Sulfotransferases (SULTs), enzymatic and genetic variation in Carnivora: Limited sulfation capacity in pinnipeds.

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Abbreviations

AAALAC: Association for Assessment and Accreditation of Laboratory Animal Care

CE: collision energies

DW: distilled water

GSTs: glutathione-S transferase

HPLC: high-performance liquid chromatography

KO: knockout

KPB: potassium phosphate buffer

LC/MS: liquid chromatography/mass spectrometry

MEGA: Molecular Evolutionary Genetics Analysis

MUSCLE: Multiple Sequence Comparison by Log-Expectation

NATs: N-acetyltransferases

NCBI: National Center for Biotechnology Information

OCPs: organochlorine pesticides

PAHs: polyhalogenated aromatic hydrocarbons

PAPS: 3′-phosphoadenosine 5′-phosphosulfate

PBDE: polybrominated diphenyl ether

PCB: polychlorinated Biphenyl

SGF29: SAGA complex associated factor 29

SNP: single nucleotide polymorphism

STS: steroid sulfatase

SULTs: sulfotransferases

UGTs: UDP-glucuronosyltransferases

Abstract

Wild carnivorans are one of the most important species due to their high positions in the food chain. They are also highly affected by numerous environmental contaminants through bioaccumulation and biomagnification. Xenobiotic metabolism is a significant chemical defense system from xenobiotics because it degrades the activity of a wide range of chemicals, generally into less active forms, resulting in their deactivation. Sulfotransferases (SULTs) are one of the most important xenobiotic metabolic enzymes, which catalyze the sulfonation of a variety of endogenous and exogenous chemicals, such as hormones, neurotransmitters, and a wide range of xenobiotic compounds. Although SULTs are of such high importance, little research has focused on these enzymes in wild carnivorans. In this study, we clarified the genetic properties of SULTs in a wide range of mammals, focusing on carnivorans, using in silico genetic analyses. We found genetic deficiencies of SULT1E1 and SULT1D1 isoforms in all pinnipeds analyzed and nonsense mutations in SULT1Cs in several carnivorans including pinnipeds. We further investigated the enzymatic activity of SULT1E1 in vitro using liver cytosols from pinnipeds. Using a SULT1E1 probe substrate, we found highly limited estradiol sulfonation in pinnipeds, whereas other mammals had relatively high sulfation. These results suggest that pinnipeds have severely or completely absent SULT1E1 activity, which importantly catalyzes the metabolism of estrogens, drugs, and environmental toxins. This further implies a high susceptibility to a wide range of xenobiotics in these carnivorans, which are constantly exposed to environmental chemicals throughout their lifetime.

97 Keywords: Wildlife, xenobiotic metabolism, in silico analysis, genome database, phase
98 II metabolism
99

1. Introduction

Cytosolic sulfotransferases (SULTs) are an essential metabolic enzyme superfamily that catalyzes sulfate conjugation for various endogenous and exogenous compounds including neurotransmitters, hormones, drugs, and environmental toxins (Falany 1991; Blanchard et al. 2004; Gamage et al. 2006; Coughtrie 2016; Suiko et al. 2017). Using 3'-phosphoadenosine 5'-phosphosulfate (PAPS) as a sulfonate donor, SULTs transfer sulfuric moieties to acceptor compounds and alter their bioactivity, typically towards less active and more water-soluble forms, thus accelerating their excretion. SULTs are primarily major phase II xenobiotic detoxification enzymes, which catalyze conjugations after phase I reactions (oxidation, reduction, and hydrolysis), together with UDP-glucuronosyltransferases (UGTs), N-acetyltransferases (NATs), and glutathione-S transferase (GSTs) (Almazroo et al., 2017; Jancova et al., 2010; Oda et al., 2015).

The mammalian SULT superfamily consists of at least seven families, SULTs 1–7. The SULT1 family, also known as phenol-SULTs, is well characterized and is responsible for the metabolism of xenobiotics and a variety of endogenous chemicals (Blanchard et al. 2004, Coughtrie 2016). The SULT1 family is further divided into five different subfamilies including SULT1A, 1B, 1C, 1D, and 1E. Each subfamily has distinct substrate specificities, although some overlap exists. The substrate specificities of the SULT subfamilies are generally considered to be as follows: SULT1A members for simple phenols, 1B members for thyroid hormones, 1C members for hydroxyaryl amines, 1D members for catecholamines, and 1E members for estrogens (Kester et al., 2003; Sakakibara et al., 1998; Shimada et al., 2004; Suiko et al., 2017; Carrie Tsoi et al., 2001). Although SULT1 isoforms and their function have

been well characterized in humans, rodents, and a few other experimental animal models (Liu et al., 1999; Teramoto et al., 2008; Carrie Tsoi et al., 2001; Tsoi et al., 2002; Wilson et al., 2004), information is still limited in other mammalian species including wild mammals.

Wild mammals are continuously exposed to a vast variety of environmental chemicals, such as polyhalogenated aromatic hydrocarbons (PAHs) and organochlorine pesticides (OCPs) (Nomiyama et al., 2014; Noyes and Lema, 2015). Due to their high position in their respective food chains, carnivorans (the order of the placental mammals includes dogs, pinnipeds, weasels, racoons, bears, felines, mongooses, and hyenas) accumulate severe amounts of lipophilic environmental contaminants more so than other species (Huang et al., 2018; Johnson, 2019; Rodríguez-Estival and Mateo, 2019). Therefore, the toxicological assessment of carnivorans is critically needed. However, xenobiotic metabolic enzymes in wild mammals are not clearly understood. We recently reported the genetic deficiencies and in vitro enzymatic dysfunction of some UGTs (UGT1A6s and 2B31s) in feline and pinniped species, which suggest that these species may poorly metabolize chemical compounds (Kakehi et al. 2015; Kondo et al. 2017). Since UGTs and SULTs are known to have similar substrate specificities, and some excreted polyphenols and chemicals are glucuronide-sulfate double conjugated, UGTs and SULTs may play concerted roles in xenobiotic metabolism (Böhmdorfer et al., 2017; Suiko et al., 2017). Considering the synergistic actions of UGTs and SULTs, information about SULTs in wildlife carnivorous species should be elucidated to facilitate a comprehensive understanding of xenobiotic metabolism in these mammals. The importance of the SULT1 family in xenobiotic metabolism and the lack of information about its function have led us to investigate the

genetic and enzymatic features of SULTs in wild mammals including pinnipeds and felines.

In this study, the genetic information of the SULT1 isozymes of various carnivorans including pinnipeds and Felidae were collected from the NCBI GenBank data, and in silico phylogenetic analyses were conducted. In addition, gene loci coding SULT isoforms in these species were investigated and compared to understand the evolutionary background of each isoform. Furthermore, the in vitro SULT activities of cats, rats, and pinnipeds (northern fur seal, harbor seal, stellar sea lion) were measured using liver cytosolic fractions.

2. Materials and methods

2-1. Chemicals

β-Estradiol and PAPS were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, formic acid, sodium phosphate, and potassium dichromate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). β-Estradiol 3-(β-d-sulfate) sodium salt was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, 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).

2-2. Animals

Details about the animals used for liver cytosol preparations are provided in Supplementary Table S1. Liver samples were collected from northern fur seals (Callorhinus ursinus), harbor seals (Phoca vitulina), cats (Felis catus), and rats (Rattus

norvegicus; Sprague–Dawley strain). Harbor seal livers from Erimo were collected from individuals accidently captured by fishing nets and drowned. Northern fur seal livers were provided by the Environmental Specimen Bank (es-BANK: http://esbank-ehime.com/) of Ehime University. Eight-week-old rats were used as controls. Sprague–Dawley rats were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). Cats (Narc: Catus, 24–28 months old, male, weight: 1 kg) were purchased from Kitayama Labes Co., Inc. (Nagano, Japan). Seven-week-old rats were housed at a constant temperature (23°C ± 1°C) and constant humidity (55% ± 5%) with automatically controlled lighting (lights on from 07:00–19:00) and were given food and water ad libitum for one week prior to sacrifice. Rats and cats were kept in a 12-hour light/dark cycle (7:00–19:00 light, 19:00–7:00 dark) at 20 ± 1°C with 35 ± 5% humidity. Food (Royal Canin, Japan) and water were given appropriately twice a day. Cat livers were collected following anesthesia with pentobarbital and euthanasia by KCl injection. Dissections were performed by a qualified veterinarian. Liver samples from all five species were immediately frozen in liquid nitrogen and stored at –80°C until further use. All experiments and animal care for rats and cats were performed in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and under the supervision and with the approval of the Institutional Animal Care and Use Committee of Hokkaido University (no. 13-0213, no. 14-0054).

2-3. Measurements of in vitro SULT activity using carnivore liver cytosols

2-3-1. Preparation of liver cytosols

Liver cytosolic fractions were prepared as previously shown by Omura and Sato (1964). Briefly, approximately 5 g of liver tissue from each of the six species were homogenized in 15 mL of potassium phosphate buffer (KPB: 0.1 M, pH 7.4). Homogenates were transferred into tubes and centrifuged at 9,000 × g at 4°C for 20 minutes. The supernatants were further centrifuged at 105,000 × g for 70 minutes to separate microsomal and cytosolic fractions. The cytosolic fraction (supernatant) was transferred and stored at –80°C until further analysis. Protein concentrations in the cytosol were measured using a BCA (Bicinchoninic acid) protein assay reagent kit (Pierce, Rockford, IL, USA).

2-3-2. In vitro sulfation assay

SULT activities for each of the five substrates were assessed. First, 25 µL of hepatic cytosolic solution was mixed with 22.5 µL of KPB (0.1 M, pH 7.4). The cytosol preparation was mixed with 2.5 µL of 1% sodium cholate solution and incubated on ice for 30 minutes. 50 µL of cytosolic solution was mixed with KPB (0.1 M, pH 7.4), 5 µL of 100 mM MgCl2, and estradiol dissolved in methanol, resulting in a final concentration of 1.25% in a total volume of 195 µL. Final substrate concentrations varied from 12.5 µM to 400 µM for estradiol. Samples were preincubated at 37°C for 5 minutes, and the sulfation reaction was initiated by adding 5 µL of 100 mM PAPS. Samples were incubated for 15 minutes, and the reaction was stopped by adding 200 µL of ice-cold methanol. Reaction samples were then placed on ice for 15 minutes before centrifugation at 750 × g for 10 minutes. The resultant supernatants were injected into a liquid chromatography/mass spectrometry (LC/MS) system.

2-3-3. Analysis of sulfate metabolite by LC/MS/MS

An HPLC system coupled with electrospray ionization ion-trap triple-quadrupole mass spectrometry (ESI/MS/MS, LC-8030, Shimadzu, Kyoto, Japan) was equipped with a Wakopak® Ultra C18-3 column (2.0 mm × 100 mm; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Mobile phase A consisted of 0.1% formic acid in distilled water (DW), and phase B consisted of 0.1% formic acid in acetonitrile in all analyses. The percentage of mobile phase B was changed linearly as follows: 2 min, 30%; 25 min, 70%; 26 min, 90%; 28 min, 90%; and 30 min, 30%. The injection volume was 5 μL, the flow rate was 0.2 mL/min, and the column temperature was 40°C. The m/z of β-estradiol-3-sulfate was 351 > 271.

2-3-4. Data analysis

All kinetic parameters, including maximum velocity (Vmax), Michaelis–Menten constant (Km), and Vmax/Km ratio, were determined using the Michaelis–Menten equation and GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). Statistical analyses were performed using JMP® 12 (SAS Institute, Inc., Cary, NC, USA). Tukey's HSD test was used for the Vmax/Km of each substrate for each species; differences of $P < 0.05$ were considered statistically significant in all analyses.

2-4. In silico genetic analysis of SULTs in carnivores

2-4-1. Phylogenetic analysis of SULT genes

Phylogenetic analyses were performed on the SULT1 genes (SULT1As, 1B1, 1Cs, 1D1, 1E1) of human, rat, mouse, dog, red fox, domestic ferret, ermine, American river

243 otter, sea otter, polar bear, giant panda, brown bear, meerkat, striped hyena, cat, Amur
244 tiger, cheetah, puma, Canada lynx, leopard, Weddell seal, harbor seal, gray seal,
245 Hawaiian monk seal, northern fur seal, southern elephant seal, Stellar sea lion,
246 California sea lion, and Pacific walrus origins. Sequences were retrieved using
247 National Center for Biotechnology Information (NCBI) BLAST searches using human
248 and dog SULT1A1, 1B1, 1C1, 1C2, 1C3, 1E1 and SULT1D1 as the query sequence.
249 BLAST searches have been conducted for database Nucleotide collection (nr/nt) for
250 each species using Blastn (Optimize for somewhat similar sequences). The gene
251 sequences used are listed in Supplementary Table S2, and the protein coding region
252 of each isozyme was analyzed. The deduced amino acid sequences were aligned
253 using MUSCLE (Multiple Sequence Comparison by Log-Expectation) and were used
254 for model selection (model showing minimal set of BIC and AICc were chosen) and
255 construction of maximum likelihood trees (bootstrapping = 100) using MEGA X
256 (Molecular Evolutionary Genetics Analysis) (Kumar et al., 2018). The JTT+G model
257 was used. All positions containing gaps and missing data were eliminated, and total
258 924 bp length of protein-coding sequence alignment are used for phylogenetic
259 analysis. The results of phylogenetic analyses for human, mouse, rat, and dog SULT1
260 genes were examined in reference to the phylogenic analysis of published papers (C.
261 Tsoi et al. 2001; Blanchard et al. 2004) to ensure that the analysis was conducted
262 successfully.
263
264 *2-4-2. Synteny analysis of SULT1 genes*
265 Sequence data from genome projects are freely available. NCBI's genome data
266 viewer (https://www.ncbi.nlm.nih.gov/genome/gdv/) was used to visualize the

chromosomal synteny maps for each species. The following latest genome assemblies were used: human Annotation Release 106, rat Annotation Release 105, mouse Annotation Release 105, dog Annotation Release 103, cat Annotation Release 102, Weddell seal Annotation Release 100, red fox Annotation Release 100, domestic ferret Annotation Release 101, ermine Annotation Release 100, American river otter Annotation Release 100, sea otter Annotation Release 100, polar bear Annotation Release 101, giant panda Annotation Release 103, brown bear Annotation Release 101, meerkat Annotation Release 100, striped hyena Annotation Release 100, Amur tiger Annotation Release 100, cheetah Annotation Release 101, puma Annotation Release 100, Canada lynx Annotation Release 102, leopard Annotation Release 100, harbor seal Annotation Release 100, gray seal Annotation Release 100, Hawaiian monk seal Annotation Release 100, northern fur seal Annotation Release 100, southern elephant seal Annotation Release 100, Stellar sea lion Annotation Release 100, California sea lion Annotation Release 100, and Pacific walrus Annotation Release 101. UCSC (University of California, Santa Cruz) BLAT (BLAST-like alignment tool) (http://genome.ucsc.edu/index.html) was used for the additional confirmation of missing genes.

3. Results

3-1. In silico genetic analysis of the SULT1 family in carnivores

3-1-1. SULT1 family in carnivorans and phylogenetic analysis of SULT1s

Potential SULT1 family isoforms in carnivorans were retrieved using BLAST searches, and candidate isoforms equivalent to UGT1A1, 1B1, 1C1, 1C2, 1C3, 1C4, 1D1, and 1E1 were found in almost all carnivorans analyzed. Several genes were automatically annotated, making their identification and naming confusing. Phylogenetic analyses were conducted to clarify SULT isoforms in carnivorans and were tentatively renamed based on their phylogeny. As shown in Figure 1, carnivoran SULT1A1s were in the same clade as human and rodent SULT1A. Although humans had several SULT1A isoforms (SULT1A1, 1A2, 1A3/4), carnivorans only had one isoform in the SULT1A family (SULT1A1). Carnivoran SULT1B, 1D, and 1E genes were also in the same clades as rodents and humans, respectively, and all mammals analyzed had either one or no isoforms of SULT1B1, 1D1, or 1E1, with some pseudogenes, such as human SULT1D1. Moreover, carnivoran SULT1Cs were also grouped into the same clade as human and rodent SULT1Cs. Carnivoran SULT1C2s and 1C4s were classified into the same clades as human or rodent SULT1C2s and human SULT1C4, respectively. SULT1C1s in carnivorans were in the same clade as rat SULT1C3 and mouse SULT1C1, whereas human SULT1C3 was not in the same clade as carnivorans and rodents. According to the review by Coughtrie (2016), SULT1C3s are only present in primates, which suggests that rat SULT1C3, mouse 1C1, and carnivoran SULT1C1s are not orthologs of human SULT1C3 and are tentatively named SULT1C1s in this article.

3-1-2. SULT1 coding loci in mammals

SULT1 coding loci in rodents, humans, and carnivorans were analyzed and compared (Figure 2). SULT1A coding loci were highly conserved among Mammalia, and almost all isozymes were coded next to SGF29 (SAGA Complex Associated Factor 29) (data not shown). SULT1B1, 1D1, and 1E1 coding loci were also conserved, and SULT1B1, 1D1, 1E1 were coded in the same loci between UGT2A1 (UDP Glucuronosyltransferase Family 2 Member A1 Complex Locus) and CSN1S1 (Casein Alpha S1) or CSN2 (Casein 2). Despite most mammals having the same genetic loci, pinnipeds displayed different features. Almost all pinnipeds had SULT1D1 pseudogenes like the human SULT1D1 pseudogene. Some pinnipeds, such as Weddell seals and harbor seals, had SULT1D1 protein coding genes. However, these genes coded very short and low-quality proteins, suggesting that they encoded dysfunctional SULTs. Moreover, SULT1E1s were not registered in any analyzed pinnipeds (Weddell seal, harbor seal, gray seal, Hawaiian monk seal, northern fur seal, southern elephant seal, Stellar sea lion, California sea lion, and Pacific walrus). To investigate the existence of SULT1E1 in these species further, BLAST searches were conducted using a human SULT1E1 query sequence (NM_005420.3) with datasets from the Refseq Genome Database. No potential SULT1E1 sequences were observed in any pinnipeds. All SULT1C isoforms were coded on conserved regions between SLC5A7 (Solute Carrier Family 5 Member 7) and GCC2 (GRIP and coiled-coil domain containing 2) in humans and carnivorans or SLC5A7 and SGOL1 (Shugoshin like 1) in rodents. Rats had six isoforms equivalent to SULT1C1 and 1C2s (five isoforms), whereas mice had two isoforms (SULT1C1 and 1C2). Carnivorans and humans had three SULT1Cs isoforms each, whereas carnivorans had SULT1C1s, 1C2s, and 1C4s,

and humans had 1C3, 1C2, and 1C4 (Figures 1 and 2). In addition, phocids like the Hawaiian monk seal, southern elephant seal, and Weddell seal had pseudogenes or low-quality protein coding SULT1C1 isoforms. The low-quality protein coding genes had stop codons within their sequences, suggesting dysfunctional genes, although there were several gaps of scaffolded assembly in this locus in Weddell seals. Further variations were observed in SULT1C2s in carnivorans, such as nonsense mutations in residues 54, 131, and 264 (a PAPS binding site) of SULT1C2s in the Panthera genus and in some pinnipeds. These mutations were present in residue 54 for Hawaiian monk seals, gray seals, and harbor seals (Phocidae clade); residue 131 for lions and leopards, but not tigers (Panthera) and walruses (Odobenidae); and residue 264 for California sea lions, Stellar sea lions, northern fur sealsm, and walruses (Otariidae and Odobenidae) (Supplementary Figure S2).

3-2. In vitro activity of SULTs in the liver cytosols of pinnipeds

Enzymatic properties including Vmax, Km, and Vmax/Km of estradiol sulfation are shown in Table 1, and a Michaelis-Menten plot of estradiol sulfation activity is shown in Figure 3. In vitro analysis of cat liver cytosols revealed a relatively high Vmax/Km compared to that of rats and pinnipeds. Data obtained from cat liver cytosols were fit for a substrate-inhibition model in a high dose range, which is commonly observed for SULT activity. Estradiol-sulfate metabolites in Stellar sea lion and Harbor seal liver cytosols were not detected. We detected UGT activity or CYP450 concentration using same liver samples of these pinniped animals, and we detected certain amount of their activity to make sure their liver samples were not degraded.

4. Discussion

4-1. SULT1As are highly conserved in mammals

In this study, we analyzed the phylogeny of SULT1 family members and found that most isoforms were highly conserved in mammals. SULT1As in carnivorans were all named SULT1A1 (or 1A1-like). Based on phylogenetic analyses, these isoforms appeared to be orthologs of rodent SULT1A1s. Humans have two other isoforms of SULT1A, SULT1A2, and 1A3/4. Like SULT1A1, human SULT1A2 is known to catalyze the sulfation of simple and neutral phenols like nitrophenol. However, previous studies have shown that SULT1A2 transcripts have a splicing defect and may not be translated. No protein has been detected in any human tissues with a SULT1A2 antibody (Nowell et al., 2005). Therefore, it is unlikely that this isoform is functionally active or that it affects differences in xenobiotic metabolism between humans and carnivorans. SULT1A3/4 shows catecholamine sulfation activity, is highly expressed in the intestines of humans, cynomolgus macaques, and common marmosets, and plays important roles in neurotransmitter biosynthesis and metabolism in the intestines (Riches et al., 2009). To date, these isoforms have only been found in higher-order primates (New World monkeys, Old World monkeys, apes, and humans), suggesting that they were originally duplicated and diverted during primate evolution. This may explain the lack of SULT1A3/4 orthologs in carnivorans.

4-2. SULT1Bs are highly conserved in carnivorans

The SULT1B1 isoform was known to be highly conserved in mammals. Surprisingly, in our further investigation, even platypus and marsupials had orthologs of SULT1B1 (Supplementary Figure S2). Avian SULT1B1 and xenopus SULT1B isoforms equivalent to mammalian SULT1B1 have also been characterized. SULT1B1 has a

similar substrate specificity as SULT1A1 but with lower affinity (for simple phenols and thyroid hormones). Selective probe substrates for 1B1 still remain to be elucidated. Interestingly, no endogenous substrate for xenopus 1B1 has been found, and it does not catalyze the sulfation of thyroid hormones, which is a common substrate for mammalian and avian SULT1B1s (Wilson et al., 2004; Yamauchi et al., 2019). Therefore, the physiological functions of SULT1B1 isoforms are unclear, even though these isoforms are highly conserved in tetrapods. Together with SULT1A1s, SULT1B1 may have evolved for exogenous metabolism and important xenobiotic defense systems, yet affinity for their exogenous substrate is also low.

4-3. SULT1D1 defects in pinnipeds suggest unique catecholamine metabolism

SULT1D1 is another isoform that showed interspecies differences. Like humans, all pinnipeds had SULT1D1s pseudogenes. However, carnivorans, rodents, and avian species had orthologous SULT1D1 isoforms in conserved regions. Canine and mouse SULT1D1 was cloned and characterized and had a high affinity for dopamine, naphtha-1-ol, and PNP (Liu et al., 1999; Carrie Tsoi et al., 2001). Previous studies using immunoblots have found that canine SULT1D1 was highly expressed in the intestines and kidneys but lowly expressed in the liver (C. Tsoi et al., 2001). In rats, SULT1D1 mRNA was highly expressed in the kidneys, followed by the intestines and lungs, and lowly expressed in the liver. Thus, SULT1D1s were suggested to play significant roles in sulfating catecholamines in the kidneys rather than in the liver. Some reports have suggested that primate SULT1A3 could compensate for catecholamine sulfation (Dajani et al., 1999, 1998) and may explain the presence of the SULT1D1 pseudogene in primates. BLAST searches have suggested that the SULT1D1 gene was only present in Strepsirrhini and Tarsiidae but not in higher

405 primates (data not shown), which is consistent with SULT1A3 expression in these
406 species. However, in pinnipeds, both SULT1A3 and 1D1 were missing from the
407 genome, indicating that sulfation of catecholamine in these animals may be limited.
408 Catecholamine sulfates are mainly found in the blood and may be precursors of active
409 molecules that are later deconjugated by sulfatases in peripheral tissues. SULTs may
410 be essential to regulate catecholamine function in other mammals. From our findings,
411 pinnipeds might have completely different pathways to regulate neurotransmitter
412 function and estrogen metabolism (this will be discussed later).

413 **4-4. Physiological significance of 1E1 defects in pinnipeds**

414 Surprisingly, one of the most important and well-characterized isoforms, SULT1E1,
415 was completely absent in pinnipeds including Phocidae, Otariidae, and Odobenidae.
416 In vitro enzymatic analysis also suggested remarkably limited SULT1E1 activity in the
417 liver of harbor seals, northern fur seals, and Stellar sea lions. This is the first report of
418 innate SULT1E1 deficiency in placental mammals. SULT1E1s are critically important
419 for the metabolism of sulfate estrogens (estradiol and estrone) and have a very high
420 affinity for a vast range of xenobiotics including some environmental pollutants, such
421 as hydroxylated-polychlorinated biphenyls (OH-PCBs) and hydroxylated-
422 polybrominated diphenyl ether (OH-PBDEs). A previous report by Tong et al. (2005)
423 suggested that SULT1E1 ablation in mice caused severe thrombosis in the placenta,
424 resulting in fetal loss in the knock out (KO) mice because of the excessive estrogen
425 levels in the placenta. Moreover, Gershon et al. (2007) showed that excessive
426 estrogen resulted in the low expression of COX-2, reduced cumulus expansion, and
427 impaired ovulation in SULT1E1 KO mice. In addition, single nucleotide polymorphisms
428 (SNP) in human SULT1E1 may be a risk factor for breast or endometrial cancer

development (Yi et al., 2021). Like catecholamine, estrogen sulfates are also mainly found in the blood and are precursors of active steroids, utilizing steroid sulfatase (STS) to resume their actions in peripheral tissues. Hence, SULT1E1 is an essential estrogen-modulating factor in mammals. The detailed mechanism of estrogen modulation in pinnipeds has not yet been described but pinnipeds may not utilize estrogen sulfation to modulate estrogenic activity. Currently, SULT1E1 orthologs have only been discovered in mammals including placental mammals and platypuses, but not in marsupials or other vertebrates, indicating that SULT1E1 diverged after the evolutional emergence of mammals (Coughtrie, 2016). However, in chicken and turtle eggs, biosynthesis of estrogen sulfate was observed, suggesting the existence of estrogen-sulfotransferases in these species (Paitz et al., 2020; Paitz and Bowden, 2013), despite there being no SULT1E1 orthologs in reptiles or birds in our analyses. Phylogenetic analysis of avian SULTs showed one clade of avian SULTs (tentatively named SULT1D/1E), which was located closely to mammalian 1E1 and 1D1 groups in the phylogenetic tree, suggesting that avian SULT1E/1D may have similar substrate specificity as mammalian SULT1D1 or 1E1. In addition, since SULTs have a vast overlap in their substrate specificities, other SULTs could also catalyze estrogen conjugation with lower affinity, suggesting a possible role for these isoforms. However, these reactions were not observed in vitro using pinniped liver cytosols. Previously, Browne et al. (2006) reported the detection of estrone-sulfate in the blood of some pinnipeds using radioimmunoassays followed by HPLC separation, indicating that estrogen sulfation may not be completely absent in pinnipeds. However, the involvement of other SULT isoforms or activity in other organs is still unclear.

452 This in vitro analysis has limitation and didn't completely reflect the SULT1E1 activity
453 because we didn't investigate substrate specificity for other isoforms in Carnivorans
454 and studies using recombinant SULTTs in carnivora is highly important for further
455 discussion. Also in this in vitro analysis, we utilized environmental samples and we
456 didn't conduct chemicals analysis to detect environmental pollutants in these
457 specimens. Thus, some contaminants such as persistent organic pollutants (POPs)
458 might have effect on SULT expression or activity (Kodama and Negishi, 2013).
459 Although this in vitro analysis had such limitation, we considered the result in this study
460 suggested important species-differences of SULT activity in Carnivora.
461
462 **4-5. SULT1Cs in carnivorans and genetic deficiency**
463 Along with other SULT isoforms, SULT1Cs were highly conserved, with some
464 differences between rodents, human, and carnivorans. Phylogenetic analysis revealed
465 that SULT1C1, 1C2, and 1C4 in carnivorans were in the same clade of the
466 phylogenetic tree as rodent SULT1C1, 1C2s and human SULT1C4, respectively. Rat
467 SULT1C3 is considered to be an ortholog of mouse SULT1C1 and not an equivalent
468 of human SULT1C3, suggesting that a comprehensive nomenclature system remains
469 unestablished. In rats, several isoforms in the SULT1C2 clade were observed while
470 mice, humans, and carnivorans had only one isoform in this clade. Human and rodent
471 SULT1Cs are known to conjugate xenobiotics, such as p-nitrophenol, 1-naphthol, 2-
472 ethylphenol, 2-n-propylphenol, and 2-sec-butylphenol (Allali-Hassani et al., 2007);
473 they also conjugate procarcinogen hydroxyaryl amines, such as N-hydroxy-2-
474 acetylaminofluorene, resulting in the metabolic activation of their carcinogenicity
475 (Kurogi et al., 2017; Meinl et al., 2008; Sakakibara et al., 1998; Stanley et al., 2005).

In humans, SULT1Cs were mainly detected in fetal tissues and were thought to play a possible role in terminating several signaling pathways during fetal development (Runge-Morris and Kocarek, 2013; Stanley et al., 2005), whereas rat SULT1Cs were still detected in adults and played important roles in xenobiotic metabolism into adulthood (Lu et al., 2013; Nagata et al., 1993). In Carnivora, only canine SULT1C4 has been cloned and characterized as a phenol-preferring SULT (Kurogi et al., 2010). Furthermore, Kurogi et al. revealed that SULT1C4 was expressed in the kidneys, stomach, testes, ovaries, and thyroid glands but not in the liver, suggesting a significant role of SULT1C4-mediated detoxification in non-liver organs in adult dogs and possibly other carnivorans.

Interestingly, SULT1C1s were detected as pseudogenes or low-quality protein coding genes in Hawaiian monk seals, southern elephant seals, and Weddell seals, indicating that SULT1C1s in these species may not be functionally expressed. These species are classified as Monachinae (southern seals) (Berta et al., 2018), suggesting low SULT activity in this group of animals. Moreover, several variations of SULT1C2s were found in carnivorans. Many species had nonsense mutations in SULT1C2s, including pinnipeds, lions, and leopards (Panthera genus). Overall, SULT1Cs are highly diverse, and some 1Cs, like 1C1 and 1C2, were absent in pinnipeds and some carnivoran species, indicating a possible lack of sulfation for some xenobiotics in these animals.

4-6. Balance between UGTs and SULTs

Many chemicals have been shown to be simultaneously glucuronidated and sulfated, suggesting that UGTs and SULTs may compensate for each other, with some regioselective differences (Böhmdorfer et al., 2017; Saengtienchai et al., 2014; Wu et al., 2011). Previous reports have shown very limited function for UGTs in felines and

pinnipeds, suggesting the compensatory activity of SULTs in these species (Kakehi et al., 2015; Kondo et al., 2017). Our present in vitro analysis suggests that feline livers have high SULT activity towards estrogens compared to rats. Limited or no SULT activity was detected in pinnipeds. These findings indicate that SULTs may compensate for limited activity of UGTs in felines, but not in pinniped species. Together with low UGT activity, our present findings suggest that pinniped species have very limited phase II metabolic processes, resulting in poor degradation of numerous chemicals including environmental estrogens, such as Bisphenol A, 4-n-octylphenol, 4-n-nonylphenol, and OH-PCBs (Grimm et al., 2017; Suiko et al., 2005).

Conclusion

This is the first comprehensive report of the genetic characteristics of SULT isoforms in wild, non-laboratory mammals. In this study, we found that some pinnipeds may have an extremely limited capacity to sulfonate both exogenous and endogenous chemicals, such as estrogens, medicines, and environmental chemicals. These findings improve our knowledge of the genetic variation of SULT genes in carnivorans and, importantly, improve our understanding of xenobiotic metabolism as carnivorans' defense system for numerous anthropogenic chemicals.

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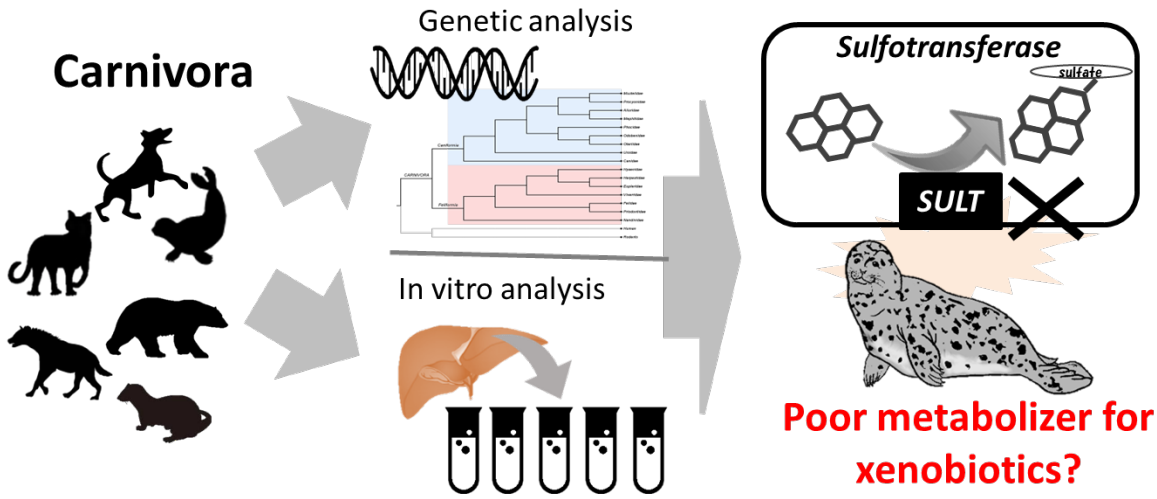
Graphical abstract



Tables

	Rat	Cat	Steller Sea Lion	Harbor Seal
Vmax/Km (µl/min/mg)	5.17 ± 0.77 a	563 ± 37.4 b	N.D.	N.D.
Vmax (pmol/min/mg)	54.3 ± 6.93	46.7 ± 5.83	N.D.	N.D.
Km (µM)	10.5 ± 2.23 a	0.0829 ± 0.0390 b	N.D.	N.D.

Table 1. Kinetic parameters of the SULT estradiol activity for each species

Data presented for rats, cats, and pinnipeds as means ± SD. Vmax/Km values that were significantly different ($P < 0.05$) within a substrate, based on Tukey's HSD tests for each Vmax/Km, are indicated by “a” and “b”.

N.D.: not determined.

Figures

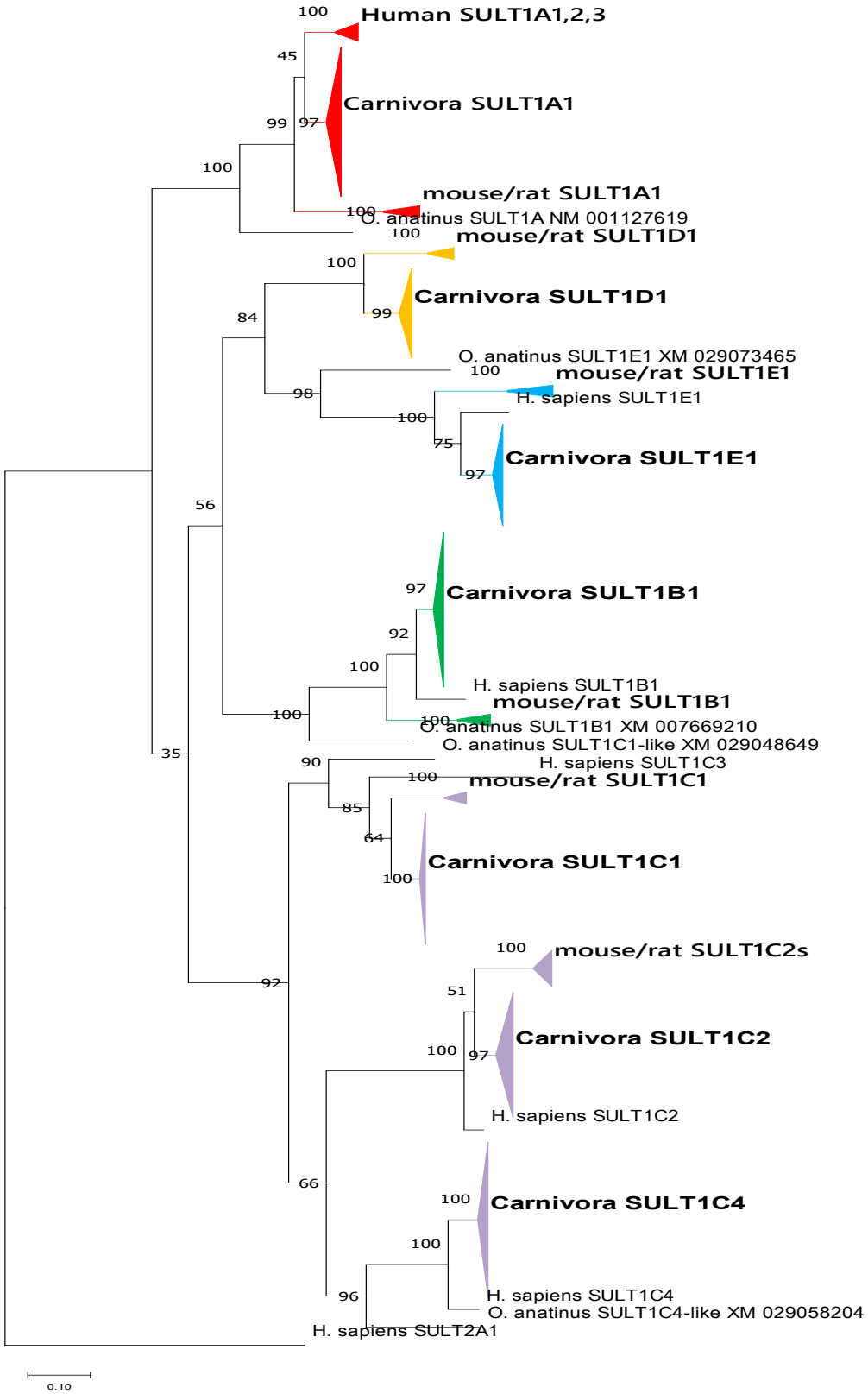


Figure 1. Phylogenetic tree of SULT1s in mammals including carnivorans.

Phylogenetic tree of SULT1 amino acid sequences in humans, mice, rats, platypuses, and carnivorans. Gene sequences of protein-coding regions for each isozyme were analyzed. The JTT + G model was used. The numbers next to the branches indicate the number of occurrences per 100 bootstrap replicates. Gene names and clade names are tentatively named for carnivoran SULTs in this article along with their phylogeny. Clades of carnivorans, mouse, and rat SULTs in the phylogenetic tree are shown as triangles with the following colors: red for SULT1As, green for SULT1B1s, pale purple for SULT1Cs, yellow for SULT1D1s, and light blue for SULT1E1s. Human SULT2A1 is shown as an outgroup of SULT1s.

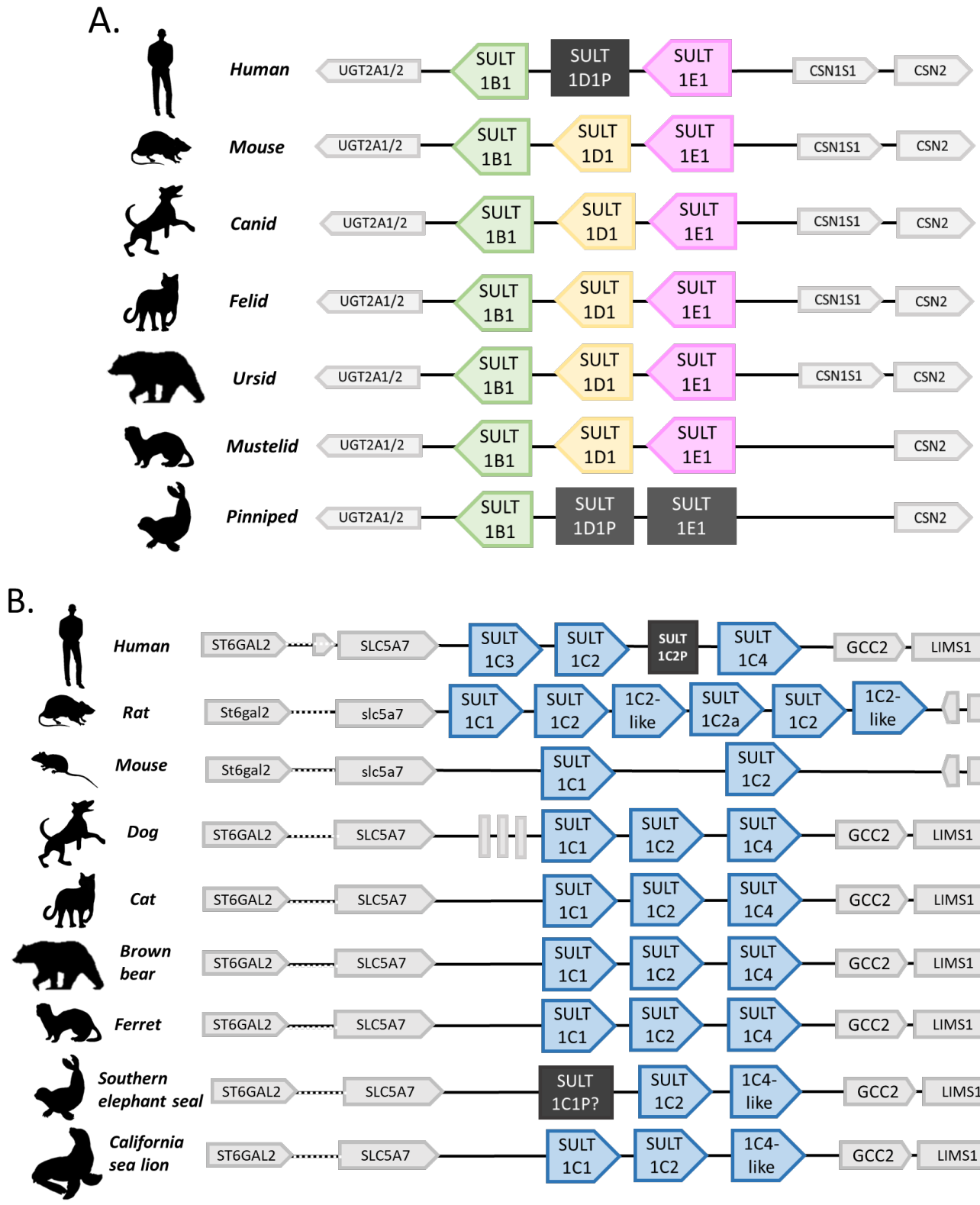


Figure 2. Genetic loci of SULTs in mammals.

A. Gene loci of SULT1B1s, 1D1s, and 1E1s in humans, mice, and carnivorans are described. B. Gene loci of SULT1Cs in mammals are shown. Black blocks indicate pseudogenes. Gray blocks show other non-SULT genes. Dotted lines represent long omitted gene loci. P stands for Pseudogene.

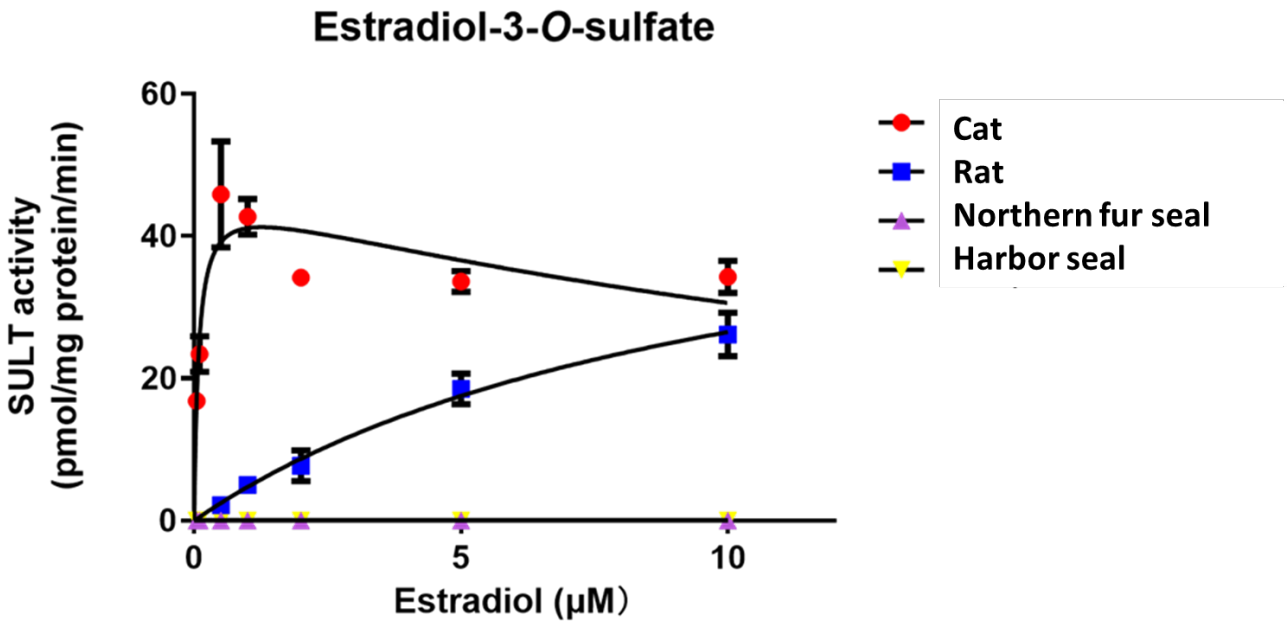


Figure 3. Michaelis-Menten plot for the in vitro SULT activity of estradiol.

In vitro SULT enzymatic activity is shown in the Michaelis-Menten plot. Cats (circle), rats (square), northern fur seal (triangle), and Harbor seal (reverse triangle) cytosols and estradiol substrates were used for in vitro analyses. Cat data were fit for a substrate-inhibition model.

Supplementary Data

Supplementary Table 1. Animal liver samples used in this study

Species	Steller Sea Lion	Harbor Seal	Cat	SD Rat
Scientific name	Eumetopias jubatus	Phoca vitulina	Felis catus	Rattus norvegicus
Number	4	4	3	4
Gender	Male	Male	Male	Male
Sampling year	2003	2016	2017	2014
Location	Rausu (Japan)	Erimo (Japan)	Kitayama Labes Co., Inc	Sankyo Labo Service Corporation, Inc.
Age class	Mature	Mature	24–28 months	8 weeks

Supplementary Table 2. SULT genes used in the phylogenetic analysis and sequence comparative analysis.

Family	Common name	Species name	Gene name (with tentative name)	Accession number
Hominidae	Human	Homo sapiens	SULT1A1	NM001055.3
			SULT1A2	NM001054
			SULT1A4	NM001017390
			SULT1B1	NM014465
			SULT1C2	NM001056
			SULT1C3	NM001008743.3
			SULT1C4	NM001321770.2
			SULT1E1	Y11195.1
			SULT2A1 as outer group	NM003167
Muridae	Rat	Rattus norvegicus	sult1a1	AF394783.1
			ST1b1 (sult1b1)	D89375.1
			RGD1559960 (sult1c2)	XM039084411.1
			sult1c2a	NM001013177.2

			sult1c2	NM133547.5
			LOC120093086 (sult1c2-like)	XM039084404.1
			sult1c3 (sult1c1)	NM031732.2
			sult1d1	NM021769.1
			sult1e1	NM012883.1
	Mouse	Mus musculus	sult1a1	NM133670
			sult1b1	BC024361
			sult1c1	NM018751.2
			sult1c2	NM026935.4
			sult1d1	NM016771.3
			sult1e1	BC034891.1
Canidae	Dog	Canis lupus familialis	SULT1A1	AY069922.1
			SULT1B1	NM001195835.2
			SULT1C3 (SULT1C1)	XM038680151.1
			SULT1C2	XM038680149.1

			SULT1C4	XM038680150.1
			SULT1D1	XM038685809.1
			SULT1E1	XM038685811.1
	Red fox	Vulpes	LOC112929198 (SULT1A1)	XM026011091
			SULT1B1	XM026003470
			SULT1C2	XM026011035
			SULT1C4	XM026010893
			SULT1D1-like: LOC112923601 (SULT1D1)	XM026003469
			LOC112923635 (SULT1E1)	XM026003484
Mustelidae	North American river otter	Lontra canadensis	LOC116858063 (SULT1A1)	XM032842614
			SULT1B1	XM032845783
			LOC116857948 (SULT1C1)	XM032842428
			SULT1C2	XM032842417.1

		SULT1C4-like: (SULT1C4) LOC116857940	XM032842416
		SULT1D1-like: (SULT1D1) LOC116859864	XM032845591
		SULT1E1-like: (SULT1E1) LOC116859692	XM032845460
Sea otter	Enhydra lutris kenyoni	SULT1A1: LOC111162234 (SULT1A1)	XM022526502.1
		SULT1B1: LOC111149182 (SULT1B1)	XM022506124
		SULT1C1-like: (SULT1C1) LOC111155904	XM022516369.1
		SULT1C4-like: (SULT1C4) LOC111155885	XM022516341.1
		SULT1D1-like: (SULT1D1) LOC111149106	XM022506029
		SULT1E1: LOC111149178 (SULT1E1)	XM022506117

Ermine	Mustela erminea	SULT1A1: LOC116581510 (SULT1A1)	XM032328707
		SULT1B1	XM032334482
		SULT1C1: LOC116595393 (SULT1C1)	XM032351657
		SULT1C2: LOC116595392 (SULT1C2)	XM032351655
		SULT1C4-like: LOC116595391 (SULT1C4)	XM032351652
		SULT1D1: LOC116585153 (SULT1D1)	XM032334485
		SULT1E1-like: LOC116585151 (SULT1E1)	XM032334481
Domestic ferret	Mustela putorius furo	SULT1A1	XM 004773964
		SULT1B1	XM013063435.1
		SULT1C2	XM004764352.2
		SULT1C3	XM004764354.2
		SULT1C4	XM004764349.2
		SULT1D1	XM013063434.1

			SULT1E1	XM004766333.2
Ursidae	Brown bear	Ursus arctos horribilis	SULT1A1	XM026515715
			SULT1B1	XM026509010
			SULT1C1-like: LOC113269027 (SULT1C1)	XM026517699
			SULT1C2: LOC113267746 (SULT1C2)	XM026515916
			SULT1C4	XM026515929
			SULT1D1: LOC113262555 (SULT1D1)	XM026509011
			SULT1E1	XM026509014
	Polar bear	Ursus maritimus	SULT1A1: LOC103657825 (SULT1A1)	XM008685238.2
			SULT1B1	XM008683684
			SULT1C1:LOC103670621 (SULT1C1)	XM040622914.2
			SULT1C2	XM008699075.2
			SULT1C4	XM 008699073.2
			SULT1D1: LOC103656319 (SULT1D1)	XM008683686

			SULT1E1: LOC103656321 (SULT1E1)	XM008683687.1
	Giant panda	Ailuropoda melanoleuca	LOC100479867: SULT1A1 (SULT1A1)	XM002927364.4
			SULT1B1	XM002929461.4
			SULT1C1: LOC100466952 (SULT1C1)	XM002930285.4
			SULT1C2: LOC100467202 (SULT1C2)	XM002930286.4
			SULT1C4	XM002929382.4
			SULT1D1: LOC100468789 (SULT1D1)	XM011237499.3
			SULT1E1: LOC100469041 (SULT1E1)	XM019810143.2
Phocidae: Monachinae	Weddell seal	Leptonychotes weddellii	SULT1A1: LOC102733278 (SULT1A1)	XM006733938.2
			SULT1B1: LOC102727575 (SULT1B1)	XM006729321.2
			SULT1C1-like: LOC102725762 (SULT1C1)	XM031022251.1
			SULT1C2: LOC102747532 (SULT1C2)	XM006728933
			SULT1C4: LOC102747824 (SULT1C4)	XM006728934.2

Southern elephant seal	Mirounga leonina	SULT1A1: LOC117999643 (SULT1A1)	XM034988959
		SULT1B1: LOC118010478 (SULT1B1)	XM035004869
		SULT1C1-like: LOC118008308 (SULT1C1)	XM035001943.1
		SULT1C2	XM035001944.1
		SULT1C4-like: LOC118008292 (SULT1C4)	XM035001900.1
Hawaiian monk seal	Neomonachus schauinslandi	SULT1A1: LOC110589844 (SULT1A1)	XM021700477.1
		SULT1B1: LOC110591481 (SULT1B1)	XM021702403
		SULT1C1-like: LOC110572024 (SULT1C1P)	
		SULT1C2	XM021680275.1
		SULT1C4	XM021680274.1

Phocidae: Phocinae	Harbor seal	Phoca vitulina	SULT1A1: LOC116648763 (SULT1A1)	XM032431006.1
			SULT1B1: LOC116628089 (SULT1B1)	XM032398193.1
			SULT1C1: LOC116641753 (SULT1C1)	XM032419668
			SULT1C2	XM032419671
			SULT1C4: LOC116641754 (SULT1C4)	XM032419669.1
	Gray seal	Halichoerus grypus	SULT1A1: LOC118535031 (SULT1A1)	XM036091215.1
			SULT1B1: LOC118525390 (SULT1B1)	XM036076112
			SULT1C1: LOC118553409 (SULT1C1)	XM036120449
			SULT1C2	XM036120462.1
			SULT1C4	XM036087224
Otariidae	California sea lion	Zalophus californianus	SULT1A1: LOC113933049 (SULT1A1)	XM027612717
			SULT1B1: LOC113924953 (SULT1B1)	XM027599339.2
			SULT1C1: LOC113937879 (SULT1C1)	XM027622796.1
			SULT1C2: LOC113937590 (SULT1C2)	XM027622119.1

			SULT1C4-like: LOC113937589	XM027622118.2
	Stellar sea lion	Eumetopias jubatus	SULT1A1: LOC114214438 (SULT1A1)	XM028110090
			SULT1B1: LOC114206800 (SULT1B1)	XM028100710
			SULT1C1: LOC114199385 (SULT1C1)	XM028091278.1
			SULT1C2-like: LOC114199388 (SULT1C2)	XM028091279
			SULT1C4	XM028091284
	Northern fur seal	Callorhinus ursinus	SULT1A1: LOC112810457 (SULT1A1)	XM025854081.1
			SULT1B1: LOC112835901 (SULT1B1)	XM025887547
			SULT1C1: LOC112821508 (SULT1C1)	XM025868772
			SULT1C2	XM025869098.1
			SULT1C4-like: LOC112821718 (SULT1C4)	XM025869099.1
Odobenidae	Pacific walrus	Odobenus rosmarus divergens	SULT1A1: LOC101380863 (SULT1A1)	XM012561271.1

			SULT1B1		XM004392889.1
			SULT1C1-like: (SULT1C1)	LOC101376188	XM012567311.1
			SULT1C2		XM004414460.2
			SULT1C4		XM004414459.1
Falidae	Domestic cat	Felis catus	SULT1A1		XM019820729.1
			SULT1B1		XM023253069
			SULT1C3 (SULT1C1)		XM003983864.5
			SULT1C2		XM011281074
			SULT1C4		XM003983869.5
			SULT1D1-like: (SULT1D1)	LOC101086557	XM023253066.1
			SULT1E1		XM003985305.3
	Cheetah	Acinonyx jubatus	SULT1A1: LOC106966152 (SULT1A1)		XM027053118.1
			SULT1B1		XM015074496.2

		SULT1C1-like: LOC106977307 (SULT1C1)	XM015074791.2
		SULT1C2: LOC106977290 (SULT1C2)	XM027069399.1
		SULT1C4: LOC106977305 (SULT1C4)	XM015074790.2
		SULT1D1-like: LOC106977024 (SULT1D1)	XM027064350.1
		SULT1E1	XM015074488.2
Puma	Puma concolor	SULT1A1: LOC112855996 (SULT1A1)	XM025919525.1
		SULT1B1	XM025921642.1
		SULT1C1-like: LOC112868851 (SULT1C1)	XM025931831.1
		SULT1C2	XM025932749.1
		SULT1C4-like: LOC112869068 (SULT1C4)	XM025932133

		SULT1D1-like: (SULT1D1) LOC112858721	XM025922137.1
		SULT1E1-like: (SULT1E1) LOC112858407	XM025921805
Leopard	Panthera pardus	SULT1A1: LOC109256521 (SULT1A1)	XM019431898
		SULT1B1: LOC109278167 (SULT1B1)	XM019468233
		SULT1C1-like: (SULT1C1) LOC109258064	XM019434615.1
		SULT1C2-like: (SULT1C2) LOC109258069	XM019434617.1
		SULT1C4	XM019434612.1
		SULT1D1-like: (SULT1D1) LOC109278146	XM019468226.1
		SULT1E1	XM019468175.1
Tiger	Panthera tigris	SULT1A1: LOC102972472 (SULT1A1)	XM007084062.3

			SULT1B1	XM007086908.3
			SULT1C1: LOC102952335 (SULT1C1)	XM007097761.3
			SULT1C2: LOC102952042 (SULT1C2)	XM042979753.1
			SULT1C4	XM007097759.3
			SULT1D1: LOC102969965 (SULT1D1)	XM007086913.3
			SULT1E1	XM007086910.3
	Lynx	Lynx canadensis	SULT1A1: LOC115504563 (SULT1A1)	XM030301602
			SULT1B1	XM030313745
			SULT1C1: LOC115510437 (SULT1C1)	XM030310292.2
			SULT1C2: LOC115509692 (SULT1C2)	XM030309021.1
			SULT1C4	XM030310291
			SULT1D1-like: LOC115512581 (SULT1D1)	XM030313743
			SULT1E1	XM030313739
Hyaenidae	Striped hyena	Hyaena hyaena	SULT1A1: LOC120242277 (SULT1A1)	XM039247942.1

			SULT1B1		XM039251765.1
			SULT1C1-like: (SULT1C1)	LOC120227542	XM039225639.1
			SULT1C4		XM039222195
			SULT1D1-like: (SULT1D1)	LOC120244963	XM039251783.1
			SULT1E1-like: (SULT1E1)	LOC120244962	XM039251782.1
Herpestidae	Meerkat	Suricata suricatta	SULT1A1: LOC115297986 (SULT1A1)		XM029946269
			SULT1B1		XM029931052.1
			SULT1C1-like: (SULT1C1)	LOC115288947	XM029936197.1
			SULT1C4		XM029937066

			SULT1D1-like: LOC115301877 (SULT1D1)	XM029951883.1
			estrogen sufotransferase-like: LOC115296737 (SULT1E1)	XM029945225.1
Monotremata:				
Ornithorhynchidae	Platypus	Ornithorhynchus anatinus	SULT1A	NM001127619
			SULT1B1	XM007669210
			SULT1C1-like: LOC100090962	XM029048649
			SULT1C4-like: LOC114809133 (SULT1C4)	XM029058204
			SULT1E1	XM029073465

Supplementary Figures

a.

number of aligned nucleotide	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176
translated amino acid from human SULT1C2		P			K			A			G			T			T			W			I			Q			E			V			
H. sapiens SULT1C2 NM 001056	C	C	T	A	A	A	G	C	A	G	G	G	A	C	A	A	C	G	T	G	G	A	T	T	C	A	G	G	A	A	A	T	T	G	T
R. norvegicus Sult1c2a NM 001013177	C	C	T	A	A	A	T	C	A	G	G	G	A	C	A	A	C	A	T	G	G	A	T	T	C	A	A	G	A	A	A	T	T	G	T
R. norvegicus SULT1C2-like LOC100910526 XM 006244254	C	C	T	A	A	A	T	C	A	G	G	G	A	C	A	A	C	A	T	G	G	A	T	T	C	A	A	G	A	A	A	T	T	G	T
R. norvegicus SULT1C2 XM 039084411 RGD1559960	C	C	T	A	A	A	T	C	A	G	G	G	A	C	A	A	A	A	T	G	G	A	T	T	C	A	A	G	A	A	A	T	A	G	T
R. norvegicus Sult1c2 NM 133547.4	C	C	T	A	A	A	T	C	A	G	G	G	A	C	A	A	C	A	T	G	G	A	T	T	C	A	A	G	A	A	A	T	T	G	T
M. musculus Sult1c2 NM 026935.4	C	C	T	A	A	A	T	C	A	G	G	G	A	C	A	A	C	A	T	G	G	A	T	T	C	A	A	G	A	A	A	T	T	G	T
C. lupus familiaris SULT1C2 XM 038680149.1	C	C	T	A	A	A	T	C	A	G	G	G	A	C	T	A	C	A	T	G	G	A	T	T	C	A	G	G	A	A	A	T	T	G	T
V. vulpes SULT1C2 XM 026011035	C	C	T	A	A	A	T	C	A	G	G	G	A	C	T	A	C	A	T	G	G	A	T	T	C	A	G	G	A	A	A	T	T	G	T
V. lagopus SULT1C2 XM 041754093 LOC121490422	C	C	T	A	A	A	T	C	A	G	G	G	A	C	T	A	C	A	T	G	G	A	T	T	C	A	G	G	A	A	A	T	T	G	T
U. arctos horribilis SULT1C2 XM 026515916	C	C	T	A	A	A	T	C	A	G	G	G	A	C	C	A	C	A	T	G	G	A	T	T	C	A	G	G	A	G	A	T	T	G	T
U. maritimus SULT1C2 XM 008699075.1 LOC103670620	C	C	T	A	A	A	T	C	A	G	G	G	A	C	C	A	C	A	T	G	G	A	T	T	C	A	G	G	A	G	A	T	T	G	T
A. melanoleuca SULT1C2 XM 002930286.4 LOC100467202	C	C	T	A	A	A	T	C	A	G	G	G	A	C	C	A	C	A	T	G	G	A	T	T	C	A	G	G	A	G	A	T	T	G	T
M. putorius furo SULT1C2 x3 XM 004764352.2	C	C	T	A	A	A	T	C	A	G	G	C	A	C	C	A	C	A	T	G	G	A	T	T	C	A	G	G	A	A	A	T	T	G	T
N. vison SULT1C2 XM 044262435 LOC122915683	C	C	T	A	A	A	T	C	A	G	G	C	A	C	C	A	C	A	T	G	G	A	T	T	C	A	G	G	A	A	A	T	T	G	T
M. erminea SULT1C2 XM 032351655 LOC116595392	C	C	T	A	A	A	T	C	A	G	G	C	A	C	C	A	C	A	T	G	G	A	T	T	C	A	G	G	A	A	A	T	T	G	T
F. catus SULT1C2 x1 XM 011281074.3	C	C	T	A	A	A	T	C	A	G	G	G	A	C	C	A	C	G	T	G	G	A	T	T	C	A	G	G	A	A	A	T	T	G	T
L. canadensis Lynx SULT1C2 XM 030309021 LOC115509692	C	C	T	A	A	A	T	C	A	G	G	G	A	C	C	A	C	G	T	G	G	A	T	T	C	A	G	G	A	A	A	T	T	G	T
P. yagouaroundi SULT1C2-like XM 040454146 LOC121016479	C	C	T	A	A	A	T	C	A	G	G	G	A	C	C	A	C	G	T	G	G	A	T	T	C	A	G	G	A	A	A	T	T	G	T
P. bengalensis SULT1C2 XM 043553279 LOC122466971	C	C	T	A	A	A	T	C	A	G	G	G	A	C	C	A	C	G	T	G	G	A	T	T	C	A	G	G	A	A	A	T	T	G	T
P. tigris SULT1C2 XM 042979753 LOC102952042	C	C	T	A	A	A	T	C	A	G	G	G	A	C	C	A	C	G	T	G	G	A	T	T	C	A	G	G	A	A	A	T	T	G	T
P. pardus SULT1C2-like XM 019434617.1 LOC109258069	C	C	T	A	A	A	T	C	A	G	G	G	A	C	C	A	C	G	T	G	G	A	T	T	C	A	G	G	A	A	A	T	T	G	T
P. leo SULT1C2-like XM 042930191 LOC122214989	C	C	T	A	A	A	T	C	A	G	G	G	A	C	C	A	C	G	T	G	G	A	T	T	C	A	G	G	A	A	A	T	T	G	T
O. rosmarus divergens SULT1C2 XM 004414460.2	C	C	T	A	A	A	T	C	A	G	G	G	A	C	C	A	C	A	T	G	G	A	T	T	C	A	G	G	A	A	A	T	T	A	T
Z. californianus SULT1C2 XM 027622119	C	C	T	A	A	A	T	C	A	G	G	G	A	C	C	A	C	A	T	G	G	A	T	T	C	A	G	G	A	A	A	T	T	A	T
E. jubatus SULT1C2-like XM 028091279 LOC114199388	C	C	T	A	A	A	T	C	A	G	G	G	A	C	C	A	C	A	T	G	G	A	T	T	C	A	G	G	A	A	A	T	T	A	T
C. ursinus SULT1C2 XM 025869098	C	C	T	A	A	A	T	C	A	G	G	G	A	C	C	A	C	A	T	G	G	A	T	T	C	A	G	G	A	A	A	T	T	A	T
P. vitulina SULT1C2 XM 032419671	C	C	T	A	A	A	T	C	A	G	G	G	A	C	C	A	C	A	T	**A**	G	A	T	T	C	A	G	G	A	A	A	T	T	G	T
H. grypus SULT1C2 XM 036120462	C	C	T	A	A	A	T	C	A	G	G	G	A	C	C	A	C	A	T	**A**	G	A	T	T	C	A	G	G	A	A	A	T	T	G	T
N. schauinslandi SULT1C2 XM 021680275.1	C	C	T	A	A	A	T	C	A	G	G	G	A	C	C	A	C	A	T	**A**	G	A	T	T	C	A	G	G	A	A	A	T	T	G	T

b.

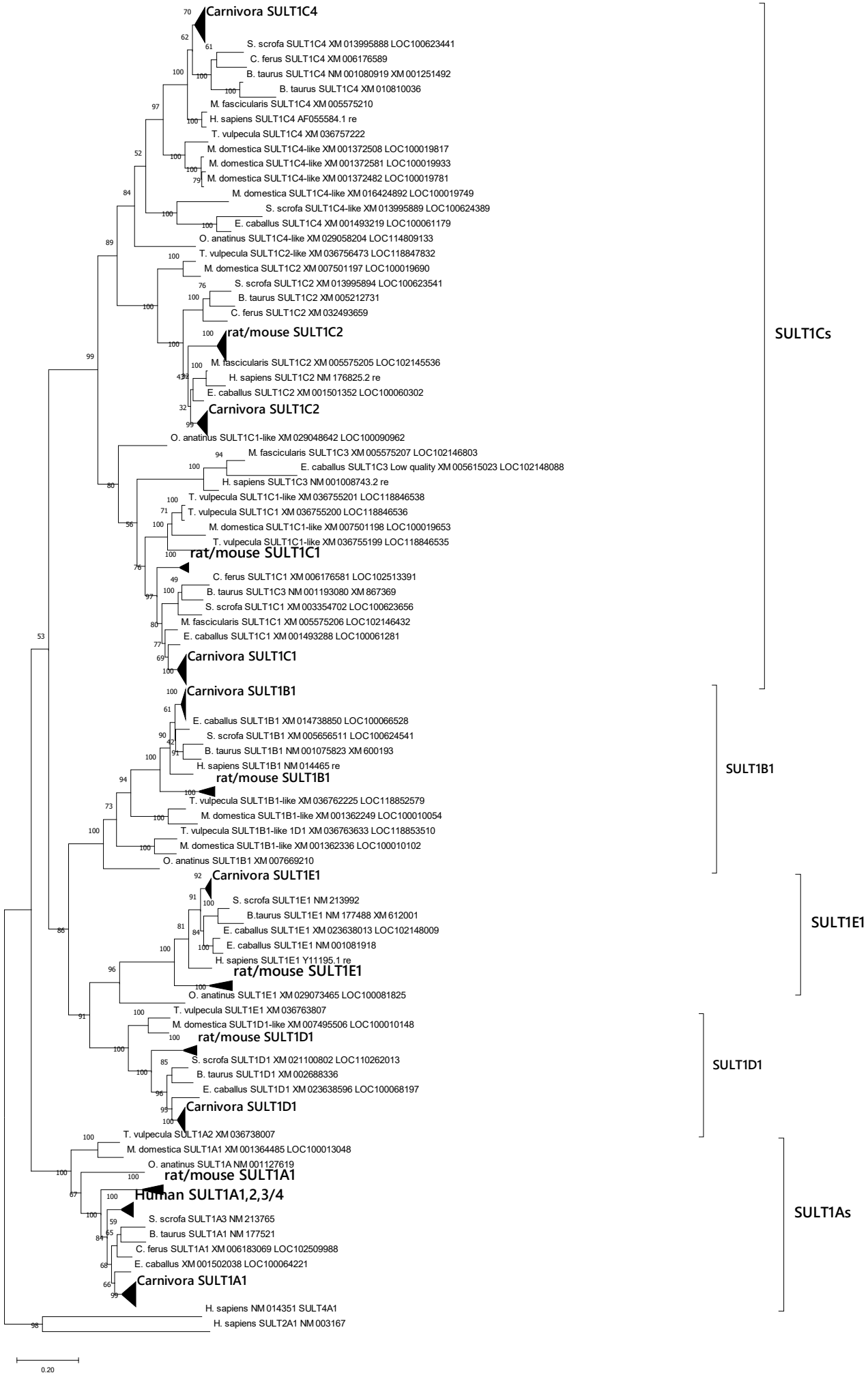
number of aligned nucleotide	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408
translated amino acid from human SULT1C2	L			Y			V			A			R			N			A			K			D			C		
H. sapiens SULT1C2 NM 001056	C	T	T	T	A	T	G	T	A	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	T
R. norvegicus Sult1c2a NM 001013177	C	T	T	T	A	T	G	T	G	G	C	T	C	G	A	A	A	C	G	C	C	A	A	A	G	A	C	T	G	C
R. norvegicus SULT1C2-like LOC100910526 XM 006244254	C	T	T	T	A	T	G	T	G	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
R. norvegicus SULT1C2 XM 039084411 RGD1559960	C	T	T	T	A	T	G	T	G	G	C	T	C	G	A	A	A	C	G	C	C	A	A	A	G	A	C	T	G	C
R. norvegicus Sult1c2 NM 133547.4	C	T	T	T	A	T	G	T	G	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
M. musculus Sult1c2 NM 026935.4	C	T	T	T	A	T	G	T	A	G	C	T	C	G	A	A	A	T	G	C	T	A	A	A	G	A	C	T	G	C
C. lupus familiaris SULT1C2 XM 038680149.1	C	T	T	T	A	T	G	T	A	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
V. vulpes SULT1C2 XM 026011035	C	T	T	T	A	T	G	T	A	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
V. lagopus SULT1C2 XM 041754093 LOC121490422	C	T	T	T	A	T	G	T	A	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
U. arctos horribilis SULT1C2 XM 026515916	C	T	T	T	A	T	G	G	A	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
U. maritimus SULT1C2 XM 008699075.1 LOC103670620	C	T	T	T	A	T	G	T	A	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
A. melanoleuca SULT1C2 XM 002930286.4 LOC100467202	C	T	T	T	A	T	G	T	A	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
M. putorius furo SULT1C2 x3 XM 004764352.2	C	T	T	T	A	T	G	T	A	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
N. vison SULT1C2 XM 044262435 LOC122915683	C	T	T	T	A	T	G	T	A	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
M. erminea SULT1C2 XM 032351655 LOC116595392	C	T	T	T	A	T	G	T	A	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
F. catus SULT1C2 x1 XM 011281074.3	C	T	T	T	A	T	G	T	G	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
L. canadensis Lynx SULT1C2 XM 030309021 LOC115509692	C	T	T	T	A	T	G	T	G	G	C	T	C	A	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
P. yagouaroundi SULT1C2-like XM 040454146 LOC121016479	C	T	T	T	A	T	G	T	G	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
P. bengalensis SULT1C2 XM 043553279 LOC122466971	C	T	T	T	A	T	G	T	G	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
P. tigris SULT1C2 XM 042979753 LOC102952042	C	T	T	T	A	T	G	T	A	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
P. pardus SULT1C2-like XM 019434617.1 LOC109258069	C	T	T	T	A	T	G	T	A	G	C	T	**T**	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
P. leo SULT1C2-like XM 042930191 LOC122214989	C	T	T	T	A	T	G	T	A	G	C	T	**T**	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
O. rosmarus divergens SULT1C2 XM 004414460.2	C	T	T	T	A	T	G	T	A	G	C	T	**T**	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
Z. californianus SULT1C2 XM 027622119	C	T	T	T	A	T	G	T	A	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
E. jubatus SULT1C2-like XM 028091279 LOC114199388	C	T	T	T	A	T	G	T	A	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
C. ursinus SULT1C2 XM 025869098	C	T	T	T	A	T	G	T	A	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
P. vitulina SULT1C2 XM 032419671	C	T	T	T	A	T	G	T	A	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
H. grypus SULT1C2 XM 036120462	C	T	T	T	A	T	G	T	A	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C
N. schauinslandi SULT1C2 XM 021680275.1	C	T	T	T	A	T	G	T	A	G	C	T	C	G	A	A	A	T	G	C	C	A	A	A	G	A	C	T	G	C

c.

number of aligned nucleotide	781	782	783	784	785	786	787	788	789	790	791	792	793	794	795	796	797	798	799	800	801	802	803	804	805	806	807	808	809	810	811	812	813
translated amino acid from human SULT1C2	G			T			V			G			D			W			K			N			H			F			T		
H. sapiens SULT1C2 NM 001056	G	G	A	A	C	T	G	T	G	G	G	G	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	C	A	C	T
R. norvegicus Sult1c2a NM 001013177	G	G	A	A	T	T	G	T	G	G	G	T	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	T	A	C	T
R. norvegicus SULT1C2-like LOC100910526 XM 006244254	G	G	A	A	C	T	G	T	G	G	G	T	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	T	A	C	T
R. norvegicus SULT1C2 XM 039084411 RGD1559960	G	G	A	A	T	T	G	T	G	G	G	T	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	T	A	C	T
R. norvegicus Sult1c2 NM 133547.4	G	G	A	A	C	T	G	T	G	G	G	T	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	T	A	C	T
M. musculus Sult1c2 NM 026935.4	G	G	A	A	C	T	G	T	G	G	G	T	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	T	A	C	T
C. lupus familiaris SULT1C2 XM 038680149.1	G	G	A	A	C	T	G	T	G	G	G	G	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	C	A	C	T
V. vulpes SULT1C2 XM 026011035	G	G	A	A	C	T	G	T	G	G	G	G	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	C	A	C	T
V. lagopus SULT1C2 XM 041754093 LOC121490422	G	G	A	A	C	T	G	T	G	G	G	G	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	C	A	C	T
U. arctos horribilis SULT1C2 XM 026515916	G	G	A	A	C	T	G	T	G	G	G	G	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	C	A	C	C
U. maritimus SULT1C2 XM 008699075.1 LOC103670620	G	G	A	A	C	T	G	T	G	G	G	G	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	C	A	C	C
A. melanoleuca SULT1C2 XM 002930286.4 LOC100467202	G	G	A	A	C	T	G	T	G	G	G	G	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	C	A	C	C
M. putorius furo SULT1C2 x3 XM 004764352.2	G	G	A	A	C	T	G	T	G	G	G	G	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	C	A	C	T
N. vison SULT1C2 XM 044262435 LOC122915683	G	G	A	A	C	T	G	T	G	G	G	G	G	A	T	T	G	G	A	A	A	G	A	C	C	A	C	T	T	C	A	C	T
M. erminea SULT1C2 XM 032351655 LOC116595392	G	G	A	A	C	T	G	T	G	G	G	G	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	C	A	C	T
F. catus SULT1C2 x1 XM 011281074.3	G	G	A	A	C	T	G	T	G	G	G	G	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	C	A	C	T
L. canadensis Lynx SULT1C2 XM 030309021 LOC115509692	G	G	A	A	C	T	G	T	G	G	G	G	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	C	A	C	T
P. yagouaroundi SULT1C2-like XM 040454146 LOC121016479	G	G	A	A	C	T	G	T	G	G	G	G	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	C	A	C	T
P. bengalensis SULT1C2 XM 043553279 LOC122466971	G	G	A	A	C	T	G	T	G	G	G	G	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	C	A	C	T
P. tigris SULT1C2 XM 042979753 LOC102952042	G	G	A	A	C	T	G	T	G	G	G	G	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	C	A	C	T
P. pardus SULT1C2-like XM 019434617.1 LOC109258069	G	G	A	A	C	T	G	T	G	G	G	G	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	C	A	C	T
P. leo SULT1C2-like XM 042930191 LOC122214989	G	G	A	A	C	T	G	T	G	G	G	G	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	C	A	C	T
O. rosmarus divergens SULT1C2 XM 004414460.2	G	G	A	A	C	T	G	T	G	G	G	G	G	A	T	T	**A**	G	A	A	A	A	A	C	C	A	C	T	T	C	A	C	T
Z. californianus SULT1C2 XM 027622119	A	G	T	A	C	T	G	T	G	G	G	G	G	A	T	T	**A**	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. jubatus SULT1C2-like XM 028091279 LOC114199388	A	G	T	A	C	T	G	T	G	G	G	G	G	A	T	T	**A**	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. ursinus SULT1C2 XM 025869098	G	G	T	A	C	T	G	T	G	G	G	G	G	A	T	T	**A**	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. vitulina SULT1C2 XM 032419671	G	G	A	A	C	T	G	T	G	G	G	G	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	C	A	C	T
H. grypus SULT1C2 XM 036120462	G	G	A	A	C	T	G	T	G	G	G	G	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	C	A	C	T
N. schauinslandi SULT1C2 XM 021680275.1	G	G	A	A	C	T	G	T	G	G	G	G	G	A	T	T	G	G	A	A	A	A	A	C	C	A	C	T	T	C	A	C	T

Supplementary Figure S1. Several SULT1C2 nonsense mutations in pinnipeds and Panthera lineage

The figures show a: mutation at residue 55 for Phocidae, b: mutation at residue 131 in lions, leopards, and walruses, and c: mutation at residue 264 in Odobenidae and Otariidae.


Carnivora SULT1C4
S. scrofa SULT1C4 XM 013995888 LOC100623441
C. ferus SULT1C4 XM 006176589
B. taurus SULT1C4 NM 001080919 XM 001251492
B. taurus SULT1C4 XM 010810036
M. fascicularis SULT1C4 XM 005575210
H. sapiens SULT1C4 AF055584.1 re
T. vulpecula SULT1C4 XM 036757222
M. domestica SULT1C4-like XM 001372508 LOC100019817
M. domestica SULT1C4-like XM 001372581 LOC100019933
M. domestica SULT1C4-like XM 001372482 LOC100019781
M. domestica SULT1C4-like XM 016424892 LOC100019749
S. scrofa SULT1C4-like XM 013995889 LOC100624389
E. caballus SULT1C4 XM 001493219 LOC100061179
O. anatinus SULT1C4-like XM 029058204 LOC114809133
T. vulpecula SULT1C2-like XM 036756473 LOC118847832
M. domestica SULT1C2 XM 007501197 LOC100019690
S. scrofa SULT1C2 XM 013995894 LOC100623541
B. taurus SULT1C2 XM 005212731
C. ferus SULT1C2 XM 032493659
rat/mouse SULT1C2
M. fascicularis SULT1C2 XM 005575205 LOC102145536
H. sapiens SULT1C2 NM 176825.2 re
E. caballus SULT1C2 XM 001501352 LOC100060302
Carnivora SULT1C2
O. anatinus SULT1C1-like XM 029048642 LOC100090962
M. fascicularis SULT1C3 XM 005575207 LOC102146803
E. caballus SULT1C3 Low quality XM 005615023 LOC102148088
H. sapiens SULT1C3 NM 001008743.2 re
T. vulpecula SULT1C1-like XM 036755201 LOC118846538
T. vulpecula SULT1C1 XM 036755200 LOC118846536
M. domestica SULT1C1-like XM 007501198 LOC100019653
T. vulpecula SULT1C1-like XM 036755199 LOC118846535
rat/mouse SULT1C1
C. ferus SULT1C1 XM 006176581 LOC102513391
B. taurus SULT1C3 NM 001193080 XM 867369
S. scrofa SULT1C1 XM 003354702 LOC100623656
M. fascicularis SULT1C1 XM 005575206 LOC102146432
E. caballus SULT1C1 XM 001493288 LOC100061281
Carnivora SULT1C1
SULT1Cs
Carnivora SULT1B1
E. caballus SULT1B1 XM 014738850 LOC100066528
S. scrofa SULT1B1 XM 005656511 LOC100624541
B. taurus SULT1B1 NM 001075823 XM 600193
H. sapiens SULT1B1 NM 014465 re
rat/mouse SULT1B1
T. vulpecula SULT1B1-like XM 036762225 LOC118852579
M. domestica SULT1B1-like XM 001362249 LOC100010054
T. vulpecula SULT1B1-like 1D1 XM 036763633 LOC118853510
M. domestica SULT1B1-like XM 001362336 LOC100010102
O. anatinus SULT1B1 XM 007669210
SULT1B1
Carnivora SULT1E1
S. scrofa SULT1E1 NM 213992
B.taurus SULT1E1 NM 177488 XM 612001
E. caballus SULT1E1 XM 023638013 LOC102148009
E. caballus SULT1E1 NM 001081918
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rat/mouse SULT1E1
O. anatinus SULT1E1 XM 029073465 LOC100081825
SULT1E1
T. vulpecula SULT1E1 XM 036763807
M. domestica SULT1D1-like XM 007495506 LOC100010148
rat/mouse SULT1D1
S. scrofa SULT1D1 XM 021100802 LOC110262013
B. taurus SULT1D1 XM 002688336
E. caballus SULT1D1 XM 023638596 LOC100068197
Carnivora SULT1D1
SULT1D1
T. vulpecula SULT1A2 XM 036738007
M. domestica SULT1A1 XM 001364485 LOC100013048
O. anatinus SULT1A NM 001127619
rat/mouse SULT1A1
Human SULT1A1,2,3/4
S. scrofa SULT1A3 NM 213765
B. taurus SULT1A1 NM 177521
C. ferus SULT1A1 XM 006183069 LOC102509988
E. caballus XM 001502038 LOC100064221
Carnivora SULT1A1
SULT1As
H. sapiens NM 014351 SULT4A1
H. sapiens SULT2A1 NM 003167
0.20

Supplementary Figure S2. Phylogeny of SULT isoforms in mammals and marsupials

Gene sequences of SULT isoforms in several mammals (cow: Bos taurus, horse: Equus caballus, pig: Sus scorfa, camel: Camelus ferus, human, rat, mouse, and several Carnivora), marsupials (gray short-tailed opossum: Monodelphis domestica and common brushtail: Trichosurus vulpecula), and platypus were added for additional phylogenetic analysis.

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