Sequencing and analysis of the internal transcribed spacers (ITSs) and coding regions in the *EcoR* I fragment of the ribosomal DNA of the Japanese pond frog *Rana nigromaculata*

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The rDNA of eukaryotic organisms is transcribed as the 40S-45S rRNA precursor, and this precursor contains the following segments: 5' – ETS – 18S rRNA – ITS 1 – 5.8S rRNA – ITS 2 – 28S rRNA – 3'. In amphibians, the nucleotide sequences of the rRNA precursor have been completely determined in only two species of *Xenopus*. In the other amphibian species investigated so far, only the short nucleotide sequences of some rDNA fragments have been reported. We obtained a genomic clone containing the rDNA precursor from the Japanese pond frog *Rana nigromaculata* and analyzed its nucleotide sequence. The cloned genomic fragment was 4,806 bp long and included the 3'-terminus of 18S rRNA, ITS 1, 5.8S rRNA, ITS 2, and a long portion of 28S rRNA. A comparison of nucleotide sequences among *Rana*, the two species of *Xenopus*, and human revealed the following: (1) The 3'-terminus of 18S rRNA and the complete 5.8S rRNA were highly conserved among these four taxa. (2) The regions corresponding to the stem and loop of the secondary structure in 28S rRNA were conserved between *Xenopus* and *Rana*, but the rate of substitutions in the loop was higher than that in the stem. Many of the human loop regions had large insertions not seen in amphibians. (3) Two ITS regions had highly diverged sequences that made it difficult to compare the sequences not only between human and frogs, but also between *Xenopus* and *Rana*. (4) The short tracts in the ITS regions were strictly conserved between the two *Xenopus* species, and there was a corresponding sequence for *Rana*. Our data on the nucleotide sequence of the rRNA precursor from the Japanese pond frog *Rana nigromaculata* were used to examine the potential usefulness of the rRNA genes and ITS regions for evolutionary studies on frogs, because the rRNA precursor contains both highly conserved regions and rapidly evolving regions.

Key words: ITS, Japanese pond frog, *Rana nigromaculata*, ribosomal rRNA genes

INTRODUCTION

The ribosomal DNA (rDNA) found in the nuclear genome of eukaryotes is transcribed as the 40S-45S rRNA precursor and consists of tandemly repeated copies. The rRNA precursor contains the segments 5' – ETS – 18S rRNA – ITS 1 – 5.8S rRNA – ITS 2 – 28S rRNA – 3', where ETS and ITS are external and internal transcribed spacers that are removed during RNA processing (Gerbi,

1986). The length of the rDNA unit varies greatly between different species, ranging from 6.8 kbp in the protozoa (Lipps and Steinbruck, 1982) to 44 kbp in the rat (Stumph et al., 1979). This variability in length can be accounted for by differences in a so-called non-transcribed spacer (NTS) adjacent to the transcription unit for the 40S-45S rRNA precursor.

In amphibians, the nucleotide sequences of the rRNA precursor have been completely clarified in only the two tetraploid species of *Xenopus* (Ajuh et al., 1991). In the other amphibian species examined, only the short nucleotide sequences of some rDNA fragments have been

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determined (Hillis et al., 1993). While the two species of *Xenopus* share almost identical rRNA coding regions, the ITS regions differ greatly between them (Ajuh et al., 1991). Sequence data are not apt to distinguish speciation events between different ploidy levels, nor do they contain information on the allopolyploidization events themselves (Kobel et al., 1998). According to an earlier estimate, 25–50% of duplicated genes have been silenced in the tetraploid *X. laevis* (Graf and Kobel, 1991; Hughes and Hughes, 1993). Comparisons of the rates of nucleotide substitution in various gene segments suggest that sequences with reduced coding constraints, for example, the intron, may be rapid enough to produce accumulated changes on a scale warranting their study (Li, et al., 1985; Gillespie, 1986). Ribosomal RNA exhibits an unusual evolutionary pattern featuring the interspersion of rapidly evolving segments among highly conserved regions that contain some of the most evolutionarily stable macromolecular sequences known (Gerbi, 1985).

Recent studies of the Japanese pond frog *R. nigromaculata* have made some progress. The complete mitochondrial genome has now been determined (Sumida et al., 2001), and examinations of the linkage groups have led to the mapping of 34 loci on ten chromosomes (Sumida and Nishioka, 2000). Nonetheless, the rRNA genes of this species have never been studied. In amphibian phylogeny, *Xenopus laevis* in the family Pipidae is considered more primitive than *Rana nigromaculata* in the family Ranidae.

In the present study, we determined the nucleotide sequence of the rRNA precursor from the Japanese pond frog *Rana nigromaculata* in order to evaluate the potential usefulness of the rRNA gene and the ITS regions for evolutionary studies on animals, particularly those focused on frogs.

MATERIALS AND METHODS

DNA sources. Japanese pond frogs, *Rana nigromaculata*, were collected from Ushita, Hiroshima City and used for artificial crossings to produce offspring in the laboratory. Genomic DNA was extracted from the livers of the offspring and purified by CsCl-EtBr density gradient centrifugation according to the method reported by Sumida (1997). The frog sample (No. 31409) used was frozen and stored at the Institute for Amphibian Biology, Hiroshima University.

Cloning, sequencing, and data analysis. The *EcoR* I fragments of genomic DNA from *R. nigromaculata* were cloned into pBluescript II SK(+) and then subcloned into pUC19. Several series of deletion mutants were produced from this pUC19 subclone using a deletion kit (TaKaRa) according to the manufacturer's instructions. Once the cloned DNAs were purified by the alkaline lysis

method (Davis et al., 1986) for DNA sequencing, the sequencings were performed on an automated DNA sequencer (ABI) by the DyeDeoxy termination method. Several clones were sequenced from the 5' and 3' sides, including one that was found to contain the rRNA precursor. Both strands of the 4.8kbp *EcoR* I fragment containing the rDNA precursor were completely sequenced using various deleted subclones (total 99 clones). A DNA sequence analyzed using DNASIS Ver. 3.2 (Hitachi Software Engineering) revealed the nucleotide sequences of the 18S, 5.8S, and 28S rRNA genes and two ITS regions. These genes and ITS regions were aligned with counterparts in other animals using ClustalW (Thompson et al., 1994), and the alignment data were used for the current analyses. The DNA sequence of *Rana nigromaculata* was deposited in the DDBJ database (accession number AB099628).

Window analysis. A window analysis was carried out to evaluate the variable and conserved regions within the 28S rRNA gene. Initially, we created alignment data on the 28S rRNA genes of human, *Rana*, and two *Xenopus* species. The alignment contained 4,533 nucleotide sites, and the inclusion of deletion sites among them resulted in an increased length exceeding that of the actual sequenced 28S rRNA gene. Next, the nucleotide diversity (Pi: equation 10.5 or 10.6 in Nei, 1987) per 5 nucleotide sites (= 1 window) was calculated using a computer program, DnaSP Ver. 3.52 (Rozas and Rozas, 1999). The Pi values were plotted along the sequence alignment. We also checked the plot patterns at another set of window sizes (1, 3, 10 and 20 nucleotide sites) and obtained the similar pattern at each setting. Thus, we showed only the first result (1 window = 5 nucleotide sites) to simplify the description (see Fig. 3).

Phylogenetic analysis and substitution rate of the 28S rRNA gene. We conducted a phylogenetic analysis based on the 28S rRNA gene and examined the substitution rate of the sequences among anurans. In the following analyses we used 3,504 alignment sites, excluding all sites deleted from the alignment data mentioned above. Based on the alignment, genetic distances among the 28S rRNA genes of frogs and human were calculated by Kimura's two-parameter method (Kimura, 1980), and the phylogenetic tree was reconstructed by the neighbor-joining (NJ) method (Saitou and Nei, 1987) using a computer program package, PHYLIP Ver. 3.5 (Felsenstein, 1993) and ClustalW (Thompson et al., 1994). Based on the resultant tree topology, the rate homogeneity for nucleotide substitutions among frogs was tested by Tajima's method (Tajima, 1993) using MEGA2 (Kumar et al., 2001). To calibrate evolutionary time, we also conducted branch length linearization (see section 9 in Nei and Kumar, 2000) based on the resultant NJ tree by the

Fig. 1. Scheme of the sequenced ribosomal precursor unit of *R. nigromaculata*. ITS1 and ITS2 denote first and second internal transcribed spacers, respectively. The locations of the restriction sites for *EcoR* I, *Xba* I, *Kpn* I, *Sal* I, *BamH* I and *Sac* I are shown.

Table 1. Nucleotide sequence length (bp) in different segments of the ribosomal precursor unit of *R. nigromaculata*, *X. laevis*, *X. borealis* and human.

Segment	<i>R. nigromaculata</i>	<i>X. laevis</i> ^a	<i>X. borealis</i> ^a	Human ^b
5'-ETS	—	712	612	3656
18S	230*	1826	1826	1871
ITS1	506	557	554	1095
5.8S	162	162	162	157
ITS2	343	262	336	1155
28S	3577*	4115	4128	5035
3'-ETS	—	—	—	381
Total	4818	7634	7618	13350

*not complete (partial). ^aData from Ajuh et al. (1991). ^bData from Gonzalez and Sylvester (1995).

Table 2. Nucleotide composition in different segments of the ribosomal precursor unit of *R. nigromaculata*, *X. laevis*, *X. borealis* and human.

Segment	<i>R. nigromaculata</i>				<i>X. laevis</i> ^a				<i>X. borealis</i> ^a				Human ^b			
	%A	%G	%C	%T	%A	%G	%C	%T	%A	%G	%C	%T	%A	%G	%C	%T
5'-ETS	—	—	—	—	10.7	37.2	45.9	6.2	10.0	37.1	44.3	8.7	5.7	39.7	39.6	15.1
18S	22.0*	30.2*	25.9*	22.0*	23.7	28.3	25.5	22.5	23.8	28.2	25.5	22.5	22.4	29.4	26.7	21.5
ITS1	15.8	39.1	38.9	6.1	12.4	41.3	42.9	3.4	15.5	38.8	39.7	6.0	7.7	40.3	39.4	12.7
5.8S	19.1	30.2	30.2	20.4	18.5	29.6	30.2	21.6	18.5	29.6	29.6	22.2	19.7	28.7	28.7	22.9
ITS2	7.3	36.7	45.5	10.5	5.0	35.1	53.1	6.9	10.4	37.5	44.3	7.7	5.1	40.0	43.0	11.8
28S	18.3*	36.0*	30.7*	15.0*	18.4	35.5	30.2	16.0	18.6	35.2	30.1	16.1	15.9	36.0	33.2	14.9
3'-ETS	—	—	—	—	—	—	—	—	—	—	—	—	3.1	40.9	40.9	15.0

*not complete (partial). ^aData from Ajuh et al. (1991). ^bData from Gonzalez and Sylvester (1995).

Table 3. Percent nucleotide sequence divergences of ribosomal RNA genes among *Rana*, *Xenopus* and human.

Combination	5.8S rRNA (162bp)	18S rRNA (230bp*)	28S rRNA (3571bp*)
<i>R. nigromaculata</i> - <i>X. laevis</i> ^a	3.2	0.4	5.1
<i>R. nigromaculata</i> - <i>X. borealis</i> ^a	3.8	0.4	5.3
<i>R. nigromaculata</i> - Human ^b	4.6	2.7	9.5
<i>X. laevis</i> ^a - Human ^b	2.6	2.2	8.8
<i>X. borealis</i> ^a - Human ^b	1.9	2.7	9.1
<i>X. laevis</i> ^a - <i>X. borealis</i> ^a	0.6	0.4	0.9

*not complete (partial). ^aData from Ajuh et al. (1991). ^bData from Gonzalez and Sylvester (1995).

LS method using the Kitsch program in PHYLIP.

Sequence data and phylogenetic analysis based on the 5.8S rRNA gene. The 5.8S rRNA gene sequences used in the phylogenetic analyses were from the 35 vertebrate species shown in Figure 6. The method used to reconstruct the phylogenetic tree was the same as that used for the 28S rRNA genes. The nucleotide sequence

of *Giardia intestinalis* (Protista) was used as an outgroup to construct the phylogenetic tree.

RESULTS

Gene content and gene arrangement of the sequenced ribosomal precursor unit of *R. nigromaculata*. The whole *EcoR* I fragment of the ribosomal precursor

A

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1                                     60
R. nigromaculata GAATTC-CCAGTAAGTGC GGTCATAAGCTCGCGTTGATTAAGTCCCTGCCCTTTGTACA
X. laevis         GAATTC-CCAGTAAGTGC GGTCATAAGCTCGCGTTGATTAAGTCCCTGCCCTTTGTACA
X. borealis      GAATTC-CCAGTAAGTGC GGTCATAAGCTCGCGTTGATTAAGTCCCTGCCCTTTGTACA
Human            GAATTCCCAGTAAGTGC GGTCATAAGCTTGCGTTGATTAAGTCCCTGCCCTTTGTACA
                ***** * *****

61                                     120
R. nigromaculata CACCGCCCGTCGCTACTACCGATTGGATGGTTTAGTGAGGTCCTCGGATCGGCCCCGCCG
X. laevis         CACCGCCCGTCGCTACTACCGATTGGATGGTTTAGTGAGGTCCTCGGATCGGCCCCGCCG
X. borealis      CACCGCCCGTCGCTACTACCGATTGGATGGTTTAGTGAGGTCCTCGGATCGGCCCCGCCG
Human            CACCGCCCGTCGCTACTACCGATTGGATGGTTTAGTGAGGCCCTCGGATCGGCCCCGCCG
                *****

121                                    180
R. nigromaculata GGGTCGGCG-ACGGCCCTGGCGGAGCGCCGAGAAGACGATCAAACCTTGACTATCTAGAGG
X. laevis         GGGTCGGCC-ACGGCCCTGGCGGAGCGCCGAGAAGACGATCAAACCTTGACTATCTAGAGG
X. borealis      GGGTCGGCA-ACGGCCCTGGCGGAGCGCCGAGAAGACGATCAAACCTTGACTATCTAGAGG
Human            GGGTCGGCCACGGCCCTGGCGGAGCGCTGAGAAGACGGTCGAACCTTGACTATCTAGAGG
                *****

181                                    232
R. nigromaculata AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATT
X. laevis         AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATT
X. borealis      AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATT
Human            AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATT
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B

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1                                     60
R. nigromaculata GCAAGACTCTTAGCGGTGGATCACTCGGCTCGCGCGTCGATGAAGAACGCAGCTAGCTGC
X. laevis         TCGCGACTCTTAGCGGTGGATCACTCGGCTCGTGCGTCGATGAAGAACGCAGCTAGCTGC
X. borealis      TCGCGACTCTTAGCGGTGGATCACTCGGCTCGTGCGTCGATGAAGAACGCAGCTAGCTGC
Human            ---CGACTCTTAGCGGTGGATCACTCGGCTCGTGCGTCGATGAAGAACGCAGCTAGCTGC
                *****

61                                     120
R. nigromaculata GAGAATTAGTGTGAATTGCAGGACACATTGATCATCGACACTTCGAACGCACCTTGCGGC
X. laevis         GAGAATTAGTGTGAATTGCAGGACACATTGATCATCGACACTTCGAACGCACCTTGCGGC
X. borealis      GAGAATTAGTGTGAATTGCAGGACACATTGATCATCGACACTTCGAACGCACCTTGCGGC
Human            GAGAATTAATGTGAATTGCAGGACACATTGATCATCGACACTTCGAACGCAC-TTGCGGC
                *****

121                                    162
R. nigromaculata CCCGGGTTCCCTCCCGGGGCGCGCCTGTCTGAGGGTCGCTCC
X. laevis         CCCGGGTTCCCTCCCGGGGCCACGCCTGTCTGAGGGTCGCTCC
X. borealis      CCCGGGTTCCCTCCCGGGGCTACGCCTGTCTGAGGGTCGCTCC
Human            CCCGGGTTCCCTCCCGGGGCTACGCCTGTCTGAGCGTCGCTT-
                *****

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C

	1		60
<i>R. nigromaculata</i>	GATTGGCTCTAAGGGCTGGGTTCGGTCGGGCTGGGGCGCGAAGCGGGGCTGGGCGCGCGCC		
<i>X. laevis</i>	GATTGGCTCTAAGGGCTGGGTTCGGTCGGGCTGGGGCGCGAAGCGGGGCTGGGACGCGCC		
<i>X. borealis</i>	GATTGGCTCTAAGGGCTGGGTTCGGTCGGGCTGGGGCGCGAAGCGGGGCTGGGACGCGCC		
Human	GATTGGCTCTAAGGGCTGGGTTCGGTCGGGCTGGGGCGCGAAGCGGGGCTGGGCGCGCGCC		
	*****		*****
	61		120
<i>R. nigromaculata</i>	GCGGCTGGACGAGGCGC-----C-----GCCCCCGCC		
<i>X. laevis</i>	GCGGCTGGACGAGGCGC-----CCC GGCGGCCTCCCTC		
<i>X. borealis</i>	GCGGCTGGACGAGGCGC-----CCC GGCGGCCTCCCTC		
Human	GCGGCTGGACGAGGCGCGCGCCCCCCCCACGCCCGGGGCACCCCCCTCGCGGCCTCCCC		
	*****		* *** *
	121		180
<i>R. nigromaculata</i>	CCCCC-----ACCGTCC---TCGCCC---		
<i>X. laevis</i>	CTCCC-----GCCCCC-TCTCTCTC---		
<i>X. borealis</i>	CTCCC-----GCCCCCCTCTCTCTC---		
Human	CGCCCCACCCGCGCGCGCCGCTCGCTCCCTCCCCACCCGCGCCCTCTCTCTCTCTCTCT		
	* **		** * ** *
	181		240
<i>R. nigromaculata</i>	-----CCGCCTCCCCCCTCCCCGGGGG-----GGCGAGGCG		
<i>X. laevis</i>	-----CGG-----CCCCCCTCGCGGGGGG-----CCGCGGG		
<i>X. borealis</i>	-----CGGTCCCCCCTCGCGGGGGGGG-----GGCGGGGCG		
Human	CCCCCGCTCCCCGTCTCCCCCCTCCCCGGGGGAGCGCCGCGTGGGGGCGCGGGGGGGG		
	* * ***** *		* * *
	241		300
<i>R. nigromaculata</i>	-----GGCGGCCGGGGCCG--GGCGGCGG--GGCGCGGGC-----		
<i>X. laevis</i>	-----GGCGGGGGGGGCGCG--GGGGGCCG--GGAGCCGCCCGC-----		
<i>X. borealis</i>	-----GGCGGGGGGGGACGCGGGGGGGGCG--GGAGCCGCCCGC-----		
Human	AGAAGGGTCGGGGCGGCGAGGGGCGCGCGGGCGGCCCGCGGGGCGGCCGGCGGGGGCAGGT		
	***** ** *		** ** *
	301		360
<i>R. nigromaculata</i>	-----GGCGGCGGCG--ACTCTGGACGCGC		
<i>X. laevis</i>	-----CGGCGGCGGCG--ACTCTGGACGCGC		
<i>X. borealis</i>	-----CGGCGGCGGCG--ACTCTGGACGCGC		
Human	CCCCGCGAGGGGGGCCCCGGGGACCGGGGGGCGGCGGCGGCGGACTCTGGACGCGA		

	361		400
<i>R. nigromaculata</i>	GCCGGGCCCTTCCCGTGGATCGCCCCAGCTGCGGCGGGCG		
<i>X. laevis</i>	GCCGGGCCCTTCCCGTGGATCGCCCCAGCTGCGGCGGGCG		
<i>X. borealis</i>	GCCGGGCCCTTCCCGTGGATCGCCCCAGCTGCGGCGGGCG		
Human	GCCGGGCCCTTCCCGTGGATCGCCCCAGCTGCGGCGGGCG		
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Fig. 2. Aligned sequences of a 3'-side 232-bp segment of the 18S rRNA gene (A), a complete 162-bp segment of the 5.8S rRNA gene (B), and 400-bp segment of the 28S rRNA gene (C) of *R. nigromaculata*, *X. laevis*, *X. borealis* and human. Asterisks indicate nucleotide sequences that are conserved among four taxa. The 28S rRNA gene sequence of *R. nigromaculata* corresponds to nucleotide sites 2391-2621.

unit of *R. nigromaculata* was 4,818 bp in length and contained the following segments: 5' – 18S rRNA – ITS 1 – 5.8S rRNA – ITS 2 – 28S rRNA – 3' (Fig. 1, Table 1). The 3'-side segment of the 18S rRNA gene was 230 bp and the 5'-side segment of the 28S rRNA gene was 3,577 bp. The 5.8S rRNA gene was completely sequenced with a length of 162 bp. The ITS 1 and ITS 2 were 506 bp and 343 bp,

respectively. While these ITS segment lengths were not different from those of the two *Xenopus* species, they were very different from those of human.

Base composition and nucleotide divergences. Table 2 shows the nucleotide compositions in different segments of the ribosomal precursor unit. The frequen-

cies of nucleotides G and C were 56–67% in the segments of the three genes, whereas they were more than 78% in the two ITS segments. The ITS regions of *Rana* had a high nucleotide bias, as did those of the two *Xenopus* species for ribosomal regions, where the secondary structure requires the formation of strong, double-stranded stems due to the stronger hydrogen bonding of the base pairs formed by G and C (Larson and Wilson, 1989). The nucleotide sequence divergences between *Rana* and *Xenopus* were 3.2–3.8%, 0.4% and 5.1–5.3% in 5.8S, 18S and 28S rRNA genes, respectively, and those between human and *Rana* or *Xenopus* were 1.9–4.6%, 2.2–2.7% and 8.8–9.5% in 5.8S, 18S and 28S rRNA genes, respectively (Table 3). In the 5.8S rRNA gene, the nucleotide sequence divergences between *Rana* and *Xenopus* (3.2–3.8%) were larger than those between *Xenopus* and human (1.9–2.6%). The large divergences between two frog genera were caused by a few nucleotide changes found only in *Rana* (i.e., autapomorphic substitutions). Because the length of 5.8S rRNA gene is very short (approx. 160 bp), a slight number of autapomorphic changes affect the divergence values. Thus, the values of nucleotide sequ-

ence divergences among frogs and human shown here do not represent directly their phylogenetic relationships (The 5.8S rRNA sequence data support the monophyly of anurans; see below and Fig. 6). Finally, the nucleotide sequences in the ITS regions of human could not be aligned with those of *Rana* and *Xenopus*.

Comparison of the nucleotide sequences of *Rana* with those of *Xenopus* and human.

(i) **18S rRNA gene.** Nucleotide sequences were determined in a 230-bp segment of the 3' end of the 18S rRNA gene (Fig. 2A). There was no variation between the sequenced 230-bp segment of *Rana* and that of *Xenopus*, whereas 7 nucleotide substitutions and 2 gap sites were identified in the comparison between *Rana* and human.

(ii) **5.8S rRNA gene.** The nucleotide sequences of the 5.8S rRNA gene were aligned in the complete 162-bp segment among *Rana*, the two *Xenopus* species, and human (Fig. 2B). Five nucleotide substitutions were found between *Rana* and *Xenopus*, and 4 gap sites were identified in the comparison between *Rana* and human. (Fig. 2B).

Fig. 3. Distribution of conserved and variable regions and the window analysis in the 28S rRNA genes of *R. nigromaculata*, *X. laevis*, *X. borealis* and human. (A) The variable regions deduced from sequencing are shown as black tracts (V–V10), and the conserved regions are in white. These tracings are drawn in the 5'→3' orientation from the 28S rRNA segments shown in Ajuh et al. (1991) (*X. laevis* and *X. borealis*) and Gonzalez and Sylvester (1995) (human). Thin bars show lengthy deletions (> 20 bases). Asterisks indicate the lengthy deleted region only shared in *Rana* and Human. (B) Window analysis. The genetic diversities (Pi) per 5 nucleotide sites are indicated along the nucleotide sequences. The portions corresponded to the variable regions and lengthy deletions are indicated by thick and thin bars, respectively. Deleted nucleotides were not counted as nucleotide changes, hence the Pi of the portions with lengthy deletions are low.

(iii) 28S rRNA gene. Based on nucleotide homology, the 3,577-bp segment of the 28S rRNA gene of *Rana nigromaculata* corresponded to sites 1-3608 in the 28S rRNA gene sequence of *Xenopus laevis* and sites 1-4453 in that of human. The 28S rRNA gene segment of *Rana* was aligned with the counterparts of the two *Xenopus* species and human (Figs. 2C and 3A). Compared with the 28S rRNA gene of human, that of *Rana* had an abundance of lengthy (> 20 bases) deletions (thin bars in Fig. 3A). As most of these deletions were also observed in the two *Xenopus* species, the lengths of the 28S rRNA genes of the three amphibian species were much shorter than that of human. At the same time, however, it should be noted that the two deleted regions indicated by asterisks were only common in *Rana* and human (Fig. 3A). These sites could have been caused by nucleotide insertion only in the *Xenopus* lineage. A previous investigation showed that the 28S rRNA gene of vertebrates had 11 “variable regions” with numerous polymorphic and gap sites between human and *Xenopus* (Ajuh et al., 1991). When examined under electron microscopic, these regions were thought to correspond to the loop regions in secondary structures of 28S rRNA (Ajuh et al., 1991). In the present study, a window analysis was carried out to assess the presence of these variable regions in the 28S rRNA gene of *Rana*. Based on the result of this window analysis (Fig. 3B), the nucleotide diversities (P_i , see Nei, 1987) were plotted in each window (5 nucleotide sites) along the 28S rRNA gene alignment. Similar plot patterns were obtained when another set of window sizes (1, 3, 10, 20 nucleotide sites) was attempted (data not shown). The analysis showed 9 regions where the nucleotide sites with high diversities were concentrated (>0.2). The positions of these regions closely corresponded with those of the “variable regions (V1, 2a, 2b, 3, 5, 7, 8, 9, and 10)” previously reported. Though the values of nucleotide divergences were somewhat low (< 0.2), a throng of polymorphic sites were also packed into the sites corresponding to the remaining two variable regions (V4 and V6). Furthermore, lengthy deletions observed in the 28S rRNA gene of *Rana* were concentrated in the highly polymorphic regions (V2a, 2b, 5, 7, 8 and 9). These results showed that the “variable regions” observed in human and *Xenopus* are also present in the 28S rRNA gene of *Rana* (Fig. 3B). Thus, the secondary structure of the 28S rRNA of *Rana* seemed to be similar to those of the other known vertebrates.

(iv) ITS regions. There were seven short tracts of completely or almost completely conserved sequences embedded in the largely divergent sequences of the two ITS regions, ranging in length from 5 to 27 nucleotides (Fig. 4). Two of the seven were embedded in the ITS 1 (Fig. 4A) and the other five were embedded in the ITS 2 (Fig. 4B). Marked tendencies towards ‘simple sequence’ patterns such as homopolymeric tracts, pyrimidine tracts,

and purine tracts were observed (Fig. 4).

Phylogenetic analysis.

(i) 28S rRNA gene. Although partial sequences of the 28S rRNA gene have been reported in many amphibians, the complete sequences of this gene are known in only two *Xenopus* species. To examine the usefulness of the 28S rRNA gene sequences for evolutionary studies of anurans, we conducted a phylogenetic analysis by adding the long 28S rDNA sequence of *Rana*. Figure 5 shows the NJ tree based on the 3,504 alignment sites of the 28S rRNA gene. As shown in this figure, the long 28S rRNA gene sequence data reconstructed the monophyly clade of *Xenopus* with a high BP value (1000) and deep divergence between *Rana* and *Xenopus*. Moreover, the branch lengths of the tree seemed to be equal between the frog species, suggesting that the rate of the 28S rRNA gene was constant in these animals. The relative rate test (Tajima, 1993) was used to investigate this likelihood. For all tested frog lineages, the rate constancy hypothesis did not reject with high statistical values (Table 4).

(ii) 5.8S rRNA gene. The nucleotide sequence divergences of the 5.8S rRNA gene were calculated among various phyla to evaluate the utility of this gene for phylogenetic analysis. The nucleotide sequence divergences of the 5.8S rRNA gene were 3.2~3.8% between two frog genera (*Rana* and *Xenopus*), 4.0~4.6% between *Rana* and mammals, 4.6~12.2% between *Rana* and fishes, 17.0~34.3% between *Rana* and invertebrates, and 60.3~70.3% between *Rana* and Protista. The mean sequence divergences of the 5.8S rRNA gene within kingdoms were 62.9% in Protista and 21.0% in Animalia, and those within individual phyla were 6.1~7.5% in the three phyla Platyhelminthes, Mollusca, and Chordata, and 29.7% in the phylum Arthropoda (Table 5). The mean sequence divergences of the 5.8S rRNA gene were 67.9% between the two kingdoms Protista and Animalia, 26.8% among the four invertebrate phyla, and 26.1% between the phylum Chordata and the four invertebrate phyla (Table 5). The phylogenetic analysis was carried out using the nucleotide sequences of the 5.8S rRNA gene of various taxa. A phylogenetic tree based on the complete set of nucleotide sequences of the 5.8S rRNA gene showed that each phylum formed a cluster, and that the divergences within the Protista, invertebrates, and Chordata were the largest, medial, and smallest, respectively (Fig. 6).

DISCUSSION

The ITS regions. The internal transcribed spacer (ITS) regions diverge extensively among the one *Rana* and two *Xenopus* species, but several short sequences that seem to be important in function are conserved among these species. As discussed previously, a difference noted in

the spacing between the boxed tracts (see Fig. 4) among the one *Rana* and two *Xenopus* species implies evolutionary divergence by insertions and deletions in the intervening ITS tracts (Furlong and Maden, 1983; Furlong et al., 1983). The four tracts in the ITS 2 are well conserved between the two *Xenopus* and one *Rana* species. Underlined sequences of *Rana* denote tracts of 10 or more

nucleotides in the spacers consisting of purines only, pyrimidines only, or a single type of base. These simple sequence tracts are not conserved in the two *Xenopus* species. When we try to account for their conservation in *Rana*, it remains unclear whether they have been left intact merely by chance or whether they are under some degree of functional constraint. Large parts of the tran-

A

	1	60
<i>X. borealis</i>	ACGAGAGAG-----	
<i>X. laevis</i>	ACGAGA-----CCCCCTCACCCGGAGAGAGG	
<i>R. nigro.</i>	<u>CCGAGAGAGACAGGGAGGCGGCCCGCCGGCCCGTCCTCGCCTCGTCCGACCCGAGACCCC</u> *****	
	61	120
<i>X. borealis</i>	-----GGCGAGAGGCTCACG	
<i>X. laevis</i>	AAGGCGCCCGCGCACCCCTCCCCGCGGAGAGAGAGAGACGCCCGCCCGGAGCGGAGA	
<i>R. nigro.</i>	CACGAGCCGCGGGGCGCGTGGGAGAGGGAGGCGCGGGCGCGCGGCCCCCGGCGCCC	*
	121	180
<i>X. borealis</i>	TGCGAGAGCGCGGCTCTGCGCGACCCCCGCCACCGTCCCGGCTCCCCCGCGGACGACGA	
<i>X. laevis</i>	CGCCCCCCCCCACGGGGGGGGGGCGGCCGCCCGAAAGGGACGACGAGGAACCCCCAGA	
<i>R. nigro.</i>	CTCCCCCGCAACCAGCCCCGCGGGACCCAGAGGAACGCGCGCGGGGGAGGGGGAGGCGC	* * *
	181	240
<i>X. borealis</i>	AGGTCCGGGCGCGGGCTCCCGCCCGCCCCGACACGAGGAGAGAGCGGAAAAGGAACAACA	
<i>X. laevis</i>	GGCCCCGGCGAGGGGGGCGCGGCGGCCCGGTCCACCCCGGGCCCGCCCGCCCGCTCC	
<i>R. nigro.</i>	GGAGTCGCGGCGAGGGGGGGGGCATCCCCCTCCGCGCCCGGCACCCGCGAGCCCTCCC	* ** * *
	241	300
<i>X. borealis</i>	CAAAAAAACGAGCCCGGGTACCTAGCCGGGGGGGGGCGAGGGCGGGGACGGGGGGCAGA	
<i>X. laevis</i>	CGCCGCGGGCCCGCCCGGGTACCTAGCCGGGGCGGGGGCGCGGGGGCTGGCGCGGGAGCG	
<i>R. nigro.</i>	GCCGGCAGGCCGAGCCGGGTACCGCTCGGGCCCGCGTCCGCCCCGACCCGGGGGGGGCGG	* * * * *
	301	360
<i>X. borealis</i>	GGC-----CCTCCGACCCCCCTCCCTCCTCCCGGGCGGT	
<i>X. laevis</i>	GGCGGCCCCAGGGCCGTCCGGCCTCCCCGCGTCCGCTCCCGGACCCCGCCGGGCGGT	
<i>R. nigro.</i>	AGGGGCCCGGAAGGTTTGAAGCCTCGAGACGGGAGCCCTTCGCGGGGGCTCCCGGCC	* ** *
	361	420
<i>X. borealis</i>	CGAAGACCCCGGCCACGGCCGGCGGCGGCGACGAGGGGCGAGCCG--TCCGGAGGCGGG	
<i>X. laevis</i>	CGAAGACCCCGCCCGCCGGGCGGCGGGAGGGCCGGGAGG--GAGCCGGGGAGGG--GAGGG	
<i>R. nigro.</i>	AGCGCCCGGACGCTCACGGCCGGTAGGGG--CGGGCCCGCCCGGTTCG-----	* * * * *
	421	480
<i>X. borealis</i>	GG--CGGGCGCGGAGGGGGGACGGGGGGGAGGCGGAGGTACGGCCGGACGCGGCCGCGTC	
<i>X. laevis</i>	GGGAGGCGGCGGCGAGCCCGGCCGGGCGCGCGCGCGCAGGACCCCCGTCCCCGTCCCGC	
<i>R. nigro.</i>	-----	
	481	540
<i>X. borealis</i>	CTCCGCCCCCCCCCCCCGTCTGTCCGCGCCCCGCCCCGCGCGCGGTTCGCGTCAGTCC	
<i>X. laevis</i>	CCGCCCCCGCCGGCCC-----	
<i>R. nigro.</i>	-----	
	541	600
<i>X. borealis</i>	CGCCGCCCCGTCGGCGGCGCCCCGGGGCGCCCGGGGCCCCGGGGGCCCCCGTCCAGCT	
<i>X. laevis</i>	-----GGGGCGCCCGGGCCGC	
<i>R. nigro.</i>	-----ACCGCGGCGGCCCCGCTCGGC	** *
	601	660
<i>X. borealis</i>	CCACGGCGCCCGCGCGCGCGCGGTACCCCTGCCGCGA--GGCAACGAGA--GAGAAAAACAA	
<i>X. laevis</i>	ACCGCCTCAGCGGCAGCACCGGTAGCCCTGCCGAGACCGAAAAGGAAAACCGACCGACGC	
<i>R. nigro.</i>	CAGGGCGAGAAACCACGACGTCCCCCTTGTCGACCT--TACCGAAAGGAAAAAAAAAAAA	* * * * *
	661	
<i>X. borealis</i>	CGAAAAAAATC	
<i>X. laevis</i>	TCGGCGAGAGC	
<i>R. nigro.</i>	CGAAAAAA--GA	*

B

	1	60
<i>X.borealis</i>	GACGTCCATCGCCCCCGCCGGGTCCC	CGACCCGGGGAAAACGGGC
<i>X.laevis</i>	GACGTCCATCGCCCCCGCCGGGTCCC	GTCCCGG-----CGCGGAGGCGCGGCTGGGGCC
<i>R.nigro.</i>	TCCGTTCGGTTCGCCCC-GCCGGG-----	CGCAGCTGGGGCG
	****	*****
	61	120
<i>X.borealis</i>	TTCGAGGGGTCGAGGGACGCCCCCACC GCCCTTCCGGGGGAGGTGAGGGGGCCCCGC	
<i>X.laevis</i>	TTCGAGGGGTCGAGGGACGCCCCCACC GCCCTTCCGGGGGAGGTGAGGGGGCCCCGC	
<i>R.nigro.</i>	TTCGAGGGGTCGAGGGACGCCCCCACC GCCCTTCCGGGGGAGGTGAGGGGGCCCCGC	

	121	180
<i>X.borealis</i>	-----	ACCCCTTCGTC
<i>X.laevis</i>	-----	CTCCCTTCGTC
<i>R.nigro.</i>	TCCGGGGCCCCGCGCCCGACGCCGCGCCCTCCTCCCTCTCCCGCCGGG	CCCTTCGTC

	181	240
<i>X.borealis</i>	CCCCAAGGCCAGACCCCGACCATCCCGGCGGCCCCCCC-----	
<i>X.laevis</i>	CCCCAAGGCCAGACCCCGGCCCCGCGCCCGGGCCCCCG-----	
<i>R.nigro.</i>	CCCGAAGGCCAGACCCCTTCGCGCTCGGGGTCCCCCTCCGCGAGGGCTCCGTCCCCCT	
	***	*****
	241	300
<i>X.borealis</i>	-----CAGGGCCCCGGCGCGGCTGTCTGTGGGAAACCGAT	TTCACGGCTGCCGCGCGCG
<i>X.laevis</i>	-----CCCGGCCGGCG-GCGGCTGTCTGTGGATCCC-----	TTCACGGCTGCCGCCCCGG
<i>R.nigro.</i>	CTCCCG-AAGGGGGCCGCGCGGCTGTCTGTGGCTCA--GGC	AGCAGGGCTGACCGCGCGG
	**	*****
	301	360
<i>X.borealis</i>	GGGCGCTGGGGACCC-----GCCGGGGCGGGAAGAGCCCCGGGCGGGCCGGGGCCGGGGA	
<i>X.laevis</i>	CGGCCCCCGGGGCCCCGGCCCCGCGGCGGGAGCGGGCCCGGC-----	
<i>R.nigro.</i>	CCCCGC-----CCCTCGGGCGCGGGAGACGGC-----	
	* *	* *
	361	413
<i>X.borealis</i>	GGCGCGCTTGAACCCGCGCCCCCCCCGCGTCCCCCGCCCGCCCCCGCT	CGAC
<i>X.laevis</i>	-----CCCCCCCCCGGGCCGCGCCCCGCGCCCCCCCCCCCCACGAC	
<i>R.nigro.</i>	-----TCCCCCTCCGCGCCGCGGGCGGGCCCCC-----	AACTCTCCGAC
	***	*****

Fig. 4. Aligned sequences of internal transcribed spacers ITS1 (A) and ITS2 (B) of *R. nigromaculata*, *X. laevis* and *X. borealis*. Boxes denote nucleotide sequences that are conserved between two *Xenopus* species. Asterisks indicate the nucleotides that are conserved among three taxa. Lines indicate tracts of eight or more nucleotides of which show strong bias towards a single type of base.

Fig. 5. A phylogenetic tree constructed by the NJ method based on the nucleotide sequence divergences of the 28S rRNA gene among *R. nigromaculata*, *X. laevis*, *X. borealis* and human. The 3,504 sites without gaps were used for phylogenetic tree reconstruction, and human was used as an outgroup. The scale bar represents branch length in terms of nucleotide substitutions per site. The branch lengths of linearized tree were indicated above nodes. The bootstrap value (1000) of *Xenopus* lineage was calculated by 1,000 replicates.

Table 4. Tajima's rate test (Tajima, 1993) for the 28S rRNA gene of frogs.

Species			Chi-square*	<i>P</i>
Outgroup	Ingroup			
Human	<i>R. nigromaculata</i>	<i>X. laevis</i>	0.17	0.69
Human	<i>R. nigromaculata</i>	<i>X. borealis</i>	0.11	0.74
Human	<i>X. laevis</i>	<i>X. borealis</i>	0.07	0.80
<i>R. nigromaculata</i>	<i>X. laevis</i>	<i>X. borealis</i>	0.00	1.00

*The Chi-square value has 1 degree of freedom. When the value is >3.841, the constant rate hypothesis between the pair of sequences is rejected by $P=0.05$ level. All tested pairs of the 28S rRNA gene sequences did not reject the hypothesis.

Table 5. Percent nucleotide sequence divergences of the 5.8S rRNA gene among 35 species at the various taxonomic levels.

Taxa (No. of phylum or class / No. of species)			Nucleotide sequence divergences	
			Range	Average
Within kingdom	Protista	(2 / 3)	25.8 ~ 86.9	62.9
	Animalia	(5 / 32)	0 ~ 48.2	21.0
Within phylum	Platyhelminthes	(1 / 12)	0 ~ 12.9	7.5
	Mollusca	(1 / 4)	2.0 ~ 11.1	6.1
	Arthropoda	(2 / 3)	1.8 ~ 44.4	29.7
	Echinodermata	(1 / 1)	—	—
	Chordata	(3 / 12)	0 ~ 17.6	6.9
Between kingdoms	Protista - Animalia		59.9 ~ 85.1	67.9
Between phyla	Invertebrata (4 phyla)		15.1 ~ 40.0	26.8
	Invertebrata (4 phyla) - Chordata		14.1 ~ 48.2	26.1

scribed spacers evidently undergo rapid phylogenetic change, including insertions and deletions.

The homology in the conserved tracts and their occurrence in the same linear order in the ITS 2 of *Xenopus* and *Rana* (Fig. 4B) indicate that these tracts are indeed

the unchanged remnants of common ancestral ITS sequences, as proposed by Furlong and Maden (1983). The considerable interspecific and intergeneric differences in spacing between some of the conserved tracts (Fig. 4B) may indicate a history of insertions and / or deletions and

Fig. 6. A phylogenetic tree constructed by the NJ method based on the nucleotide sequence divergences of the 5.8S rRNA gene among 35 species belonging to two kingdoms. The 5.8S rRNA gene sequences used in the phylogenetic analyses are from the following 35 species: *Giardia intestinalis* (Protista; X05396; Boothroyd et al., 1987), *Tetrahymena thermophila* (Protista; X54512; Engberg and Nielsen, 1990), *Nyctotherus cordiformis* (Protista; AJ009574; van Hoek et al., unpublished), *Poracanthium furcatum* (Digenia; AJ241791; Jousson et al., 1999), *Ixodes scapularis* (black-legged tick; L22276; Wesson et al., 1993), *Bombyx mori* (domestic silkworm; M36302; Fujiwara et al., 1987), *Samia cynthia* (ailanthus silkworm; M10367; Feng et al., 1982), *Lytechinus variegatus* (green urchin; X00350; Hindenach and Stafford, 1984), *Oncomelania hupensis* (Mollusca; U93226; Lo, unpublished), *Arianta arbustorum* (Mollusca; AF124052; Lo, unpublished), *Arion rufus* (Mollusca; X00131; Ursi et al., 1983), *Stagnicola caperata* (Mollusca; AF013140; Remigio and Blair, 1997), *Poracanthium furcatum* (Digenia; AJ241791; Jousson et al., 1999), *Nicolla elongata* (Digenia; AJ241792; Jousson et al., 1999), *Cainocreadium labracis* (Digenia; AJ241795; Jousson et al., 1999), *Pseudopycnadena fishtali* (Digenia; AJ241798; Jousson et al., 1999), *Gaeuskajatrema perezii* (Digenia; AJ241800; Jousson et al., 1999), *Macvicaria crassigula* (Digenia; AJ241803; Jousson et al., 1999), *Pachycreadium carnosum* (Digenia; AJ241799; Jousson et al., 1999), *Macvicaria alacris* (Digenia; AJ241801; Jousson et al., 1999), *Schistosoma matthei* (Digenia; Z2171; Kane and Rollinson, 1994), *Plagiorchis vespertilionis* (Digenia; AF151949; Tkach et al., 2000), *Echinostoma trivolvis* (Digenia; AF067852; Sorensen et al., 1998), *Plagiorchis elegans* (Digenia; AF151952; Tkach et al., 2000), *Petromyzon marinus* (sea lamprey; AF061798; Mallatt and Sullivan, 1998), *Misgurnus fossilis* (loach; M28025; Kupriy-anova et al., 1984), *Cyprinus carpio* (carp; U87963; Vera et al., 1997), *Xenopus laevis* (African clawed frog; K01376; Ford and Mathieson, 1978), *Xenopus borealis* (Kenyan clawed frog; K01375; Ford and Mathieson, 1978), *Rana nigromaculata* (Japanese pond frog; AB099628; present study), *Hydrolagus collei* (spotted ratfish; AF061799; Mallatt and Sullivan, 1998), *Oncorhynchus keta* (salmon; D45855; Naganuma et al., unpublished), *Oncorhynchus mykiss* (rainbow trout; V01346; Nazar and Roy, 1978), *Mus musculus* (house mouse; K01365; Goldman et al., 1983), *Rattus norvegicus* (rat; V01270; Chan et al., 1984) and *Homo sapiens* (human; U13369; Nazar et al., 1976). Numbers on the tree represent local bootstrap values (above 600) from 1,000 replicates. The scale bar represents branch length in terms of nucleotide substitutions per site.

multiple changes in the intervening divergent regions.

In summary, the sequence contents of the divergent regions may have undergone extensive changes through the cumulative effects of many individual occurrences, including insertions, deletions, and point mutations.

The results of this study supported the following conclusions: 1) the rate of substitutions for the ITS regions was high (Li et al., 1985; Gillespie, 1986), thus making it difficult to compare sequences between *Xenopus* and *Rana*; 2) the short tracts in the ITS regions were strictly conserved between the two *Xenopus* species, and there were corresponding sequences for *Rana*. These results suggest that the conserved short tracts may be probably related to some important function such as the processing, although further examinations will be necessary for elucidating the function of these short tracts.

The coding regions. A comparison of the nucleotide sequences of the coding regions among *Rana*, human, and the two species of *Xenopus* revealed the following: (1) the 18S and 5.8S rRNA gene sequences were highly conserved among these taxa. (2) the regions corresponding to the stem and loop of the secondary structure of the 28S rRNA gene were conserved between *Xenopus* and *Rana*, and the rate of substitutions for the loop was higher than that for the stem. (3) The human loop regions had large insertions not seen in amphibians. Figure 3 summarizes the differences among the four 28S rRNA gene sequences and the distribution of these differences between the conserved and variable regions. According to the known data on the two *Xenopus* species, that overall rate of divergence in the variable regions can be estimated to have been at least 20-fold more rapid than that in the core regions (Ajuh et al., 1991). V8 corresponds to the 'double hairpin' seen by electron microscopy (Wellauer and Dawid, 1973; Wellauer et al., 1974; Wakeman and Maden, 1989). The variable regions, V2a, V5 and V8, of human are much longer than those in *Rana* and *Xenopus* due to large insertion sequences. By contrast, there is almost no length difference in these regions among frogs. The cumulative effects of insertions and deletions of sequence blocks in the variable regions could eventually generate a major length difference in the loop region of the gene found between *Xenopus/Rana* and human. Such length variation will make difficult to compare the nucleotide sequence between taxa. Thus, the variable regions in 28S rRNA gene are thought to be most informative for elucidating phylogenetic relationships among anuran families.

Phylogenetic utility of 28S and 5.8S rRNA gene sequences. Sequence studies of the rRNA genes from the conserved regions to the rapidly evolving regions, the so-called divergent domains or expansion segments, have proven useful for investigating the evolutionary diver-

gences that have occurred over the evolution of the metazoans (Hillis et al., 1996). The more conserved regions have been useful for examining relationships among major phyla (Hillis et al., 1996). The relatively variable regions within the rRNA genes make them useful for examining relationships within more closely related groups, such as various groups of vertebrates (Larson and Wilson, 1989; Hillis et al., 1991, 1993, 1996). The ITS regions are useful for examining relationships among closely related species.

The 28S rRNA gene contains regions with both rapid and slow nucleotide substitution rates, hence gene sequences of 28S rRNA have been frequently used in phylogenetic analyses for various organisms, from deep to recently diverged taxa. The present phylogenetic tree constructed from 28S rRNA gene sequences closely corresponded with the generally accepted phylogenetic relationship of these two frog groups (e.g. Kobel et al., 1998). This result may imply that this gene is also suitable for elucidating the phylogenetic relationships among extensive anuran families, including the primitive Pipidae and more evolved Ranidae. The present result also suggested that the substitution rate of the 28S rRNA gene was constant in the *Rana* and *Xenopus* species. The constant rate allowed us to estimate the divergence times of the frogs using a molecular clock of the gene sequence. Thus, we estimated the per-site number of nucleotide changes on each branch in the linearized tree by using the LS method (Fig. 5). Based on the values and a diverged time, 360 Myr, between amphibians and other tetrapods (see Kumar and Hedges, 1998), the nucleotide substitution rate, 0.117% per 10 Myr, was estimated. This scale indicated that the two *Xenopus* species diverged about 26 Myr ago, and that *Rana* and *Xenopus* diverged about 214 Myr ago. According to mitochondrial rRNA gene sequence data collected by Kobel et al. (1998), the two *Xenopus* species diverged about 30 Myr ago (close to our estimate of 26 Myr), while the two anuran families diverged about 130 Myr ago (much smaller than our estimate of 214 Myr). In the mtDNA data, multiple substitutions may have occurred between the two families (Sumida et al., unpublished), resulting in the estimation of a shorter divergence time. The constant rate between two families confirms that multiple substitutions may be excluded from the present data based on the nuclear rRNA gene. For this reason, we conclude that our present estimate for the divergence time may be reasonable.

The 5.8S rRNA gene region evolves relatively slowly, at a speed similar to that of the other coding regions, but its location within the ITS has precluded its use as anything but an alignment tool. Recently, however, this region of DNA was shown to contain considerable phylogenetic information, particularly with respect to deep basal branches (Cullings and Vogler, 1998). In the present

phylogenetic tree constructed by the NJ method based on the 5.8S rRNA gene sequence data, the ingroup Animalia, in which the monophyly was supported in 60% of 1000 bootstrap iterations, were grossly divided into several clades, including five phyla. The phylum Chordata, in which monophyly was supported in 61% of 1000 bootstrap iterations, did not show any distinct divergences among fishes, frogs, and mammals. While the 5.8S rRNA gene sequence data may be helpful for examining the monophyly of each phylum, the sequence data (162 bp) is short for a tree that includes so many taxa, limiting its utility for elucidating relationships among phyla or within phylum.

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