Molecular Cloning of Novel Cytochrome P450 1A Genes from Nine Japanese Amphibian Species

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ABSTRACT. Novel cytochrome P450 1A (CYP1A) cDNA fragments were isolated from the liver of nine Japanese amphibian species using reverse transcription polymerase chain reaction (RT-PCR). Degenerate PCR primers were used to amplify 122-bp fragments of CYP1A cDNAs. Construction of a phylogenetic tree revealed that urodele and anuran amphibians formed two branches. Within the anuran species, three branches were formed: 1) Ranidae and Rhacophoridae, 2) *Bufo japonicus formosus* and *Hyla japonica*, and 3) *Xenopus laevis*. The cDNA nucleotide sequence of these CYP1A fragments showed identities ranging 72–98% (all), 72–78% (Anura vs. Urodela), 75 to 98% (Anura), 81% (Urodela), 74–80% (*Xenopus laevis* vs. nine Japanese amphibians).

KEY WORDS: amphibian, Anura, cytochrome P450 1A, phylogenetic tree, Urodela.

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It was reported that up to 120 amphibian species have almost or completely disappeared from the earth since 1980 [15]. Currently, 21 of 62 amphibian species in Japan are listed as ‘endangered’ (Red List, Ministry of the Environment, 2006). Thus, amphibians face extinction of species and declines in population all over the world. Furthermore, this may affect the ecosystem.

Numerous environmental factors such as habitat loss, UV light, disease, mineral depletion, and chemicals in the environment have the potential to cause declines of amphibian populations [11, 17]. Amphibians are reported to be more sensitive to chemicals than other species due to the high permeability of their skin [1], and thus, they are known as environmental sentinels [12]. Many chemicals concentrate on the environment, especially the hydrosphere, and accumulate in the water, sediment, and living body through the food chain. Amphibians uniquely require a habitat that includes both aquatic and terrestrial environments. Therefore, they are strongly affected by the concentrations of biological pollutants in both environments compared with other animals.

All living bodies have defense mechanisms that metabolize and excrete various xenobiotics such as environmental chemicals. The main pathways of these metabolisms are phase I and phase II. The cytochrome P450 enzymes (CYPs) play the most important role in the phase I pathway. CYPs comprise a unique superfamily of heme-containing proteins that are bound to the membranes of the endoplasmic reticulum and play a crucial role as the oxidation-reduction component of the monooxygenase system. CYPs metabolize not only xenobiotics such as drugs, carcinogens,

and environmental chemicals but also steroids, fatty acids, and vitamins *in vivo*.

Of these CYPs, CYP1As are potently induced via the aryl hydrocarbon receptor (AhR) by the polyaromatic hydrocarbons (PAHs) and dioxins (a general term for polychlorinated dibenzo-*p*-dioxin, polychlorinated dibenzofuran, and coplanar polychlorinated biphenyl), which are the most toxic of the persistent organic pollutants (POPs). CYP1A has been suggested as a reliable biomarker of exposure of planar halogenated aromatic hydrocarbons [13].

Many studies of CYP1A in many mammals and fishes have been done. In amphibians, two forms of CYP1A, named CYP1A6 and 1A7, have been cloned only in *X. laevis* [5]. In other amphibian species, the characteristics of CYP1A are still unclear. Currently, extinctions of species and declines of populations of amphibians are so serious that it is important to study amphibian CYP1As, which might play an important role in the metabolism of numerous environmental chemicals [2, 5]. The aim of this study was to sequence Japanese amphibian CYP1As. This is the first paper to present the molecular phylogeny that covers the CYP1A genes of various amphibian species.

The Japanese amphibian species collected and used were *Cynops pyrrhogaster* (collected in Miyagi, body weight (BW) 5.15 g), *Hynobius retardatus* (collected in Hokkaido, larva), *Bufo japonicus formosus* (collected in Kyoto, BW 183 g), *Hyla japonica* (collected in Kanagawa, BW 2.90 g), *Rana japonica* (collected in Chiba, BW 4.45 g), *Rana tagoi* (collected in Gifu, BW 6.60 g), *Rana ornativentris* (collected in Gifu, BW 6.70 g), *Rhacophorus arboreus* (collected in Chiba, BW 17.5 g), and *Buergeria buergeri* (collected in Chiba, BW 5.10 g) (Fig. 1). All amphibians except for *B. j. formosus* were euthanized using a high concentration of anesthetic, A-5040-3 aminobenzoic acid ethyl ester (0.01%). *B. j. formosus*, a large frog, was euthanized

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Fig. 1. The locations of the sampling points in Japan.

by ice-cold water and CO_2 . The livers were excised immediately and stored at -80°C . Total RNA was prepared from each liver sample of the nine amphibian species using Isogen (Nippon Gene, Tokyo, Japan). RNA concentration and purity were determined spectrophotometrically at 260 and 280 nm [20]. The cDNA were synthesized from total RNA. The mixture of total RNA (about $5\ \mu\text{g}$), $0.5\ \mu\text{l}$ of 10 pmol/ μl oligo dT (Toyobo, Osaka, Japan) was incubated at 70°C for 10 min and then cooled on ice for 1 min. Four microliters of the 5-fold concentrated RT buffer (Toyobo, kit component), $8\ \mu\text{l}$ of 2.5 mM dNTP mixture, and $1\ \mu\text{l}$ of reverse transcriptase (ReverTra Ace, Toyobo) were added to the mixture and then incubated at 42°C for 50 min and at 99°C for 5 min. The degenerate primers selected to amplify 122-bp fragments of CYP1A cDNAs were Forward (NPADFIP): 5'-CCC TGC NGA YTT CAT CCC-3', Reverse-1 (YGLTM): 5'-GTG TTT GAT NGT NAG NCC ATA-3', and Reverse-2 (QWQV/INHD): 5'-TCR TGR TTN AYT GCC ACT-3' [9]. The first PCR was performed on a Takara PCR Thermal Cycler Personal (Takara, Tokyo, Japan) using the Forward and Reverse-1 primers. The product of the first PCR amplification was used for a nested PCR using the Forward and Reverse-2 primers. PCR conditions were: one cycle at 94°C for 2 min, 35 cycles at 94°C for 30 s, 51°C for 1 min, 72°C for 1 min, and final extension at 72°C for 5 min. The nested PCR was performed under the same conditions as the first PCR.

Then, PCR products were ligated to a TOPO pCR2.1 vector (Invitrogen, Carlsbad, CA, U.S.A.), and the plasmid was transfected into 10F competent *E. coli* cells (Invitrogen). The transformed *E. coli* was cultured at 37°C overnight. Plasmid DNA was extracted using a GenElute™ Plasmid Mini-prep kit (Sigma-Aldrich, St. Louis, MO, U.S.A.) and

kept at -20°C until use. The sequencing reaction was performed at 96°C for 7 min, and 40 cycles of 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min using a BigDye Terminator (Applied Biosystems, Tokyo, Japan). Ethanol precipitation was performed after the amplification, and the nucleotide sequence was analyzed by an automated DNA sequencer (ABI Prism 310 Genetic Analyzer, Applied Biosystems) following the manufacturer's instruments.

Genetic relationships among CYP1As were estimated based on the pairwise matrix of sequence divergences calculated by Clustal W [19]. Phylogenetic trees for nucleotide sequences were constructed by the neighbor-joining (NJ) method (Kimura's 2-parameter model, [8]) based on the CYP1A fragments of the nine amphibian species and six mammals and seven fishes. Nucleotide sequences with the following GenBank accession numbers were used in the phylogenetic analysis: AB022088 (*X. laevis* CYP1A7), GPIP451A1 (*Cavia porcellus* CYP1A1), NM_012540 (rat CYP1A1), NM_001136059 (mouse CYP1A1), EU220011 (horse CYP1A1), NM_000499 (human CYP1A1), NM_001040238 (Monkey CYP1A1), EU107276 (*Siganus canaliculatus* CYP1A), OTU14161 (*Opsanus tau* CYP1A), AB059743 (*Seriola quinqueradiata* CYP1A), EU049597 (*Chaetodon mertensii* CYP1A), FJ389918 (*Oreochromis niloticus* CYP1A), AF022433 (*Liza aurata* CYP1A1) and AY827103 (*Mugil curema* CYP1A). Bootstrap values were tested with 1000 replications for the NJ trees. Sequences of *X. laevis* CYP1A, mammalian CYP1As, and fish CYP1As were retrieved from the GenBank database.

In this study, the molecular phylogeny of CYP1A genes was constructed and analyzed, focusing on the CYP1As of Japanese amphibians. Two amphibian CYP1A forms, named CYP1A6 and 1A7, have been cloned only in *X. lae-*

vis. A sequence comparison showed that both *X. laevis* CYP1As were more similar to mammalian CYP1A1 than to mammalian CYP1A2 [5]. Consequently, we predicted that Japanese amphibian CYP1A corresponds to mammalian CYP1A1. To determine whether Japanese amphibian CYP1A fragments correspond to CYP1A6 or 1A7, cloning of full-length Japanese amphibian CYP1A cDNAs is necessary. However, short fragments are useful for the analysis of the differences in CYP1As and construction of a phylogenetic tree [18].

In the phylogenetic tree (Fig. 2), urodele and anuran amphibians formed two branches. This clustering was strongly supported in 94.0% of 1000 bootstrap iterations. Within the Anura, three branches were formed: 1) Ranidae and Rhacophoridae, 2) *B. j. formosus* and *H. japonica*, and 3) *X. laevis*, which was supported in only 67.0% of the bootstrap iterations. Within Ranidae, *R. tagoi* differentiated earlier than *R. japonica* and *R. ornativentris* (78.5% of bootstrap iterations). The sister relationship in-group of *R. japonica* and *R. ornativentris* was supported in 76.3% of bootstrap iterations. Ranidae in Japanese amphibians is a large family that contains 24 species. The phylogenetic classification of 11 Japanese Ranidae species based on the mitochondrial cytochrome b gene has been reported [16].

The first cluster contained three subspecies of *R. tagoi* and *R. sakuraii*. The second cluster contained *R. tsushimensis* and *R. okinavana*. The third cluster contained *R. japonica* and three species with $2n=24$ chromosomes: *R. ornativentris*, *R. pirica*, and *R. dybowskii*. Among brown frogs, *R. tagoi* and *R. sakuraii* are the most distinct in their reproductive ecology [10]. Both species lay a small number of large eggs in small subterranean streams (*R. tagoi*) or under the stones of mountain streams (*R. sakuraii*), in contrast to other brown frogs that breed in still open water. Thus, according to the classification based on the mitochondrial cytochrome b gene, *R. tagoi* differentiated earlier than *R. japonica* and *R. ornativentris*. This report corresponds to the result of this study.

According to the pairwise matrix (Table 1), the cDNA nucleotide sequence of these CYP1A fragments showed identities ranging 72–98% (all), 72–78% (Anura vs. Urodela), 75–98% (Anura), 81% (Urodela), and 74–80% (*X. laevis* vs. nine Japanese amphibians). Within Ranidae (*R. japonica*, *R. tagoi*, and *R. ornativentris*), CYP1A fragments showed identities ranging 97–98%. Two species within Rhacophoridae (*R. arboreus* and *B. buergeri*), CYP1A fragments also showed high identities (94%). In the comparisons of different families, CYP1A fragment identities

Fig. 2. Phylogenetic tree constructed by the neighbor-joining method based on the nucleotide sequences of 122-bp fragments of the CYP1A gene (Kimura's 2-parameter model, [8]). Numbers on the trees represent the local bootstrap values from 1000 replicates. The scale bars represent branch length in terms of nucleotide substitutions per site. (a) and (b) indicate anuran and urodele species, respectively.

Table 1. Pairwise identity matrix for amphibian CYP1A

	1	2	3	4	5	6	7	8	9
1 <i>C. pyrrhogaster</i>									
2 <i>H. retardatus</i>	81								
3 <i>X. laevis</i>	74	74							
4 <i>B. j. formosus</i>	78	74	80						
5 <i>H. japonica</i>	77	73	79	91					
6 <i>R. tagoi</i>	76	77	77	89	82				
7 <i>R. japonica</i>	74	75	77	87	81	97			
8 <i>R. ornativentris</i>	76	75	77	87	81	97	98		
9 <i>R. arboreus</i>	72	73	75	87	81	91	91	91	
10 <i>B. buergeri</i>	74	75	75	81	79	92	92	94	94

To compare the identities (%) of amphibian CYP1As, a pairwise identity matrix was generated by ClustalW [19].

ranged 91–94% (Ranidae vs. Rhacophoridae) and 91% (*B. j. formosus* vs. *H. japonica*). These values were high within Anura. On the other hand, CYP1A identities of Japanese Anura vs. *X. laevis* showed 75–80%. This value was also low between Anura and Urodela (72–78%). In comparisons of the identities of different orders such as amphibian vs. fish, and amphibian vs. mammals, CYP1A fragments ranged 40–69% (amphibian vs. fish) and 54–75% (amphibians vs. mammals) (data not shown).

Thus, this study shows that CYP1A sequence identity values between *X. laevis* and Japanese amphibians are low, and that *X. laevis* is independent in the phylogenetic tree. One reason is that, in contrast to other Japanese Anura, only *X. laevis* lives exclusively in water and has evolved in distinct habitats. Until now, chemical toxicity in amphibians has been studied primarily in *X. laevis* [3, 4, 6, 7, 14]. However, according to the sequence analysis in this study, there may be large differences in CYP1A-dependent metabolism between *X. laevis* and other Japanese amphibians. This species difference of CYP1A might cause the difference of sensitivity for environmental chemicals among amphibian species because CYP1A plays important role in metabolic activation and detoxification of xenobiotics. Therefore, not only *X. laevis* but also other amphibian CYP1As require further study to reveal the high-risk amphibian species against environmental chemicals. In the future, characterization of CYP1A, such as measurement of tissue distribution of mRNA expression, determination of full-length sequence, characterization of enzyme activities and specificity of substrate in each amphibian species will be needed.

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