–	14.77	30.42
74/48/9	81/44/8	76/43/8	78/46/6	81/40/7	81/40/7	82/40/7	–	29.02
116/110/62	114/106/63	119/103/61	113/113/59	113/110/60	115/108/60	114/109/60	99/106/57	–

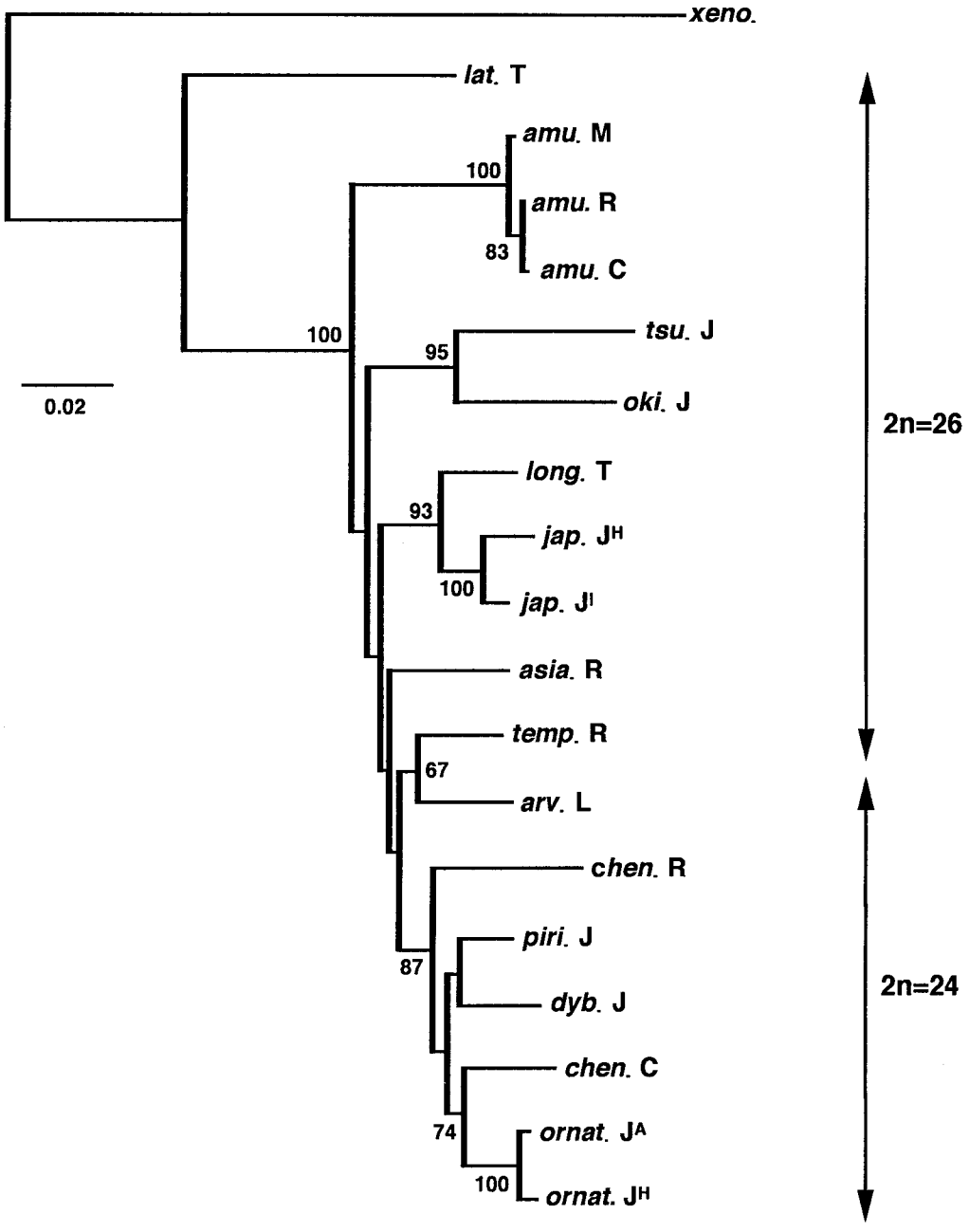

Fig. 6. Phylogenetic tree constructed by the neighbor-joining (NJ) method based on the nucleotide sequences of 411-bp segment of the 12S rRNA gene and 546-bp segment of the 16S rRNA gene from 18 haplotypes of the brown frogs distributed in the Palearctic and Oriental regions and *Rana latouchii* (*lat.*T) and *Xenopus laevis* (*xeno.*) as outgroup. Numbers on the tree are bootstrap probabilities (above 60%) based on 1000 replications. Scale bar represents branch length in terms of nucleotide substitutions per site. Abbreviations of haplotypes are given in Table 1.

clades (Fig. 6). The first clade contained three populations of *R. amurensis*. The second clade included *R. tsushimensis* and *R. okinavana*, the third clade consisted of *R. longicrus* and two populations of *R. japonica*, the fourth clade contained *R. asiatica*, and the fifth clade contained *R. temporaria* and *R. arvalis*. The sixth clade included four species with 2n=24 chromosomes, *R. chensinensis* from Russia and China, *R. pirica*, *R. dybowskii*, and *R. ornativentris* from Hiroshima and Aomori (Fig. 6).

DISCUSSION

Reproductive isolation of Palearctic and Oriental brown frogs

Mayr (1940) defined species as “groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups.” Dobzhansky (1950) defined the species as “the largest and most inclusive ... reproductive community of sexual and cross-fer-

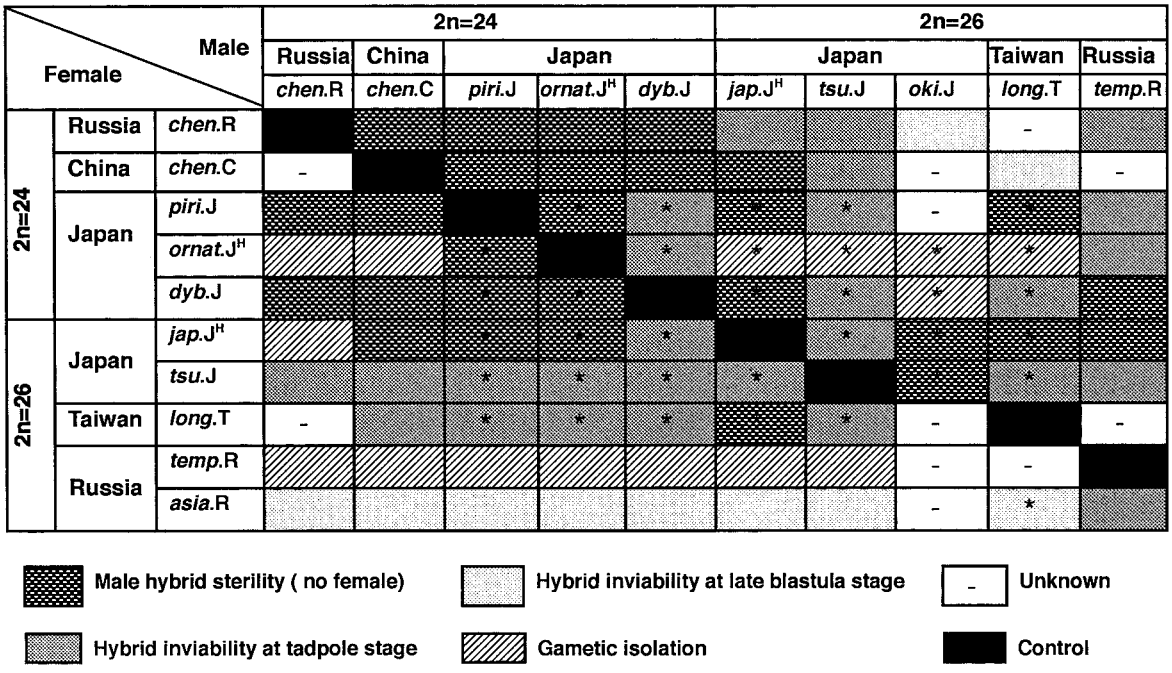

Fig. 7. Reproductive isolating mechanisms found in various combinations among 11 brown frog species distributed in the Palearctic and Oriental regions. The mark of each section shows the reproductive isolating mechanism by which two species are completely isolated from each other. The data from Kawamura *et al.* (1981) are asterisked.

tilizing individuals which share in a common gene pool." If two taxa are reproductively isolated from each other and cannot interbreed, they are considered different species.

Brown frog species and populations from Japan, Taiwan, China, and Russia were found to be reproductively isolated from one another by gametic isolation, hybrid inviability at the embryonic or tadpole stage, and male hybrid sterility. The reproductive isolating mechanisms among the Palearctic and Oriental brown frog species and populations are summarized from the present results and the data provided by Kawamura *et al.* (1981) (Fig. 7). This figure shows that there was complete gametic isolation in 15 hybrid combinations, from which no cleaved eggs were obtained. Gametic isolation is related to the difficulty with which the eggs are inseminated with frog sperm, that is, the difficulty with which sperm can penetrate jelly envelopes. This figure also shows that all the hybrids derived from 10 and 27 hybrid combinations died during embryonic and tadpole stages, respectively. The hybrid inviability is attributed to the incompatibility between the two genomes. Furthermore this figure clarifies that all viable hybrids derived from the 28 hybrid combinations became completely sterile males. The complete sterility of mature male hybrids has also been reported in other hybrid combinations among various brown frog species (Dürken, 1935, 1938; Kawamura, 1940, 1943, 1950; Kawamura and Kobayashi, 1959, 1960; Kawamura and Nishioka, 1962, 1973, 1977). The hybrid sterility is attributed to structural differences in chromosomes between the parental species. Thus, it may be that different brown frog species in the Palearctic and Oriental regions are first isolated from each other, either completely or incompletely, by gametic isolation: when the gametic isolation is incomplete, the two species are completely or incompletely isolated by hybrid inviability; when the hybrid inviability is incomplete, the two species are completely isolated by hybrid sterility.

Relationship among *R. pirica* and Russian and Chinese *R. chensinensis*

Assignment of the brown frog from Hokkaido to *R. chensinensis* was made provisionally by Kawamura (1962). The original description of *R. chensinensis* (David, 1875) is brief and uninformative, and nothing is reported for the topotypic population. Liu and Hu (1961) and Hu *et al.* (1966) noted that *R. chensinensis*, the so-called Chinese brown frog, is itself highly polymorphic and assuredly comprises several distinct forms. Kawamura *et al.* (1985) proposed that Japanese *R. chensinensis* should be described as *R. ezoensis,* based on a preliminary report of crossing experiments. From the available morphological information about the nominate population of *R. chensinensis* (Hu *et al.*, 1966), Matsui (1991) regarded the brown frog occurring in Hokkaido as an undescribed form and designated this frog as a new species, *R. pirica*. In a morphological comparison of *R. chensinensis* from China with three Japanese relatives, *R. pirica*, *R. ornativentris*, and *R. dybowskii,* Matsui *et al.* (1993) found that *R. pirica*, a species long regarded as conspecific with *R. chensinensis,* is more remote from topotypes of *R. chensinensis* morphologically than are *R. ornativentris* and *R. dybowskii*. The present results of the crossing experiments showed that *R. pirica* occurring in Hokkaido is reproductively isolated from *R. chensinensis* from Russia and China by complete hybrid sterility. The

hybrids between these three taxa usually showed incomplete gametic isolation and hybrid inviability, but a small number of hybrids attained sexual maturity. While this seems to show that the three taxa are somewhat related to one another, all the hybrids became completely sterile males. As these three taxa are reproductively isolated by complete hybrid sterility and morphologically distinguished from each other (Figs. 2 and 7), each should be a different valid species. Thus, it was reasonable of Matsui (1991) to describe the brown frog from Hokkaido as a new species *Rana pirica*, even though the species name *Rana ezoensis* had been proposed by Kawamura *et al*. (1985) as a new species name based on preliminary data from crossing experiments. It is also very interesting to note that Russian *R. chensinensis* is a separate valid species which is morphologically distinguished and reproductively isolated from Chinese *R. chensinensis*.

Molecular phylogeny of brown frogs

Recently, molecular phylogenies of Asian brown frogs have been clarified by analyzing nucleotide sequences of the mitochondrial cytochrome *b* gene (Tanaka *et al*., 1994, 1996, 1998a, b, c, 1999; Matsui *et al*., 1998; Sumida and Ogata, 1998). The present study analyzed the nucleotide sequences of mitochondrial 12S and 16S rRNA genes to elucidate phylogenetic relationships among the brown frogs distributed in the Palearctic and Oriental regions. Regarding the relationships of Asian brown frogs, our results are partially consistent with those obtained in the previous studies of mitochondrial cytochrome *b* gene sequences by Tanaka *et al*. (1994, 1996, 1998a, b, c, 1999) and Matsui *et al*. (1998), and the inclusion of additional taxa revealed some new information. The present results showed that the outgroup *R. latouchii* was remotely related to the brown frog species examined. *R. latouchii* apparently belongs to a different lineage from brown frogs according to Dubois (1992), and this proposition has been supported by allozyme analysis (Nishioka *et al*., 1992). The ingroup brown frog species, of which the monophyly was strongly supported in 100% of 1000 bootstrap iterations, was largely divided into six clades. The first clade includes three populations of *R. amurensis* (bootstrap 100%). The second clade includes *R. tsushimensis* and *R. okinavana* (bootstrap 95%). The association of *R. tsushimensis* and *R. okinavana* has also been reported based on mitochondrial cytochrome *b* gene data (Tanaka *et al*., 1996). The third clade includes *R. longicrus* and *R. japonica. Rana longicrus* was once regarded as conspecific with *R. japonica* based on the close morphological similarities between the two species. Karyological and ecological studies further supported their close phylogenetic relationships (Kuramoto *et al*., 1973, 1984), as did the present study (bootstrap 93%), although the clade was only weakly supported by mitochondrial cytochrome *b* gene data (bootstrap 47.7%, Tanaka *et al*., 1998a). The fourth clade includes *R. asiatica.* The fifth clade includes *R. temporaria* and *R. arvalis* (bootstrap 67%), both of which are European brown frog species. The sixth clade includes *R. chensinensis* from China and Russia, and *R. ornativentris, R. pirica* and *R. dybowskii* (bootstrap 87%), all of which have 24 chromosomes (Nishioka *et al*., 1987). The same groupings of Asian brown frogs with 2n=24 chromosomes were mostly supported by allozyme analyses carried out by Nishioka *et al*. (1992) and Green and Borkin (1993). Our molecular data, in which five clades comprising 7 species with 2n=26 chromosomes showed a polytomous relationship with the sixth clade comprising 4 species with 2n=24 chromosomes, could not elucidate the detailed phylogenetic relationships among brown frog species with 2n=26 chromosomes by analyzing the mitochondrial 12S and 16S rRNA gene sequences. Further examination will be necessary to infer the detailed phylogenetic relationships among Palearctic and Oriental brown frog species with 2n=26 chromosomes by analyzing other mitochondrial and nuclear genes.

Concerning the genetic variations within species, the sequence divergences among several three populations of *R. amurensis* from Russia, China and Mongolia were very small (0.11%~0.32%). A similarly low genetic differentiation among several populations of *R. amurensis* has been demonstrated by allozyme analysis (Nishioka *et al*., 1992; Green and Borkin, 1993) and mtDNA cytochrome *b* gene analysis (Tanaka *et al*., 1998c). Thus, *R. amurensis* widely found from Mongolia and northeastern China to Far East Asia is considered to have diverged within a short time rather recently. It is noteworthy that within *R. japonica*, genetic differentiation between the western and eastern populations of Honshu was rather large (1.50%), whereas it was rather small (0.53%) within *R. ornativentris* distributed sympatrically with *R. japonica*. Indeed, genetic differentiation between western and eastern populations of *R. japonica* has also been reported by crossing experiments, spermatogenesis, allozyme data and mitochondrial cytochrome *b* gene sequence data (Sumida, 1981, 1994, 1996; Sumida and Nishioka, 1994; Sumida and Ogata, 1998).

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