UGT Xenobiotic metabolizing activity and genetic evolution in Pinniped species

Running title

UGT xenobiotic metabolism in Pinnipedia

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ABSTRACT

There are various inter-species differences in xenobiotic-metabolizing enzymes. It is known that cats show slow glucuronidation of drugs such as acetaminophen and strong side effects due to the UGT1A6 pseudogene. Recently, the UGT1A6 pseudogene was found in the Northern elephant seal and Otariidae was suggested to be UGT1A6-deficient. From the results of measurements of UGT activity using liver microsomes, the Steller sea lion, Northern fur seal, and Caspian seal showed UGT activity towards 1-hydroxypyrene and acetaminophen as low as in cats, which was significantly lower than in rat and dog. Furthermore, UGT1A6 pseudogenes were found in Steller sea lion and Northern fur seal, and all Otariidae species were suggested to have the UGT1A6 pseudogene. The UGT1 family genes appear to have undergone birth-and-death evolution based on a phylogenetic and synteny analysis of the UGT1 family in mammals including Carnivora. UGT1A2–1A5 and UGT1A7–1A10 are paralogous genes to UGT1A1 and UGTA6, respectively, and their numbers were lower in cat, ferret and Pacific walrus than in human, rat, and dog. Felidae and Pinnipedia, which are less exposed to natural xenobiotics such as plant-derived toxins due to their carnivorous diet, have experienced fewer gene duplications of xenobiotic-metabolizing UGT genes, and even possess UGT1A6 pseudogenes. Artificial environmental pollutants and drugs conjugated by UGT are increasing dramatically, and their elimination to the environment can be of great consequence to cat and Pinnipedia species, whose low

xenobiotic glucuronidation capacity makes them highly sensitive to these compounds.

Key words: Carnivores, molecular evolution, UGTs, xenobiotic, wildlife,

INTRODUCTION

Xenobiotic compounds such as drugs and environmental pollutants are activated by phase I enzymes, conjugated by phase II enzymes, and eliminated in urine or bile through phase III transporters. Phase I enzymes include primarily the cytochrome P450 (CYP) superfamily, whereas phase II conjugating enzymes include many enzyme superfamilies such as UDP-glucuronosyltransferase (UGT), sulfotransferase (SULT) and glutathione S-transferase (GST) (Xu *et al.* 2005). Some xenobiotics are metabolically activated by CYP and the resulting intermediates cause health dysfunction. The conjugation reaction is essential for metabolism.

Among phase II conjugating enzymes, the UGT superfamily plays the most important role in xenobiotic metabolism, since 55% of the 200 most frequently prescribed drugs are conjugated by UGT and eliminated in urine or bile (Guillemette *et al.* 2014; Stingl *et al.* 2014). Furthermore, UGT conjugates many endogenous compounds including bilirubin, steroid hormones, thyroid hormones, bile acids, and fat-soluble vitamins. UGT catalyzes the conjugation of the glycosyl group of glucuronic acid to many lipophilic endogenous and exogenous compounds (Tukey and Strassburg, 2000). The UGT superfamily has been classified into two major families, UGT1 and UGT2, on the basis of similarities in amino acid sequences (Burchell *et al.* 1991). UGT1A and UGT2B subfamily enzymes contribute to drug metabolism. Based on sequence similarity and substrate specificity, the human and rodent UGT1 genes can also be divided into two groups, namely UGT1A1 through 1A5 (bilirubin

group) and UGT1A6 through 1A10 (phenol group) (Emi *et al.* 1995; Owens *et al.* 2005; Zhang *et al.* 2004). In human and rodent, all of the UGT1 family enzymes are encoded by a single gene locus. The UGT1 locus consists of variable first exons and four shared exons in a tandem array. Each variable exon is very similar but encodes different polypeptides and substrate-binding domains. The four shared exons encode the common C-terminal domain that binds the co-factor uridine diphosphate glucuronic acid (UDPGA). Each first exon possibly determines substrate specificity, whereas the common exons most likely determine the interaction with the common substrate, i.e. UDPGA. UGT1 genes have broad and overlapping substrate specificity due to the highly variable structure of their protein products (Maruo *et al.* 2005; Tukey and Strassburg, 2000). Gene duplications and losses are an important source of genetic complexity and diversity (Nei and Rooney, 2005). This unique genomic structure generates the enormous molecular diversity required for the survival of organisms (Zhang *et al.* 2004).

There are large inter-species differences in UGT metabolism due to this complex molecular diversity. It is well known that cats show low glucuronide conjugation of drugs and toxins. Slow glucuronidation of acetaminophen and aspirin lead to the slow clearance and high sensitivity of cats to the adverse effects of these drugs (Davis and Westfall, 1972; Savides *et al.* 1984). The primary reason for these findings has been attributed to UGT1A6, which contributes to glucuronidation of phenolic compounds in human and rodent liver, but is a

pseudogene in cats (Court and Greenblatt, 2000). Recently, UGT1A6 pseudogenization has been found in not only cats but all other Felidae, as well as the Brown hyena (*Hyaena brunnea*) and Northern elephant seal (*Mirounga angustirostris*) (Shrestha *et al.* 2011). In the same paper, it was suggested that UGT1A6 is deficient in Otariidae (fur seals and sea lions). The Northern elephant seal is the only species in the suborder Caniformia in which a UGT1A6 pseudogene has been found. In addition to cats, some species classified in the suborder Caniformia in the order Carnivora may have low glucuronidation of xenobiotics. Possible low glucuronidation is of particular concern in Pinnipedia, which includes Phocidae (seals) and Otariidae. However, studies on *in vitro* UGT metabolic activity in Pinnipedia are lacking.

The aim of this study was to clarify inter-species differences in UGT xenobiotic metabolism in Carnivora, including Pinnipedia, and the genetic backgrounds underlying UGT xenobiotic metabolism using *in vitro* experiments and *in silico* genetic analysis. First, UGT activities in rat (*Rattus norvegicus*), dog (*Canis lupus familiaris*), cat (*Felis catus*), Steller sea lion (*Eumetopias jubatus*), Northern fur seal (*Callorhinus ursinus*) and Caspian seal (*Phoca caspica*) liver microsomes were measured and compared. UGT1A genes including UGT1A6 in three Pinnipedia species were cloned and UGT1 family genes in Carnivora whose genome projects have been completed were phylogenetically analyzed.

MATERIALS AND METHODS

Chemicals.

1-Hydroxypyrene, pyrene glucuronide, acetaminophen glucuronide, β-estradiol, sodium cholate hydrate and UDPGA were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetaminophen standard, acetic acid, formic acid, sodium phosphate, and ammonium acetate solution were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). β-Estradiol 3-(β-D-glucuronide) sodium salt was obtained from Santa-Cruz Biotechnology Inc. (Dallas, TX, USA). All chemicals used for high-performance liquid chromatography (HPLC) and mass spectrometry (MS) were HPLC or MS grade and were obtained from Kanto Chemical Co. Inc. (Tokyo, Japan).

Animals.

Liver samples were collected from Steller sea lions (*Eumetopias jubatus*), Northern fur seals (*Callorhinus ursinus*), Caspian seals (*Phoca caspica)*, cats (*Felis catus*) and rats (*Rattus norvegicus*; Sprague-Dawley strain). Steller sea lion livers in Rausu, Hokkaido were kindly gifted from Dr. Kentaro Q Sakamoto (Laboratory of Physiology, Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University). Steller sea lion livers in Hamamasu and Shakotan from Hokkaido were donated from Dr. Kaoru Hattori (Hokkaido National Fisheries Research Institute, Fisheries Research Agency) and Dr. Akihiko

Wada (Hokkaido Research Organization, Fisheries Research Institute, Central Fisheries Research Institute), respectively. Caspian seal, Northern fur seal and cat livers were provided from Environmental Specimen Bank (es-BANK: http://esbank-ehime.com/) of Ehime University.

Sprague-Dawley rats were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). Rats aged 8 weeks old were used for comparison. The rats (7 weeks old) were housed at a constant temperature (23 ± 1°C) and constant humidity (55 ± 5%), with automatically controlled lighting (0700–1900), given food and water ad libitum and handled for 1 week. After euthanasia of rats by CO_2, the liver was collected and immediately frozen in the liquid nitrogen. All the liver samples from the five species were immediately frozen in liquid nitrogen and stored at –80 °C until use. All experiments using animals were performed under the supervision and with the approval of the Institutional Animal Care and Use Committee of Hokkaido University (no. 13-0213). Details of the samples are shown in Table 1.

Preparation of microsomes.

Liver microsomes were prepared following the method of Omura and Sato (1964). About 5 g of liver tissue each from the six species were homogenized in 15 ml of potassium phosphate buffer (KPB: 0.1 M, pH 7.4). Homogenates were transferred to a tube and centrifuged at 9,000 *g* at 4 °C for 20 min. The supernatant was further centrifuged at 105,000 *g* for 70 min

147 to obtain microsomal fractions. Microsomal pellets were re-suspended in 5 ml of buffer.
148 Microsomes were stored at –80 °C until analysis. Protein concentrations in microsomes were
149 measured using a BCA protein assay reagent kit (PIERCE, Rockford, IL, USA). Dog (*Canis*
150 *lupus familiaris*) liver microsomes were purchased from BD Biosciences (San Jose, CA,
151 USA).
152
153 *β-Estradiol glucuronidation assay.*
154 First, 25 µl of hepatic microsome solution was mixed with 22.5 µl of KPB (0.1 M, pH 7.4).
155 The microsome preparation was then mixed with 2.5 µl of 1% sodium cholate solution and
156 incubated on ice for 30 min. Then, 50 µl of microsome solution was mixed with KPB (0.1 M,
157 pH 7.4), 5 µl of 100 mM $MgCl_2$, and estradiol, which was dissolved in methanol, resulting in
158 a final concentration of 2.5% methanol in a total volume of 195 µl. The estradiol
159 concentration was varied between 12.5 µM and 500 µM in final concentration. Samples were
160 then pre-incubated at 37 °C for 5 min. The reaction was initiated by adding 5 µl of 100 mM
161 UDPGA. After incubation at 37 °C for 15 min, the reaction was stopped by adding 200 µl of
162 ice-cold methanol. Reaction samples were then placed on ice for 15 min before centrifugation
163 at 750 *g* for 10 min. The resultant supernatant was injected into a liquid
164 chromatography/mass spectrometry (LC/MS) system. An HPLC system coupled with
165 electrospray ionization ion-trap mass spectrometry (ESI/MS/MS, LTQ Orbitrap: LC-8030

166 Shimadzu, Kyoto, Japan) was equipped with an Inertsil ODS-3 column (2.1 mm × 150 mm;
167 GL Sciences, Inc., Tokyo, Japan). The collision energies (CE) and other MS parameters were
168 optimized and are shown in Table S1. Mobile phase A consisted of 10 mM ammonium
169 acetate buffer (pH 5.0) and phase B consisted of phase A: acetonitrile (1:9 v/v). The solvent
170 gradient was as follows: 15% mobile phase B from 2 to 10 min followed by a linear gradient
171 to 90%, 90% mobile phase B from10 to 13 min and then 15% mobile phase B from 13 to 15
172 min. An injection volume of 5 µl, a flow rate of 0.3 ml/min, and a column temperature of
173 45 °C were used throughout.
174
175 *1-Hydroxypyrene glucuronidation assay.*
176 UGT activity of 1-hydroxypyrene was assessed using the method described by Ueda *et al.*
177 (2011) with slight modifications. Initially, 25 µl of hepatic microsome solution was mixed
178 with 22.5 µl of KPB (0.1 M, pH 7.4). The microsome preparation was then mixed with 2.5 µl
179 of 1% sodium cholate solution and incubated on ice for 30 min. Then, 50 µl of microsome
180 solution was mixed with KPB (0.1 M, pH 7.4), 5 µl of 100 mM $MgCl_2$, and 1-hydroxypyrene,
181 which was dissolved in methanol, resulting in a final concentration of 1.0% methanol, in a
182 total volume of 195 µl. The 1-hydroxypyrene concentration was varied between 10 µM and
183 500 µM in final concentration. Samples were then pre-incubated at 37 °C for 5 min. The
184 reaction was initiated by adding 5 µl of 100 mM UDPGA. After incubation at 37 °C for 10

min, the reaction was stopped by adding 400 µl of ice-cold methanol. Reaction samples were then placed on ice for 15 min before centrifugation at 750 *g* for 10 min. The resultant supernatant was injected into the HPLC system. Analysis was performed on an HPLC system (Shimadzu) using a fluorescence detector (FD) equipped with an Inertsil ODS-3 column (2.1 mm × 150 mm: GL Sciences, Inc.). Mobile phase A consisted of 10 mM ammonium acetate buffer (pH 5.0): acetonitrile (9:1, v/v) and phase B consisted of acetonitrile. The solvent gradient was as follows: 10% mobile phase B from 0 to 7 min followed by a linear gradient to 90% mobile phase B from 7 to 8 min, and then 10% mobile phase B from 8 to 10 min.

Acetaminophen glucuronidation assay.

First, 25 µl of hepatic microsome solution was mixed with 22.5 µl of KPB (0.1 M, pH 7.4). The microsome preparation was then mixed with 2.5 µl of 1% sodium cholate solution and incubated on ice for 30 min. Then, 50 µl of microsome solution was mixed with KPB (0.1 M, pH 7.4), 5 µl of 100 mM $MgCl_2$, and acetaminophen in a total volume of 195 µl. The acetaminophen concentration was varied between 0.5 mM and 30 mM in final concentration. Samples were then pre-incubated at 37 °C for 5 min. The reaction was initiated by adding 5 µl of 100 mM UDPGA. After incubation at 37 °C for 15 min, the reaction was stopped by adding 200 µl of ice-cold methanol. Reaction samples were then placed on ice for 15 min before centrifugation at 750 *g* for 10 min. The resultant supernatant was injected into the

LC/MS system. An HPLC system coupled with electrospray ionization ion-trap mass spectrometry (ESI/MS/MS, LTQ Orbitrap: LC-8030, Shimadzu) equipped with a Synergi 4u Polar-RP 80A column (2.0 mm × 150 mm: Phenomenex, CA, USA) was used. The collision energies (CE) and other MS parameters were optimized and are shown in Table S1. Mobile phase A consisted of 0.1% formic acid and phase B of 0.1% formic acid in acetonitrile. The solvent gradient was as follows: 10% mobile phase B from 0 to 8 min followed by a linear gradient to 30%, 30% mobile phase B from 8 to 9 min followed by a linear gradient to 100%, 100% mobile phase B from 9 to 10 min and then 15% mobile phase B from 10 to 15 min. An injection volume of 5 µl, a flow rate of 0.2 ml/min, and a column temperature of 45 °C were used throughout.

UGT1A6 exon 1 partial cloning.

Genomic DNA (gDNA) was isolated from livers of three Pinnipedia species (n=4 for each species) using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). DNA concentrations were measured using NanoDrop ND-1000 (Thermo-Scientific, Waltham, MA, USA). Primer F447 and R448 designed by Shrestha *et al.* (2011) were slightly modified; our primer designs are shown in Table S2. UGT1A6 exon 1 was amplified by genomic PCR using the UGT1A6 specific primers. PCR amplification was performed with Tks Gflex polymerase (TAKARA BIO INC., Otsu, Japan) on 1 µl gDNA in a total PCR reaction volume

of 20 µl. PCR products were purified using a PCR purification kit (Qiagen, Valencia, CA, USA). PCR products were sequenced directly using an ABI PRISM 310 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA). PCR products were initially confirmed as UGT1A6 by phylogeny analysis of UGT1A genes.

UGT1A1/1A02 partial cloning.

Total RNA was isolated from livers of three Pinnipedia species (n=4 for each species) using NucleoSpin RNA (TAKARA BIO INC.). Total RNA concentration was measured using NanoDrop ND-1000 (Thermo-Scientific). Oligo-dT primed cDNA was synthesized using approximately 5 µg of total RNA. UGT1A1/1A02 were amplified by PCR using the UGT1A1/1A02 specific primers. Each primer design is shown in Table S2. PCR amplification was performed with Tks Gflex polymerase (TAKARA BIO INC.) on 1 µl complementary DNA (cDNA) in a total PCR reaction volume of 40 µl. PCR products were purified using a PCR purification kit (Qiagen) and subjected to an A-attachment reaction using 10× A-attachment mix (TOYOBO, Osaka, Japan). PCR products were ligated into the pCR/2.1 TOPO-TA vector using a TOPO TA Cloning Kit (Invitrogen, Waltham, MA, USA) and transformed into DH5α-competent cells (TOYOBO). Plasmids were purified using a plasmid miniprep spin kit (Qiagen). Inserts were sequenced using an ABI PRISM 310 Genetic Analyzer (Life Technologies). PCR products were initially confirmed as either

UGT1A1 or UGT1A02 by phylogeny analysis of UGT1A genes. Gene accession numbers are shown in Table S3.

Phylogeny analysis of UGT1A genes.

Phylogenetic analysis was performed on the UGT1A genes of human, rat, dog, cat, ferret, and Pacific walrus, which were retrieved using National Center for Biotechnology Information (NCBI) BLAST search. The deduced amino acid sequences were aligned using MUSCLE (Multiple Sequence Comparison by Log-Expectation) and employed for model selection and construction of maximum likelihood trees (bootstrapping = 100) using MEGA5 (Tamura *et al.* 2011). The best model (JTT+G model) was used. All positions containing gaps and missing data were eliminated. Marbled flounder (*Pleuronectes yokohamae*) UGT1B1/1B2 and human UGT2A1/2B4 genes were used as outgroup genes. Details of analyzed genes are shown in Table S4. Consensus tree of Pinnipedia was visualized using TreeView package (Page 1996)

Synteny analysis of UGT1A genes.

Sequence data from genome projects are freely available. NCBI's MapViewer (http://www.ncbi.nlm.nih.gov/projects/mapview/) was used to visualize chromosomal synteny maps for each species. The latest genome assemblies were used: human Annotation Release 106, rat Annotation Release 104, dog Annotation Release 103, cat Annotation

Release 100, ferret Annotation Release 100, Pacific walrus Annotation Release 100 and chicken Annotation Release 102. UCSC BLAT (http://genome.ucsc.edu/index.html) was used for additional confirmation of missing genes. Orthologous relationships were confirmed by NCBI BLAST search. Synteny analysis for dog and rat UGT1A genes was conducted with reference to Li and Wu (2007) and Mackenzie *et al.* (2005), respectively.

Statistical analysis.

All kinetics parameters, including maximum velocity (Vmax), the Michaelis–Menten constant (Km), and the Vmax/Km ratio were determined using the Michaelis–Menten equation and GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). Statistics were performed using JMP® 11 (SAS Institute Inc., Cary, NC, USA). In Tukey's HSD test for Vmax/Km of each substrate for five species except dog (n=1), differences at $p<0.05$ were considered statistically significant for all analyses.

RESULTS

UGT activity in Carnivora liver microsomes

The Michaelis–Menten plots of estradiol-3-glucuronide represent UGT activities in each species are shown in Fig. 1A. In general, the Vmax, Km, and intrinsic clearance (Vmax/Km) values for estradiol-3-glucuronide showed smaller differences across all six species than values for other substrates (Table 2).

In contrast to the estradiol-3-glucuronide results, there were large inter-species differences in both 1-hydroxypyrene and acetaminophen glucuronidation, as shown in Fig. 1B and Fig. 1C. In cat and Pinnipedia livers, the Vmax/Km values for 1-hydroxypyrene glucuronide ranged from 14.5 to 76.1 µl/min/mg, which was significantly lower than that for rat (456.2±208.8 µl/min/mg) (Table 2). Furthermore, the highest Vmax/Km value (662.2 µl/min/mg) was recorded in dog. The same trend was observed in acetaminophen-glucuronidation. In cat, UGT activity was too low to calculate Vmax and Km. The Vmax/Km values in the three Pinnipedia species (3.9±4.5 to 20.9±11.2 µl/min/mg) were less than one-fifth of the value measured in rat (315.0±179.7 µl/min/mg). Even the “albumin and/or fatty acid effect” were not considered in the present study, the data were prepared similarly across species (Rowland et al. 2008).

Cloning of UGT1A genes (1A1, 1A02, and 1A6) in Pinnipedia

As shown in Fig. 2, two mutation sites in UGT1A6 exon 1 in Northern fur seal and Steller sea lion were found; the former is a two-nucleotide insertion that resulted in a premature stop codon and the latter is a one-nucleotide insertion. In Caspian seal, there was no mutation in the UGT1A6 exon 1 partial sequence and UGT1A6 is considered functional. UGT1A6 pseudogenes have been found in Felidae, brown hyena, and Northern elephant seal (Shrestha *et al.* 2011). In the current study, a UGT1A6 pseudogene was found in Otariidae for the first time. Pinnipedia is divided to three families: Phocidae (true seals), Otariidae (fur seals and sea lions) and Odobenidae (walrus). The UGT1A6 of Harbor seal and Caspian seal are suggested to be functional from the results of this study and Shrestha *et al.* (2011).

Cloned sequences were confirmed as UGT1A1 and UGT1A02 homologous genes in Steller sea lion, Northern fur seal and Caspian seal from phylogenetic analysis (Supplementary figure S2, each UGT genes were named by UGT nomenclature committee; https://www.flinders.edu.au/medicine/sites/clinical-pharmacology/ugt-homepage.cfm). The amino acid 354–397 segment of UGT1A1 and UGT1A02 in the three species has been identified as the UGT signature sequence (Meech *et al.* 2012). This signature sequence is a part of the UDPGA-binding region and includes residues interacting with UDPGA (Li and Wu, 2007).

UGT1A genes were divided into four clusters based on the phylogenetic tree

312 As shown in Fig. 3, UGT1A genes were divided to 1A1, 1A6, and two other clusters, i.e.
313 UGT1A2–1A5 and UGT1A7–1A10. Gene names in dog, ferret, and Pacific walrus were
314 sometimes different within a classified cluster because the genes are registered as transcript
315 variants in the NCBI database. In the UGT1A2–1A5 and UGT1A7–1A10 cluster, genes in
316 the same species are closely related (e.g., human UGT1A3, 1A4, and 1A5). These results
317 indicate that UGT1A1 and UGT1A6 are orthologous among species; on the other hand,
318 UGT1A2–1A5 and UGT1A7–1A10 are paralogous. All species used in the phylogenetic
319 analysis have the UGT1A1 gene and at least one UGT1A2–1A5 paralogous gene. The
320 numbers of total UGT1A genes in cat, ferret, and Pacific walrus were smaller than those in
321 human, rat, and dog (Table 3).
322
323 ***UGT1 locus is highly conserved across mammalian species***
324 The UGT1A locus of all of the mammalian species was used in synteny analysis; it is located
325 between the MROH2A (Maestro Heat-Like Repeat Family Member 2A) and USP40
326 (Ubiquitin Specific Peptidase 40) genes, and the DnaJB3 [DnaJ (Hsp40) Homolog,
327 Subfamily B, Member 3] gene is in its middle (Fig. 4). The distance between MROH2A and
328 DnaJB3 is approximately 20 kb in all analyzed species, whereas the distance between USP40
329 and DndJB3 varies from 38.2 kb to 177.0 kb depending on species. The distance between the
330 USP40 and DnaJB3 genes is shorter in cat, ferret, and Pacific walrus, which have the smallest

number of UGT1A genes, compared with human, rat, and dog. This increased distance between the MROH2A and USP40 genes is reasonable because of UGT1A gene duplication events. In chicken, the UGT1A locus is located next to USP40 and has variable exon 1 and shared exons 2–5, similar to mammals. Therefore, the UGT1A locus in all mammals is considered to be conserved between the MROH2A and USP40 genes.

The number of genes in each UGT1A2–1A5 and UGT1A7–1A10 cluster varied depending on species. Some pseudogenes were observed in human, rat, and dog in both clusters. However, only one functional gene in each cluster was found form the current database in cat, ferret, and Pacific walrus, except for the UGT1A7–1A10 cluster in cat, in which there was none. Therefore, species-specific gene duplications occurred and some genes became pseudogenes subsequently.

Whereas UGT1A1 was a functional gene in all analyzed mammalian species, UGT1A6 was a pseudogene in cat. Pacific walrus may lack a functional UGT1A6, since a homologous gene to UGT1A6 was not found in its current genome data.

DISCUSSION

Determining the effects of environmental contaminants on marine mammals requires not only data on contaminant concentrations but also xenobiotic metabolism in the body.

UGT1A6 pseudogene in Pinnipedia; its Phylogenetic timing and causes.

UGT1A6 plays an important role in glucuronidation of xenobiotics, especially phenolic compounds (Maruo *et al.* 2005). In this study, glucuronidation activities for both 1-hydroxypyrene and acetaminophen in Pinnipedia were as low as those in cat and significantly lower than in rat. Furthermore, UGT1A6 became a pseudogene in Steller sea lion and Northern fur seal. The results of this study indicated that UGT1A6 pseudogene is one factor responsible for low UGT activity for xenobiotic substrates in Pinnipedia. On the other hand, based on the results of the synteny analysis, there is a possibility that UGT1A6 is also becomes pseudogene in Pacific walrus. If this species has a UGT1A6 mutation, the mutation point of UGT1A6 would then be located before the divergence between Otariidae and Odobenidae. Phylogenetic timing of UGT1A6 mutations was estimated from results of UGT1A6 cloning in Pinnipedia, and is shown in Fig. 5. Since Northern fur seal, which is supposed to be a basal split within Otariidae, has a UGT1A6 pseudogene, all species in Otariidae may have a UGT1A6 pseudogene. On the other hand, within Phocidae, the exact mutation point of UGT1A6 is still unknown. Phocidae is divided to two subfamilies;

Phocinae (northern true seals) and Monachinae (monk seals and southern true seals). Northern elephant seal is the only species in Monachinae for which UGT1A6 was cloned. There is a possibility that UGT1A6 is a pseudogene in all Monachinae species. UGT1A6 mutations occurred independently in Phocidae and Otariidae, since mutation sites in Steller sea lion and Northern fur seal were different from that in Northern elephant seal. The basal pinniped split between Phocidae and Otarioidea (Otariidae and Odobenidae) is estimated to have taken place 33 million years ago (MYA) and the split between Otariidae and Odobenidae about 20 MYA (Arnason *et al.* 2006). Fixation of Felidae UGT1A6 mutations was estimated to occur between 10.8 and 36.5 MYA (Shrestha *et al*. 2011). Therefore, UGT1A6 mutations in Pinnipedia and Felidae are considered to have occurred independently but with similar timing.

Although UGT1A6 appears highly conserved across species based on the phylogenetic and synteny analysis of the UGT1 family, UGT1A6 mutations did occur in Felidae and some Pinnipedia species. UGT1A6 plays a central role in xenobiotic metabolism (especially of phenolic compounds) in the UGT1 family. It is likely that a high-protein feeding resulted in UGT1A6 becoming a pseudogene. Shrestha *et al.* (2011) proposed that low dietary content of plant-derived phenolic intoxicants may have been one factor that primed the pseudogenization of UGT1A6 in Felidae. Since Pinnipedia species typically eat fish and squid and occupy high trophic levels in marine ecosystems (Pauly *et al.* 1998), they are

384 hardly exposed to dietary phytotoxins. Therefore, this hypothesis seems reasonable for
385 Pinnipedia as well.
386
387 ***Low glucuronidation activity observed in Caspian seal; Factors other than UGT1A6***
388 ***pseudogenation.***
389 Our results showed that Caspian seal UGT xenobiotic activity was as low as that of Steller
390 sea lion and Northern fur seal, even though Caspian seal UGT1A6 is not a pseudogene.
391 Similar contradictory results were reported for ferret: i.e., that glucuronidation of
392 acetaminophen is slow compared with other species, although a mutation of ferret UGT1A6
393 has not been found (Court. 2002). Factors other than the UGT1A6 pseudogene may affect
394 inter-species differences in UGT activity in xenobiotic metabolism.
395 Multigene families are thought to have undergone birth and death evolution, where new
396 genes are created by gene duplication and some duplicate genes remain in the genome for a
397 long time, whereas others are deleted or become pseudogenes through deleterious mutations
398 (Nei and Rooney, 2005). Some xenobiotic-metabolizing enzymes such as CYP, GST, and
399 arylamine N-acetyltransferase (NAT) genes are suggested to have undergone rapid
400 birth-and-death evolution and positive selection by environmental changes (Da Fonseca *et al.*
401 2010; Sabbagh *et al.* 2013; Thomas. 2005).
402 In the present study, the molecular evolution of the UGT1 family was estimated from the

403 results of phylogenetic and synteny analysis of UGT1 genes. The early ancestor of mammals
404 was considered to have only two UGT genes, equivalent to UGT1A1 and UGT1A6, for
405 endobiotic and xenobiotic metabolisms, respectively, and duplication of these genes may
406 have occurred. Similarly, Zhang *et al.* (2004) insisted that the variable exons of the bilirubin
407 and phenol groups appear to have duplicated separately from two ancestral variable exons.
408 Sub-functionalization should have occurred with the first gene duplication of the UGT1A1
409 orthologous gene, since only UGT1A1 in mammals can conjugate bilirubin (Bosma *et al.*
410 1994). The two duplicated genes, which are indicated as UGT1A2–1A5 and 1A7–1A10
411 paralogous genes, have functions similar to UGT1A1 and UGT1A6, respectively; therefore,
412 their mutation and change is less constrained than UGT1A1 or UGT1A6. The biological
413 stability of the duplicated gene varied in each mammalian species depending on the need for
414 xenobiotic glucuronidation. This is why gene duplication and/or loss of UGT1A2–1A5 and
415 UGT1A7-1A10 paralogous genes are species specific and have occurred easily, whereas
416 UGT1A1 and UGT1A6 are highly conserved in mammals. All of the UGT1 genes have
417 distinct but overlapping substrate specificity (Maruo *et al.* 2005; Tukey and Strassburg, 2000).
418 This suggests neo-functionalization and/or sub-functionalization occurred in the process of
419 gene duplication. Furthermore, the number of UGT1A7–1A10 genes, including pseudogenes,
420 was higher than the number of UGT1A2–1A5 genes in human and dog. This indicates that
421 gene duplication and loss have occurred more frequently for UGT1A7–1A10 than for

422 UGT1A2–1A5 (Fig. 4). These results indicated that xenobiotic metabolizing UGT genes have
423 gone through rapid birth and death evolution (frequent gene duplication and loss) in response
424 to environmental conditions similar to other xenobiotic metabolizing enzymes such as CYP
425 genes.
426 Although, the genome information of some species is not completed, and going to be updated,
427 the synteny analysis from current information (Fig. 4) indicated that human, rat, and dog had
428 more than two genes in both the UGT1A2–1A5 and UGT1A7–1A10 clusters. In contrast, cat,
429 ferret, and Pacific walrus had only one gene in each cluster; and indeed, no gene in the
430 UGT1A7–1A10 cluster could be found in cat. This difference suggests that duplication of the
431 UGT1A2–1A5 and UGT1A7–1A10 genes may not have occurred in cat, ferret, or Pacific
432 walrus.
433 In human and rat, both UGT1A6 and UGT1A7–1A10 paralogous genes can conjugate
434 acetaminophen, and they contribute UGT activity in xenobiotic metabolism generally.
435 UGT1A6–1A10 are collectively referred to as a phenol group because these genes mainly
436 conjugate xenobiotics including phenolic compounds. UGT1A9 protein expression levels in
437 human liver are reportedly similar or some time higher than UGT1A6 levels (Harbourt *et al.*
438 2012, Sato et al. 2014). The low xenobiotic glucuronidation in Caspian seal and ferret
439 observed in UGT1 genetic analysis in mammals may be related to the small number of
440 UGT1A7–1A10 genes compared with other species such as rat and dog. The small number of

441 UGT1A genes accounts for low UGT xenobiotic activity in cat and Pinnipedia. The
442 difference in the UGT1 isoform number may cause inter-species differences in the total
443 UGT1 expression level and/or the proportion of the expression level in each UGT1A isoform.
444
445 Gene duplications and losses of UGT1A in mammalian species are considered
446 species-specific and to have occurred depending on the exposure levels of natural xenobiotics
447 such as phytotoxins and animal toxins. Since some species (e.g., Felidae and Pinnipedia)
448 have been less exposed and therefore do not require a high metabolic capacity for these
449 toxicants, these species have fewer UGT1 genes and low UGT activities for xenobiotic
450 substrates. Now, all animal species, including cat and Pinnipedia, are exposed to various
451 environmental chemicals metabolized by UGT. Species with low glucuronidation capacities
452 for xenobiotics could be highly sensitive to them. Risk assessments for xenobiotics
453 metabolized by UGT1 family are needed for these species.

SUPPLEMENTARY DATA

Supplementary data (Table S1-S4 and Fig. S1-S4) are available online at http://toxsci.oxfordjournals.org/.

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588

589 **FIGURE LEGEND**

590

591 Fig.1. Enzyme kinetics for the glucuronidation of three substrates in rat, dog, cat, Steller sea
592 lion, Northern fur seal and Caspian seal liver microsomes. (A) β-Estradiol-3 glucuronidation,
593 (B) 1-hydroxypyrene glucuronidation, (C) acetaminophen glucuronidation, Data represent the
594 mean ±SD for multiple animals.

595

596 Fig. 2. UGT1A6 exon 1 mutations in Steller sea lion and Northern fur seal. (A) 2 base pairs
597 nucleotide insertion resulting in a reading frame shift and premature stop codon in Steller sea
598 lion and Northern fur seal. (B) 1 base pair insertion resulting in a reading frame shift in
599 Steller sea lion and Northern fur seal.

600

601 Fig. 3. Phylogenetic tree of UGT1A amino acid sequences from human, rat, dog, cat, ferret
602 and Pacific walrus. The numbers next to the branches indicate the number of occurrences per
603 100 bootstrap replicates. Gene names followed the registered names in the NCBI database.
604 The UGT1 family in mammalian species was divided into four clusters: UGT1A1, UGT1A6,
605 UGT1A2-UGT1A5 and UGT1A7-1A10.

606

607 Fig. 4. Shared synteny of UGT1A locus. Each locus contains multiple first exons and

608 constant exon 2-5. The question mark in the chicken gene indicates an uncharacterized
609 protein, and white background genes are different from UGT genes.
610
611 Fig. 5. Phylogenetic tree of Pinnipedia and timing of UGT1A6 mutation.
612 Phylogenetic tree was constructed based on previous studies (Tara Lynn Fulton, 2010; Jochen
613 BW Wolf, 2007; Takahiro Yonezawa, 2009). Insufficient supported nodes were collapsed into
614 polytomies.

Table 1. Details of liver samples used in this study.

Common name	Steller sea lion	Northern fur seal	Caspian seal	dog (beagle)	cat	SD rat
Scientific name	*Eumetopias jubatus*	*Callorhinus ursinus*	*Phoca caspica*	*Canis lupus familiaris*	*Felis catus*	*Rattus norvegicus*
Sample size	4	4	4	pool	2	4
Gender	Female	Female	Female	Female	Female	Female
Year	2003	1997-1998	1998	2014	2010	2014
Location	Rausu	Sanriku	Pearl island	purchased	Osaka	purchased
Age group	mature	mature	mature	≧1 year	3, 15 year	8 week

Dog liver microsomes were purchased from BD Biosciences (San Jose, CA, USA).
Sprague-Dawley rats were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan)

Table 2. Michaelis-Menten constants for glucuronidation.

	SD rat	cat	Steller sea lion	Northern fur seal	Caspian seal	dog
β-Estradiol (UGT1A1)						
Vmax/Km (μl/min/mg)	11.6±4.34 ab	4.34±1.26 abc	30.2±9.21 c	28.9±12.8 ac	6.80±0.60 b	7.04±3.4
Vmax (pmol/min/mg)	2.43×10²±53.5	2.04×10²±26.3	5.05×10²±87.1	7.19×10²±1.73×10²	2.35×10²±22.8	1.28×10²±0.03
Km (μM)	21.8±4.31	47.2±20.9	17.6±4.21	26.5±4.65	35.0±6.04	18.2±2.18
1-hydroxypyren (UGT1A6,1A7,1A9)						
Vmax/Km (μl/min/mg)	4.56×10²±2.09×10² a	14.5±6.26 b	76.1±26.1 b	74.4±19.3 b	36.6±33.3 b	6.62×10²±1.62×10²
Vmax (nmol/min/mg)	8.23±1.14	2.99±0.43	2.40±1.29	3.61±2.13	2.61±0.70	17.4±0.46
Km (μM)	20.7±8.24	2.06×10²±64.4	36.7±29.4	53.1±36.1	1.06×10²±56.9	26.3±2.83
acetaminophen (UGT1A1,1A6,1A7)						
Vmax/Km (μl/min/mg)	3.15×10² ±1.80×10² a	N.D.	20.9±11.2 b	17.4±9.4 b	3.9±4.5 b	30.0±19.8
Vmax (nmol/min/mg)	2.81±0.64	N.D.	2.73±1.16	0.93±0.66	2.60±3.33	1.35±0.28
Km (mM)	10.8±5.60	N.D.	1.63×10²±96.8	47.6±21.0	3.71×10²±2.95×10²	45.0±14.2

Values represent mean ± SD for multiple animals (SD rat, cat, Steller sea lion, Northern fur seal, Caspian seal).
The values of dog show the mean ± SD for duplicated measurement.
Due to low glucuronidation activity in Cat, fitting was not possible for acetaminophen glucuronide.
Km/Vmax values of each species that were significantly different ($P<0.05$) in Tukey's HSD tests are indicated to the different alphabets.

Table 3. UGT1A gene numbers in each species.

	human	rat	dog	cat	ferret	Pacific walrus
1A1	1	1	1	1	1	1
1A2-1A5	3	3	3	1	1	1
1A6	1	1	1	0	1	0
1A7-1A10	4	2	5	0	1	1
total	9	7	10	2	4	3

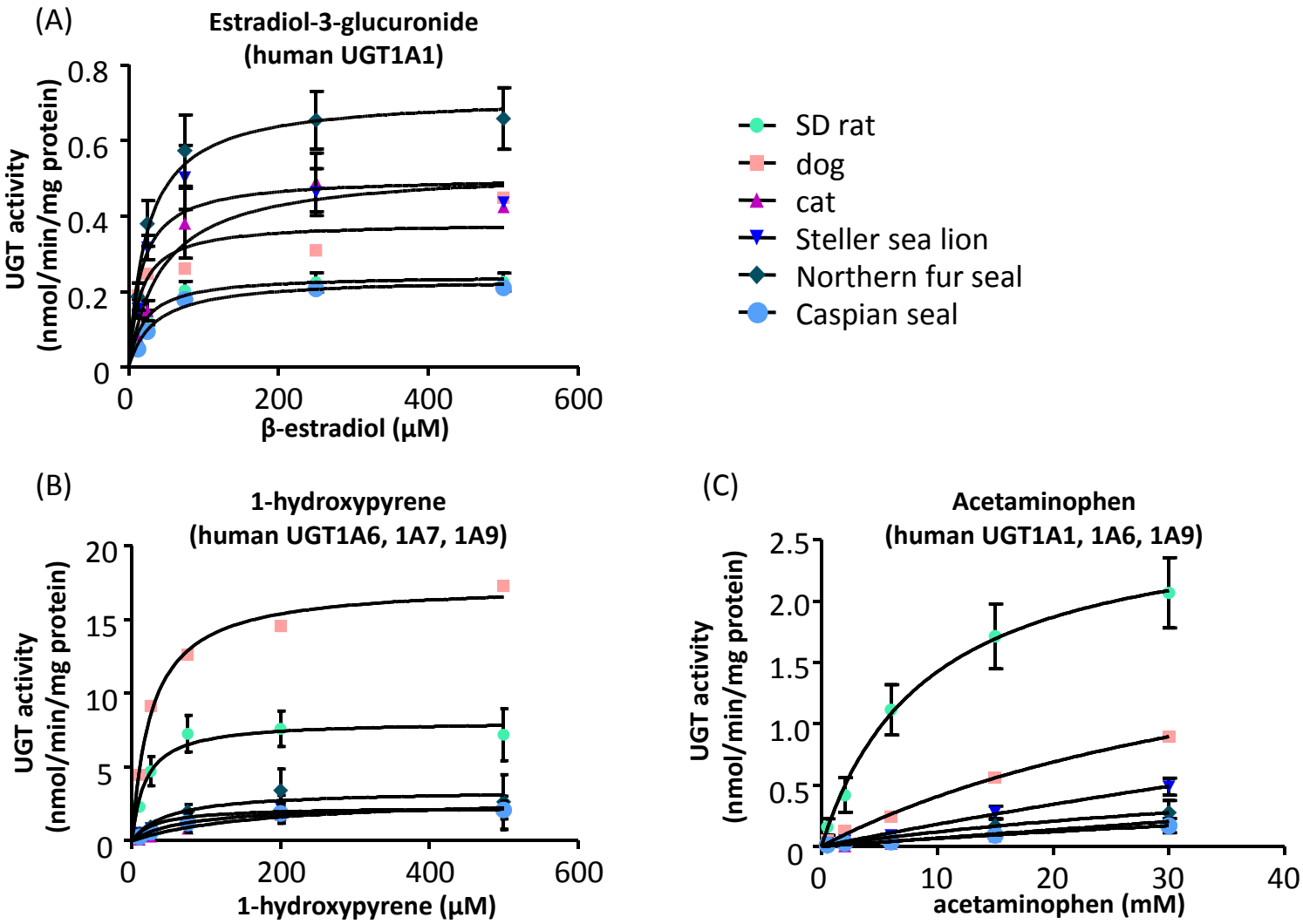

(A)
Estradiol-3-glucuronide
(human UGT1A1)
UGT activity
(nmol/min/mg protein)
0.8
0.6
0.4
0.2
0
0
200
400
600
β-estradiol (μM)
SD rat
dog
cat
Steller sea lion
Northern fur seal
Caspian seal
(B)
1-hydroxypyrene
(human UGT1A6, 1A7, 1A9)
UGT activity
(nmol/min/mg protein)
20
15
10
5
0
0
200
400
600
1-hydroxypyrene (μM)
(C)
Acetaminophen
(human UGT1A1, 1A6, 1A9)
UGT activity
(nmol/min/mg protein)
2.5
2.0
1.5
1.0
0.5
0
0
10
20
30
40
acetaminophen (mM)

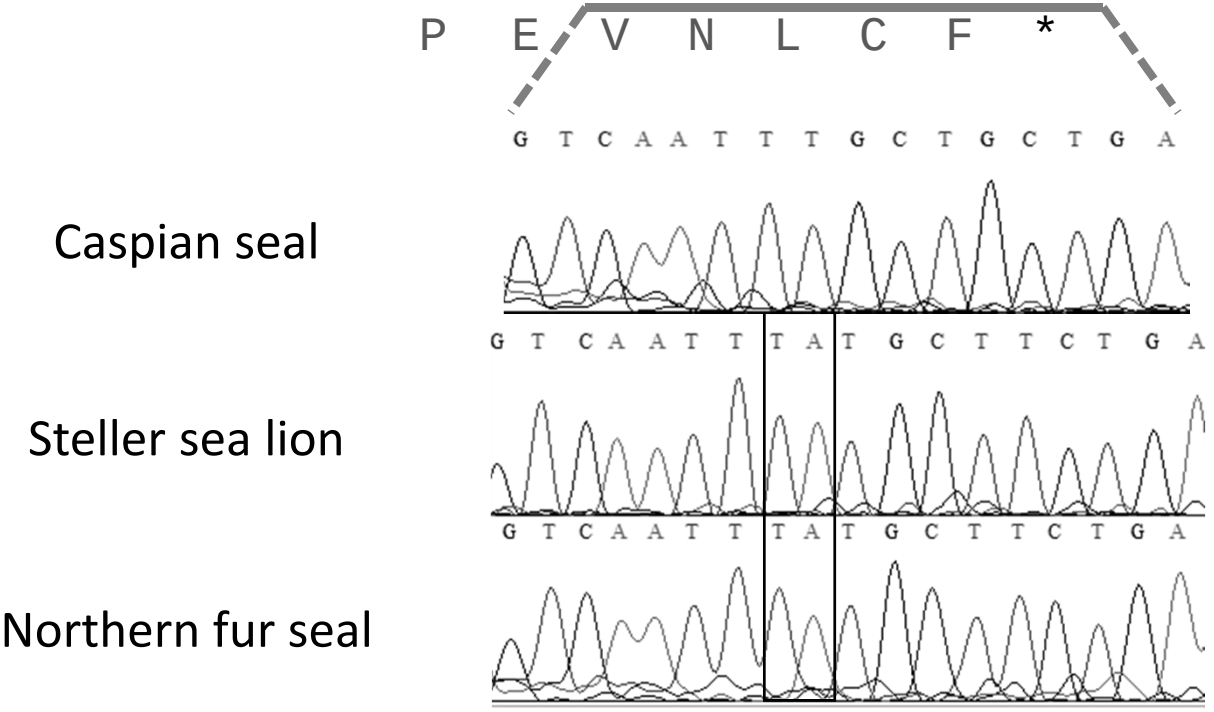
Fig. 1.

(A)

2 bp nucleotides insertion

```
                167                                        197
Dog             TGCCAGAAGTCAATT --GCTTATGAAGGAATC
                  P  E  V  N  L    L  L  K  E

Caspian seal    TACCAGAAGTCAATT--TGCTGCTGAAGGAATC
Steller sea lion TACCAGAAGTCAATTTATGCTTCTGAAGGAATC
Northern fur seal TACCAGAAGTCAATTTATGCTTCTGAAGGAATC
                  P  E  V  N  L  C  F  *
```



(B)

1 bp nucleotide insertion

```
                493                                        518
Dog             TACCTCTTCA-GGGGCTTCCCATGCTC
Caspian seal    TACCTCTTCA-GGGGCTTCCCATGCTC
Steller sea lion TACCTCTTCAGGGGGCTTCCCATGCTC
Northern fur seal TACCTCTTCAGGGGGCTTCCCATGCTC
```

Fig. 2.

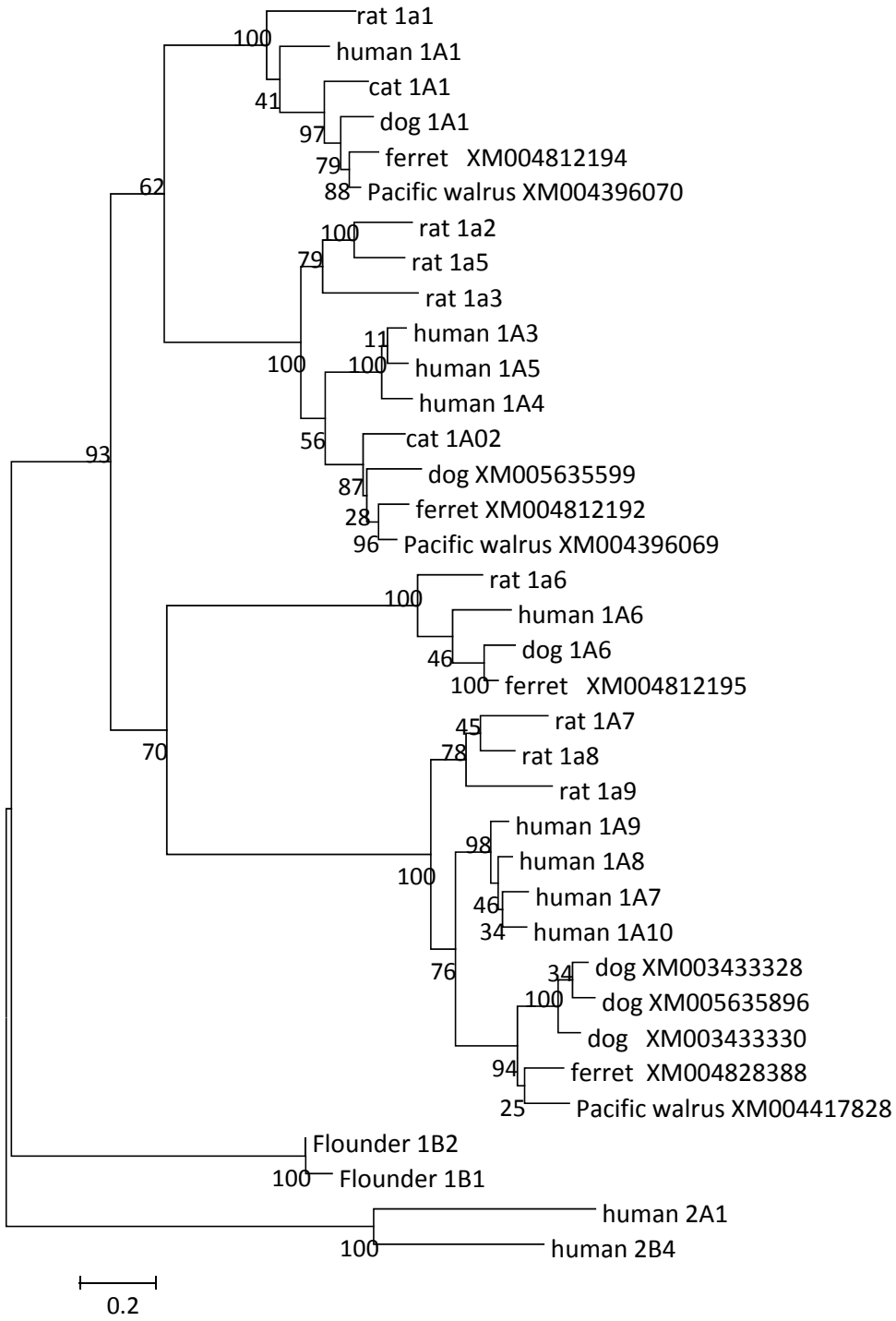

rat 1a1
100
human 1A1
cat 1A1
41
dog 1A1
97
ferret XM004812194
79
Pacific walrus XM004396070
88
62
rat 1a2
100
rat 1a5
79
rat 1a3
human 1A3
11
100
human 1A5
100
human 1A4
cat 1A02
56
93
dog XM005635599
87
ferret XM004812192
28
Pacific walrus XM004396069
96
rat 1a6
100
human 1A6
dog 1A6
46
ferret XM004812195
100
rat 1A7
45
rat 1a8
78
70
rat 1a9
human 1A9
98
human 1A8
100
human 1A7
46
human 1A10
34
dog XM003433328
34
76
dog XM005635896
100
dog XM003433330
ferret XM004828388
94
Pacific walrus XM004417828
25
Flounder 1B2
100
Flounder 1B1
human 2A1
100
human 2B4
0.2

Fig. 3.

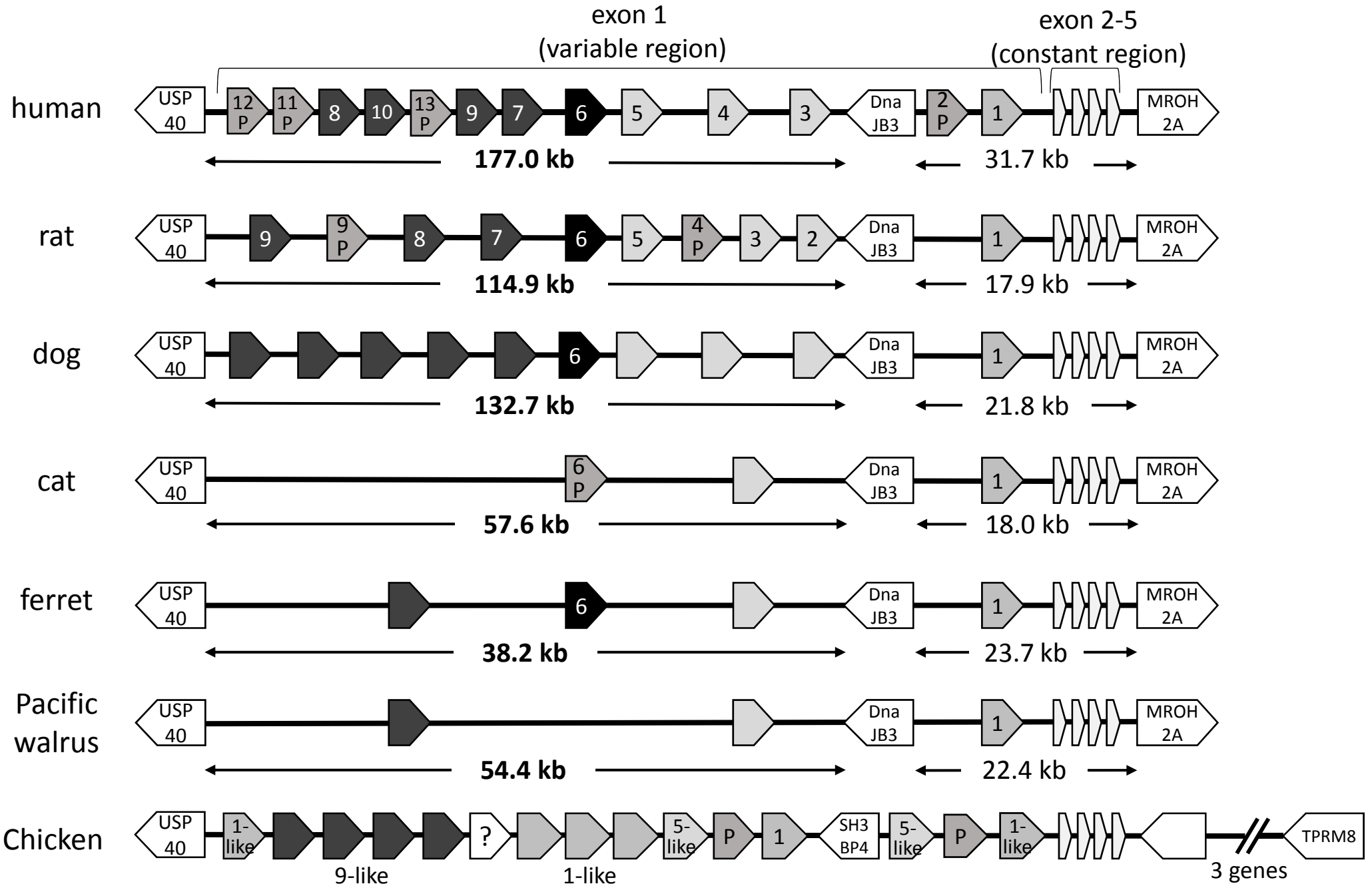

exon 1
(variable region)
exon 2-5
(constant region)
human
USP 40
12 P
11 P
8
10
13 P
9
7
6
5
4
3
Dna JB3
2 P
1
MROH 2A
177.0 kb
31.7 kb
rat
USP 40
9
9 P
8
7
6
5
4 P
3
2
Dna JB3
1
MROH 2A
114.9 kb
17.9 kb
dog
USP 40
6
Dna JB3
1
MROH 2A
132.7 kb
21.8 kb
cat
USP 40
6 P
Dna JB3
1
MROH 2A
57.6 kb
18.0 kb
ferret
USP 40
6
Dna JB3
1
MROH 2A
38.2 kb
23.7 kb
Pacific walrus
USP 40
Dna JB3
1
MROH 2A
54.4 kb
22.4 kb
Chicken
USP 40
1-like
?
5-like
P
1
SH3 BP4
5-like
P
1-like
TPRM8
9-like
1-like
3 genes

Fig. 4.

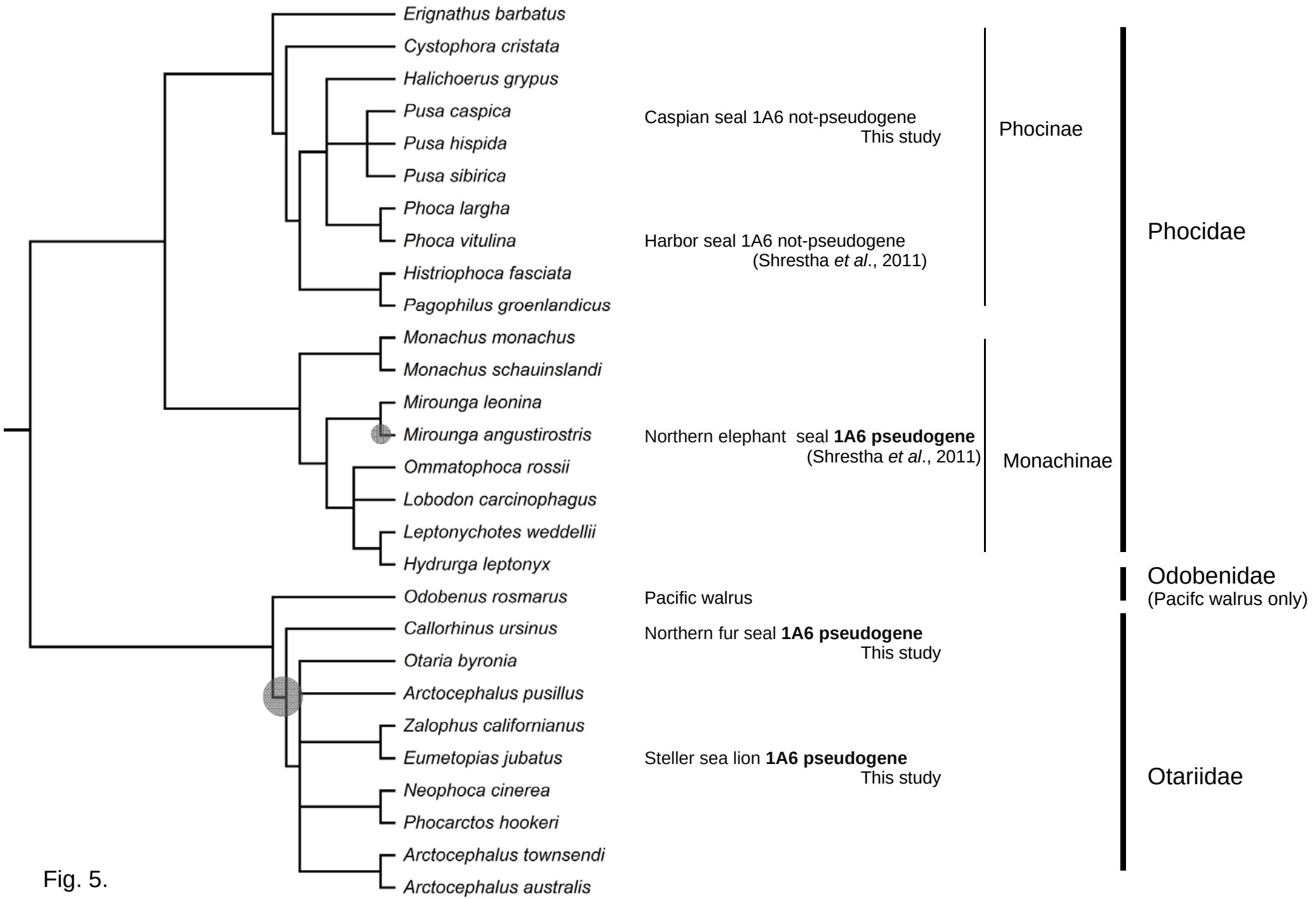

Erignathus barbatus
Cystophora cristata
Halichoerus grypus
Pusa caspica
Pusa hispida
Pusa sibirica
Phoca largha
Phoca vitulina
Histriophoca fasciata
Pagophilus groenlandicus
Monachus monachus
Monachus schauinslandi
Mirounga leonina
Mirounga angustirostris
Ommatophoca rossii
Lobodon carcinophagus
Leptonychotes weddellii
Hydrurga leptonyx
Odobenus rosmarus
Callorhinus ursinus
Otaria byronia
Arctocephalus pusillus
Zalophus californianus
Eumetopias jubatus
Neophoca cinerea
Phocarctos hookeri
Arctocephalus townsendi
Arctocephalus australis
Caspian seal 1A6 not-pseudogene
This study
Harbor seal 1A6 not-pseudogene
(Shrestha *et al*., 2011)
Northern elephant seal **1A6 pseudogene**
(Shrestha *et al*., 2011)
Pacific walrus
Northern fur seal **1A6 pseudogene**
This study
Steller sea lion **1A6 pseudogene**
This study
Phocinae
Monachinae
Phocidae
Odobenidae
(Pacifc walrus only)
Otariidae

Fig. 5.