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# Identification of novel cytochrome P450 1A genes from five marine mammal species☆

Ikuko Teramitsu [a], Yukio Yamamoto [a,1], Issei Chiba [a], Hisato Iwata [a], Shinsuke Tanabe [b], Yoshihiro Fujise [c], Akio Kazusaka [a], Fumiaki Akahori [d], Shoichi Fujita [a,*]

[a] *Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, N18, W 9, Kita-ku, Sapporo 060-0818, Japan*

[b] *Department of Environment Conservation, Ehime University, Tarumi 3-5-7, Matsuyama 790-8566, Japan*

[c] *The Institute of Cetacean Research, 4-8, Toyomi-cho, Chuo-ku, Tokyo 104-0055, Japan*

[d] *Department of Environmental Health, School of Veterinary Medicine, Azabu University, 17-71, Fuchinobe 1-Chome, Sagamihara, Kanagawa 229-8501, Japan*

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**Abstract**

Marine mammals, being endangered by the chronic exposure of hydrophobic environmental contaminants as an assorting result of global pollution, are especially focused as indicators for organochlorine pollution. The use of contaminant-induced xenobiotic metabolizers, particularly P450 (CYP) 1A, in marine mammals can be effective as potential biomarkers of the contaminant exposure and/or toxic effects. In this study, we identified the first marine mammalian CYPs. Six novel CYP1A cDNA fragments were cloned from the livers of marine mammal species, minke whale (*Balaenoptera acutorostrata*), dall's porpoise (*Phocoenoides dalli* ), steller sea lion (*Eumetopias jubatus*), largha seal (*Phoca largha*), and ribbon seal (*Phoca fasciata*) by the method of reverse transcription/polymerase chain reaction (RT/PCR); two distinct fragments were from steller sea lion and one fragment each was obtained from the other species. Five of the fragments, one from each species, were classified in the subfamily of CYP1A1, and the other fragment cloned from steller sea lion was designated CYP1A2. Degenerate PCR primers were used to amplify the fragments from liver cDNAs. The deduced amino acid sequences of these fragment CYP1As showed identities ranging from 50.0 to 94.3% with other known vertebrate CYPs in the subfamily of CYP1A, including those from fish, chicken, and terrestrial mammals. The isolated fragments were used to construct a molecular phylogeny, along with other vertebrate CYP1A cDNAs cut down in size to the corresponding region of 265 bp in which those newly determined fragments were cloned. This phylogenetic analysis by the maximum parsimony method using the PHYLIP

☆ The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequenced databases under Accession Nos. AB014354 (Minke whale CYP1A1), AB014355 (Dall's porpoise CYP1A1), AB014356 (Steller sea lion CYP1A1), AB014357 (Steller sea lion CYP1A2), AB014358 (Largha seal CYP1A1), and AB014359 (Ribbon seal CYP1A1).

* Corresponding author. Tel.: +81-11-7065102; fax: +81-11-7065105.

*E-mail address:* fujita@vetmed.hokudai.ac.jp (S. Fujita).

[1] Present address: The Fourth Department, Osaka Bioscience Institute, Suita, Osaka 565-0874, Japan.



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program suggests two distinct evolutional pathways for aquatic mammalian CYP1As, compatible to a conservative taxonomy. Pinniped genes are clustered together with dog gene, forming a carnivore group, and cetaceans form another branch. Identification of CYP1A genes in marine mammals will be an introductory step to provide new insights into the metabolic or toxicological functions of CYP1As in these animals. 

*Keywords:* Marine mammals; Cytochrome P-450 1A; Degenerate polymerase chain reaction; Phylogenetic analysis

## 1. Introduction

The cytochrome P450 enzymes (CYPs) comprise a unique superfamily of heme-containing proteins that are bound to the membranes of endoplasmic reticulum and play a crucial role as the oxidation–reduction component of the monooxygenase system. This system is involved in the oxidative metabolism of a wide variety of xenobiotics such as drugs, carcinogens, and environmental chemicals, as well as endogenous substrates such as steroids and fatty acids (Nebert and Gonzalez, 1987; Nelson et al., 1996).

The CYPs are known to exist in a wide range of organisms, from bacteria to mammals, and their genes show an extraordinary diversity. These genes are classified into over 74 families according to their amino acid sequences.

Of these various CYP isozymes, the members of CYP1A subfamily are prominent in the metabolism and activation of many hydrocarbon carcinogens and in the induction by aryl hydrocarbon receptor agonists such as polynuclear and planar halogenated aromatic hydrocarbons (Chae et al., 1993). Hence, intensive studies have been presented on the molecular bases as well as the regulations and functions of CYP1A forms in various organisms (Kawajiri et al., 1984; Shimada and Guengerich, 1990). Despite the large number of genes that have been sequenced and much effort that have been made to build a phylogenetic analysis within several subfamilies of CYPs (Morrison et al., 1995, 1998; Celander et al., 1997), little is known about P450 genes of any subfamily in marine mammals.

Marine mammals are at the top of the hierarchy of the food chain in the water ecosystem and are facing the risk of accumulating hydrophobic environmental pollutants in their bodies (Ross et al., 1996; Mossner and Ballschmiter, 1997; Wade et al., 1997). For the conservation of marine mammal populations, it is important to know the molecular and biochemical characteristics of the CYPs in marine mammals, which may be associated with the toxicokinetics of such contaminants and the adverse effects mediated by the enzyme induction or deterioration (Tanabe et al., 1994; Iwata et al., 1995). The identification of CYP genes in these animals will lead us to clarifying their involvement in xenobiotic metabolism and activation. In addition, CYP1A is being suggested as reliable biomarkers of exposures and toxicities by planar halogenated aromatic hydrocarbons in marine mammals (Tanabe et al., 1994; White et al., 1994; Troisi and Mason, 1997). In order to make quantitative and qualitative analysis of the CYP1A gene expression and function in marine mammals, studies with heterologus probes or antibodies may not be sufficient. Either protein purification or cDNA cloning studies are required in order to draw definitive conclusions.

In this study, we report the identification of novel CYP1A genes from the livers of minke whale (*Balaenoptera acutorostrata*), dall's porpoise (*Phocoenoides dalli* ), steller sea lion (*Eumetopias jubatus*), largha seal (*Phoca largha*), and ribbon seal (*Phoca fasciata*). This will be the first paper to present the molecular cloning of CYP1A genes in marine mammals with the establishment of a molecular phylogeny which covers CYP1A genes of various species, ranging widely from terrestrial to marine vertebrates.

## 2. Materials and methods

### 2.1. Animals and RNA isolation

The minke whale sample was collected by a survey of the Japanese Whale Research Program under Special Permit in the North Pacific. Dall's porpoise and steller sea lion were captured at the western coast of shakotan Peninsula facing the Sea of Japan, and largha seal and ribbon seal at the eastern coast of Rausu adjacent to the sea of Okhotsk, in Hokkaido, Japan. The porpoise and pinnipeds were collected in legally controlled hunts by local fishermen. For each species, an adult male animal was selected. All liver samples were frozen in liquid nitrogen immediately after the dissection and stored at −80°C until use. Total RNA was prepared from each liver sample by the single-step method (Chomczynski and Sacchi, 1987) using ISOGEN (NIPPON GENE, Toyama, Japan). The concentration and purity of the RNA fraction was determined spectrophotometrically at 260 and 280 nm, respectively.

Table 1
CYP1A genes used in phylogeny

| Gene | Species | Accession numbers |
|---|---|---|
| *Fish* | | |
| CYP1A | Plaice | X73631 |
| CYP1A | Oyster toadfish | U14161 |
| CYP1A | Scup | U14162 |
| CYP1A | Four-eye butterfly fish | U19855 |
| CYP1A | European sea bass | U78316 |
| CYP1A | Atlantic tomcod | L41886 |
| CYP1A | Killifish | AF026800 |
| CYP1A | Gilthead sea bream | AF011223 |
| CYP1A1V1 | Rainbow trout | S69278 |
| CYP1A1V2 | Rainbow trout | U62796 |
| CYP1A1V3 | Rainbow trout | U62797 |
| CYP1A3 | Rainbow trout | S69277 |
| *Birds* | | |
| CYP1A4 | Chicken | X99453 |
| CYP1A5 | Chicken | X99454 |
| *Mammals* | | |
| Cyp1a1 | Mouse | K02588 |
| CYP1A1 | Rat | I00732 |
| CYP1A1 | Guinea pig | D11043 |
| CYP1A1 | Hamster | D12977 |
| CYP1A1 | Rabbit | D00212 |
| CYP1A1 | Dog | PIR/C37222[a] |
| CYP1A1 | Monkey | D17575 |
| CYP1A1 | Human | K03191 |
| Cyp1a2 | Mouse | K02589 |
| CYP1A2 | Rat | K02422 |
| CYP1A2 | Guinea pig | D50457 |
| CYP1A2 | Hamster | M34446 |
| CYP1A2 | Rabbit | D00213 |
| CYP1A2 | Dog | PIR/B37222[a] |
| CYP1A2 | Human | M38504 |

[a] Only amino acid sequences were available from PIR.

### 2.2. Oligonucleotides

Degenerate primers (GIBCO BRL, Gaithersburg, MD) were designed based on the conserved regions of eight mammalian CYP1A sequences retrievable from GenBank (Table 1). The sense primer (5′-TCT TTG GRG CWG GNT TTG ACA C -3′; where R is for A or G, W for A or T, and N for all four bases) and the antisense primer (5′-TGG TTR AYC TGC CAC TGG TT-3′, where R is for A or G, and Y is for T or C) included 16- and 4-fold degeneracy, respectively and were both positioned upstream from the well-described cysteine-containing heme-binding region (Kawajiri et al., 1984; Nebert and Gonzalez, 1987; Gonzalez, 1988; Gonzalez et al., 1985).

### 2.3. cDNA amplification and isolation

Oligo(dT) primed total RNA was reverse-transcribed to cDNA using superscript II reverse transcriptase (GIBCO BRL, Gaithersburg, MD). The resultant cDNA was amplified with the aforementioned degenerate primers and the Klen Taq polymerase (CLONTECH, Palo alto, CA) by PCR on TaKaRa PCR Thermal Cycler PERSONAL (Takara, Tokyo, Japan). The thermal cycles of the reaction started with a single 1-min cycle at 94°C followed by 35 cycles for minke whale and largha seal samples and 30 cycles for the rest, of 10 s denaturation at 94°C, 30 s annealing at 56°C, and 1 min extension at 72°C. For each species, a single major band of 307 bp was detected by analytical electrophoresis on a 1.5% agarose/EtBr (0.25 μg/ml) gel. The PCR products were freshly cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA), followed by the transforming of bacterium

Table 2
Conserved regions used to design degenerate primers

| Gene | Primer regions (bp) | |
|---|---|---|
| | Upstream | Downstream |
| Mouse Cyp1a1 | 953–974 | 1240–1259 |
| Guinea pig CYP1A1 | 929–950 | 1216–1235 |
| Monkey CYP1A1 | 941–962 | 1228–1247 |
| Human CYP1A1 | 941–962 | 1228–1247 |
| Mouse Cyp1a2 | 935–956 | 1222–1241 |
| Guinea pig CYP1A2 | 941–962 | 1228–1247 |
| Hamster CYP1A2 | 935–956 | 1222–1241 |
| Human CYP1A2 | 941–962 | 1228–1247 |

DH5-α with that plasmid DNA. The bacterial cells were cultured for 14 h average. Blue–white screening was employed to identify transformed cells, and the amplified cDNA inserts of 307 bp were assured by direct PCR using M13 Primers, RV-N and M3 (TaKaRa, Tokyo, Japan), specifically targeting the multiple cloning site of the vector. The cells from the selected colonies were cultured for another 9 h in liquid Luria–Bertani's) (LB) broth. The plasmids were purified by a plasmid miniprep method using QIAprep Spin Miniprep Kit (QIAGEN, Stanford, CA).

### 2.4. DNA sequencing

Purified plasmids were directly sequenced by the method of dye terminator cycle sequencing. The cycle sequencing reactions were performed on a PROGRAM TEMP CONTROL SYSTEM PC-700 (ASTEC) with the vector-specific M13 Primers, RV-N and M3 (TaKaRa, Tokyo Japan), using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster, CA). The nucleotide sequences were determined by an automated DNA sequencer (ABI PRISM 310 genetic analyzer). A minimum of four clones for each animal species was sequenced both universal and reverse wise to avoid sequence ambiguities.

### 2.5. Phylogenetic analysis of the mammalian CYP1A genes

The nucleotide and amino acid sequences of 29 vertebrate CYP1As [fish CYP1As from plaice (*Pleuronectes platessa*), oyster toadfish (*Opsanus tau*), scup (*Stenotomus chrysops*), four-eye butterfly fish (*Chaetodon capistratus*), European sea bass (*Dicentrarchus labrax*), Atlantic tomcod (*Microgadus tomcod*), gilthead sea bream (*Pagrus major*), killifish (*Fundulus heteroclitus*), rainbow trout (*Oncorhynchus mykiss*) CYP1A1V1, 1A1V2, 1A1V3 and 1A3; chicken (*Gallus gallus*) CYP1A4 and 1A5; mammalian CYP1A1s from human (*Homo sapiens*), monkey (*Macaca irus*), dog (*Canis lupus familiaris*), rabbit (*Oryctolagus cuniculus*), hamster (*Mesocricetus auratus*), guinea pig (*Cavia cobaya*), rat (*Rattus norvegicus*), and mouse (*Mus musculus*) mammalian CYP1A2s from human (*H. sapiens*), dog (*Canis lupus familiaries*), rabbit (*Oryctolagus cuniculus*), hamster (*Mesocricetus auratus*), guinea pig (*Cavia cobaya*), rat (*Rattus norvegicus*), and mouse (*Mus musculus*)] and rat CYP3A1 were retrieved from the GenBank database (Table 1). The six novel cDNA and amino acid sequences of minke whale, dall's porpoise, steller sea lion, largha seal, and ribbon seal were aligned with these vertebrate sequences using the GENETYX-MAC 8.0 program. Phylogenetic trees for amino acid sequences were constructed by the maximum parsimony method using the PHYLIP program.

## 3. Results

Minke whale, dall's porpoise, steller sea lion, largha seal, and ribbon seal CYP1A partial cDNAs were amplified from total liver RNA by RT/PCR, two distinct fragments from steller sea lion and one fragment each from the rest, with degenerate PCR primers designed based on the well-conserved regions of eight other mammalian CYP1As (Table 2) both positioned upstream from the heme-binding site (Kawajiri et al., 1984; Gonzalez, 1988; Gonzalez et al., 1985; Nebert and Gonzalez, 1987). The binding site itself was not chosen for a primer region to increase the specificity of CYP1A primers.

Each amplified cDNA fragment was 265 bp long, excluding the 5′-end and 3′-end PCR primer regions and encoded a deduced 88 amino acid peptide. The fragments are positioned at a region corresponding to the 336–423 amino acids in rat CYP1A1 sequence. The nucleotide and the deduced amino acid sequences of those fragments are shown in Fig. 1. The sequences were sent to Dr David R. Nelson of the P450 gene superfamily nomenclature committee for classification. Five of the fragments, one from each species, were classified in the subfamily of CYP1A1, and the other fragment cloned from steller sea lion was designated CYP1A2. The deduced amino acid sequence identities between all the possible pairs within the new CYP1A1 fragments range from 89.8 to 100%, with minke whale and steller sea lion being the most distant couple, and with the two seal genes completely identical to each other (Table 3).

The predicted amino acid sequences of these novel cDNA fragments were also compared with those of other mammalian CYP1As in the corresponding region to determine sequence identifies. The novel and the know mammalian CYP1A1 sequences share 78.4–94.3% similarities, and steller sea lion CYP1A2 is 73.9–87.5% identical to other mammalian CYP1A2s. The percent identities of marine mammalian CYP1A sequences with fish and evian CYP1A amino acid sequences were within the range 50.0–78.4% (Table 4).

From the multiple alignment of the newly isolated fragments and other 29 vertebrate amino acid sequences in the corresponding region, a phylogenetic tree of CYP1A genes was constructed by the maximum parsimony method using the PHYLIP program (Fig. 2). Rat CYP3A1 was used as an outgroup species in the rooted tree. As a result, three clearly differentiated branches of fish, evian, and mammalian animals formed. Within the cluster of mammalian CYP1As, the genes are assembled in the groups of two subfamilies, CYP1A1 and CYP1A2, resembling the topology of the full-length CYP1A phylogeny (Morrison et al., 1998). The five novel CYP1A1 genes from the marine mammals were clustered in one major branch, sending out two minor branches for the cetacean and the carnivore CYP1A1s of dog and the pinnipeds. Steller sea lion CYP1A2 was placed in the group with other CYP1A2 genes in the position closest to dog CYP1A2.

Table 3
Amino acid identities within novel sequences (%)[a]

| | Dall's porpoise 1 | Stellar sea lion 1 | Spotted seal 1[b] | Stellar sealion 2 |
|---|---|---|---|---|
| Minke whale CYP1A1 | 96.9 | 89.8 | 92.0 | 78.4 |
| Dall's porpoise | | 92.0 | 94.3 | 80.7 |
| Stellar sealion CYP1A1 | | | 97.7 | 79.5 |
| Spotted seal CYP1A1[b] | | | | 80.7 |

[a] The numbers 1 and 2 in the column headings stand for CYP1A1 and CYP1A2, respectively.
[b] Ribbon seal CYP1A1 nucleotide sequence was 100% identical to that of spotted seal CYP1A1.

Table 4
Amino acid identities of the CYP1A subfamily (%)

| | Fish | Bird | Mammal CYP1A1 | Mammal CYP1A2 |
|---|---|---|---|---|
| Minke whale CYP1A1 | 59.1–62.5 | 69.3, 78.4 | 78.4–90.0 | 73.9–86.4 |
| Dall's porpoise CYP1A1 | 56.8–60.2 | 67.0, 77.3 | 78.4–92.0 | 76.1–85.2 |
| Steller sea lion CYP1A1 | 55.7–58.0 | 67.0, 75.0 | 78.4–94.3 | 76.1–81.8 |
| Spotted seal CYP1A1 | 55.7–59.1 | 67.0, 75.0 | 79.5–94.3 | 76.1–83.0 |
| Ribbon seal CYP1A1 | 55.7–59.1 | 67.0, 75.0 | 79.5–94.3 | 76.1–83.0 |
| Steller sea lion CYP1A2 | 50.0–58.0 | 62.5, 71.6 | 73.9–83.0 | 73.9–87.5 |

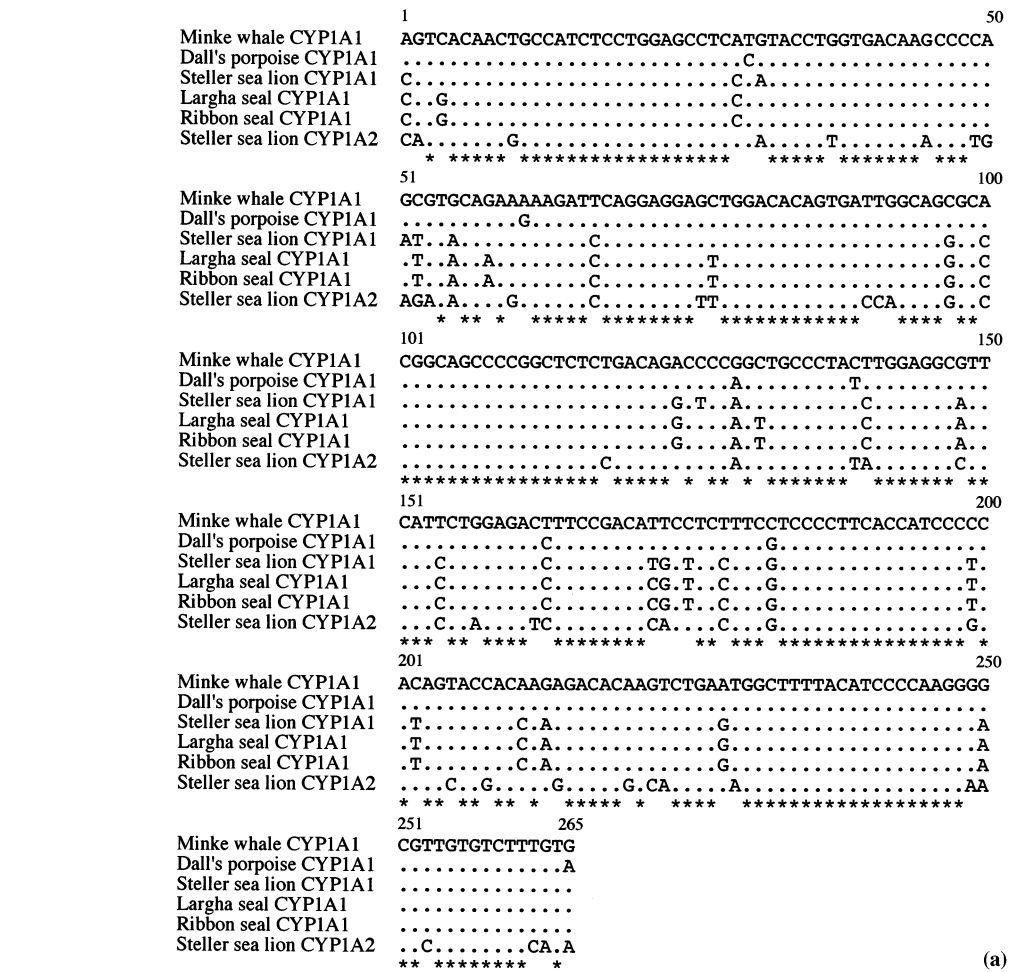


Fig. 1. Alignments of (a) the nucleotide and (b) the deduced amino acid sequences of the novel CYP1As using the GENETYX-MAC 8.0 program. (a) 265 bp nucleotide sequences of the novel CYP1As. The conserved regions are indicated with dots (.). (b) The predicted amino acid sequences are shown aligned with rat CYP1A1 sequence of the corresponding region. Underlined is the well-described heme-binding region with the cysteine positioned in the center (arrow). Sequences conserved among marine species are indicated with dots (.). Starmarks indicate the regions conserved among all the seven sequences including rat CYP1A1.

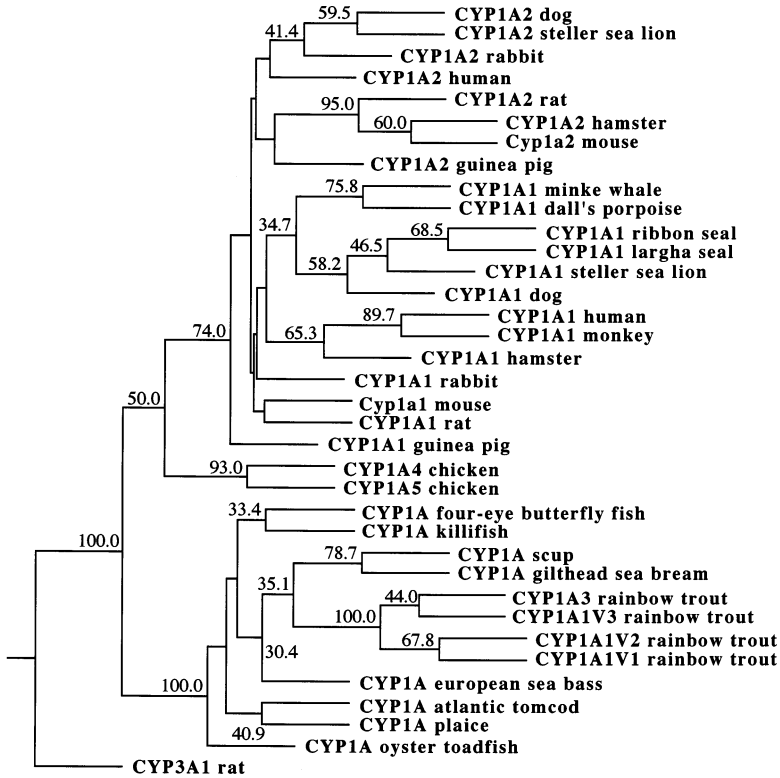


Fig. 2. Phylogenetic analysis of the vertebrate CYP1As based on the multiple alignment of the deduced amino acid sequences retrieved from the GenBank database. This is a rooted tree for amino acid sequences in the partial region constructed by the maximum parsimony method using the PHYLIP program. A number at each branch and the length of the stem indicate bootstrap value based on 100 samplings. Values under 25 are not shown. Rat CYP3A1 was used as an outgroup species.

## 4. Discussion

In this study, we renewally constructed and analyzed a molecular phylogemy of CYP1A genes, focusing on the novel marine mammalian CYP1As. Although the sequence region used in the process was only partial, which could be the reason for the incomplete divergence of mammalian CYP1A1s and CYP1A2s in the partial sequence, the tree was estimated to bear reliability to draw conclusions on the novel gene cluster.

With the generally considered idea that the CYP1A enzymes may have attained evolutionary and developmental advantages in defensive responses to xenobiotic ingestion, we preliminarily expected the outcome that the CYP1As of the marine mammals might form a distinct cluster to reflect their unique evolution to cope with the conditions of their aquatic life. The course and the speed of evolution of a gene are determined by the chance of random mutations and also by the degree of the restriction in the function of the protein it encodes. Available food resources may be one factor which has significant effects on such evolution of a gene responsible for xenobiotic metabolism. Living in a specific habitat depending on fish as most of their food resources might lead to a unique diversity of CYP1A sequence and/or enzyme function in marine mammals. It has been moderately suggested that the high accumulation of some of the hydrophobic contaminants in the bodies of marine mammals is not only the result of bioaccumulation process through food chain, but also of their properties of low or possibly no enzymatic activities of certain xenobiotic metabolizer, which are unique from those of the terres-

trial animals (Watanabe et al., 1989; Norstrom et al., 1992; White et al., 1994). Our result may not be supportive for a unique diversity of CYP1A sequence in marine mammals. The novel sequences of marine mammal CYP1As did not indicate the evidence for unique diversity within that partial region. Though solidly grouped in one major cluster of marine species as the representative candidates, the pinniped CYP1As were more closely related to the dog CYP1As than to the cetacean CYP1As. Additional CYP1A sequences from such mammals as cat (or any candidate of the order Carnivora) and bovine or sheep (or any angulates) would more clearly separate the marine mammal cluster into two clusters of carnivore genes and cetacean/artiodactyl genes, the orthodox taxonomy in mammalian species (Cao et al., 1994; Graur and Higgins, 1994). Although it is still a possibility that the phylogeny with full-length sequences might be different, our result at this point may indirectly lead to an assumption that marine mammals have responded to the xenobiotic intrusion by diversifying the enzyme function with minimal sequence alteration.

In summary, we successfully isolated partial genes of CYP1A isozymes from marine mammals. We subsequently made phylogenetic analysis of the novel marine mammalian CYP1As which showed the reflection of the orthodox taxonomy in mammalian species. Identification of CYP1A genes in marine mammals that are exposed to high risks of environmental pollution is an introductory step which will provide us with new insights into the metabolic or toxicological functions of CYP1As in these animals. Based on the results of this study, cloning and expression studies of full-length marine mammal CYP1A cDNAs are in progress. Meanwhile, the fragment sequences are being further investigated for their potency as nucleotide probes.

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