Studies on xenobiotic metabolism as a defense system in Carnivora

(食肉目動物における生体防御機構としての異物代謝に関する研究)

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Preface

The contents of *Chapter 4* are based on the article:
“Kondo M, Ikenaka Y, Nakayama S M M, Kawai Y K, Mizukawa, H, Mitani Y, Nomiyama K, Tanabe S, and Ishizuka M. Sulfotransferases (SULTs), enzymatic and genetic variation in Carnivora: Limited sulfation capacity in pinnipeds. Comp Biochem Physiol Part C Toxicol Pharmacol, in press, 2022.”.

The contents of *Chapter 2* are based on unpublished article:
“Kondo M, Ikenaka Y, Nakayama S M M, Kawai Y K, Ishizuka M. Specific gene duplication and loss of Cytochrome P450 1-3 families in Carnivora (Mammalia, Laurasiatheria). Animals, Under review. “.

The contents of *Chapter 3* are based on unpublished article:
“Kondo M, Ikenaka Y, Nakayama S M M, Kawai Y K, Ishizuka M. Duplication, loss, and evolutionary features of specific UDP-glucuronosyltransferase genes in Carnivora (Mammalia, Laurasiatheria). Animals, Under review.”.

Contents

Abstract

Chapter 1: General introduction

Chemicals, including pesticides, persistent organic pollutants, and pharmaceuticals, are sometimes released into the environment as environmental contaminants and have continuous impacts on wildlife and ecosystems. Xenobiotics metabolizing enzymes are known as "detoxifying" enzymes that alter the chemical properties of these chemicals. These are classified into Phase I through Phase III reactions. Phase I reactions include oxidation, reduction, and hydrolysis, phase II reactions include conjugation, and phase III reactions include post-metabolism efflux. Through these reactions, chemicals are often converted into less toxic forms that can be easily excreted from the body. The ability to metabolize chemical substances involving these metabolic enzymes varies greatly among animal species, and is a major factor that determines the sensitivity to chemical substances among animal species. Therefore, the evaluation of chemical-metabolizing enzymes in wild mammals, which are continuously exposed to environmental pollutants, is an urgent task.
"Carnivorans" are a group of mammals that includes cats, dogs, bears, weasels, seals, and others. They are generally located at higher levels of the ecosystem and are susceptible to bioaccumulation of highly persistent environmental contaminants. Furthermore, as an umbrella species, they are a key species group for ecosystem conservation and are important to assess their effects on environmental pollutants. In this study, I attempted to comprehensively elucidate the evolutionary, genetic, and enzymatic properties of chemical metabolizing enzymes in these carnivores by using both in vitro and in silico analyses.

Chapter 2: Specific duplication and loss of Cytochrome P450 families 1-3

This chapter details the analysis of Cytochrome P450 (CYP), the most important xenobiotics metabolizing enzyme involved in phase I reactions. CYP metabolize variety of chemicals including exogenous substances such as drugs and environmental chemicals, and endogenous substances such as steroids, fatty acids, and cholesterol. Through duplication and loss events, CYPs have created their original feature of detoxification in each mammal. I performed a comprehensive genomic analysis to reveal the evolutionary features of the main xenobiotic metabolizing family: the CYP1-3 families in Carnivora. I found specific gene expansion of CYP2Cs and CYP3As in om-nivorous animals, such as brown bear, black bear, the dog, and the badger, revealing their daily phytochemical intake as the causes of their evolutionary adaptation. Further phylogenetic analysis of CYP2Cs revealed Carnivora CYP2Cs were divided into CYP2C21, 2C41, and 2C23 orthologs. Additionally, CYP3As

phylogeny also revealed the 3As evolution was completely different to that of the Caniformia and Feliformia taxa. These studies provide us with fundamental genetic and evolutionary information on CYPs in Carnivora, which is essential for appropriate interpretation and extrapolation of pharmacokinetics or toxicokinetic data from experimental mammals to wild Carnivora.

Chapter 3: Duplication and loss of UDP-glucuronosyltransferase

This chapter focuses on glucuronosyltransferases (UGTs), the major detoxification enzymes, among the enzymes involved in phase II reactions that were not evaluated in chapter 1. UDP-glucuronosyltransferases (UGTs) are one of the most important enzymes for xenobiotic metabolism or detoxification. Through duplication and loss of genes, mammals evolved the species-specific variety of UGT isoforms. Among mammals, Carnivora is one of order include various carnivorous species yet there is huge variation of food habitat. Recently UGT1A and 2B lower activity were shown in Felidae and pinniped suggesting evolutional loss of these isoforms. However comprehensive analysis for genetic or evolutional features are still missing. This study was conducted to reveal evolutional history of UGTs in Carnivoran species. I found specific gene expansion of UGT1As in Canidae, brown bear and black bear. I also found similar genetic duplication in UGT2Bs in Canidae, and some Mustelidae and Ursidae. In addition, I discovered contraction or complete loss of UGT1A7-12 in phocids, some otariids, felids and some Mustelids. These studies highly indicated even closely related species, they have completely different evolution of UGTs and further imply the difficulty of extrapolation of the pharmacokinetics and toxicokinetic result of experimental animals into wildlife carnivorans.

Chapter 4: Enzymatic and genetic features of Sulfotransferases

This chapter focuses on sulfotransferases (SULTs), which, same as the UGTs evaluated in Chapter 3, are important for phase II reactions. Along with UGTs, it is involved in the metabolism of a variety of exogenous substances and endogenous substances such as steroid hormones, neurotransmitters such as dopamine, and thyroid hormones. In this chapter, I performed the same genetic analysis as in the previous chapters, as well as in vitro enzymatic evaluation using wild animal livers.

Genetic analysis revealed that SULT1E1, an important molecule for estrone metabolism, and SULT1D1 are genetically defective in pinnipeds. Furthermore, in vitro analysis of the metabolic activity of estradiol, the main molecule metabolized by SULT1E1, revealed that the activity of SULT1E1 is significantly lower in pinnipeds. These results suggest that the sulfate conjugation activity may be weak against estrogen, various drugs, and environmental

pollutants in pinnipeds. SULT1E1 is also involved in the metabolism of endogenous substances, suggesting that there may be significant species differences in estrogen metabolism among carnivores.

Chapter 5: Conclusion and Future investigation

These studies revealed a comprehensive characterization of the chemical metabolism enzymes important for "detoxification" in carnivores. The results indicate that a wide variety of Xenobiotics metabolism enzymes have evolved even within the evolutionarily close order Carnivora. In particular, I found characteristic duplication of CYPs in brown bears, American black bears, and badgers; genetic expansion of UGT in brown bears, black bears, and canids; genetic contraction of UGT in pinnipeds and cats; and loss of SULT1E1 in pinnipeds. Our findings will allow for more accurate characterization of xenobiotic metabolism enzymes and help to accurately extrapolate findings of xenobiotic metabolism enzymes obtained in laboratory animals. Furthermore, I believe that this research was also important in estimating animals that are sensitive to environmental chemicals by evaluating these enzymes, which are important as a defense mechanism against xenobiotics.

Abbreviation

ABC: ATP binding cassette

ADH: Alcohol dehydrogenase

ADME: Absorption, Distribution, metabolism, and Excretion

ALDH: aldehyde dehydrogenase

COMT: Catechol-*O*-methyltransferase

CYP: Cytochrome P450

DDT: Dichlorodiphenyltrichloroethane

FMO: Flavin-containing monooxygenase

GST: Glutathione-S-transferase

NAT: N-acetyltransferase

NGS: Next generation sequencing

MEGA X: Molecular Evolutionary Genetics Analysis

MRP: Multidrug resistance protein

MUSCLE: Multiple Sequence Comparison by Log-Expectation

POPs: Persistent organic pollutants

PCBs: Polychlorinated biphenyls

SLC: Solute carrier

SNP: Single nucleotide polymorphisms

SULT: Sulfotransferase

UGT: UDP-glucuronosyltransferase

XMEs: Xenobiotic metabolism enzymes

Chapter 1

General introduction

Environmental pollutants and wild mammals

Despite the benefits of anthropogenic chemicals, they are release into the environment, and there are numerous reports showing that these environmental pollutants (e.g. pesticides, industrial chemicals, metals, pharmaceuticals, and rodenticides) affect human health and wildlife at the individual and population levels [1–6]. Dichlorodiphenyltrichloroethane (DDT) may be the most widely used and notorious example of an environmental pesticide pollutant. Its toxic effects on the environment and wildlife, such as birds, were described in “Silent Spring” by Rachel Carson in 1962 [7], and are widely recognized by the public. DDT’s toxicity to wildlife mainly includes reproductive disorders (particularly eggshell thinning in birds), immune system disruption, endocrine system disruption, and neurotoxicity, and these effects sometimes lead to population declines [3,8–12]. This chemical and others such as polychlorinated biphenyls (PCBs), brominated flame retardants (including hexabromocyclododecane (HBCD) and polybrominated diphenyl ethers (PBDEs)), and organochlorine pesticides (e.g. aldrin, dieldrin, and pentachlorophenol) are known as persistent organic pollutants (POPs). POPs are highly toxic, highly persistent in environment, and accumulate and concentrate in fauna higher in the food web through biomagnification and bioaccumulation [13,14]. Although the Stockholm convention on POPs (which entered into force in 2004) banned and restricted the

use of highly accumulative and toxic chemicals, these chemicals have been detected in mammals from higher trophic levels, such as polar bears, pinnipeds, and killer whales [15–18]. Since the 1980s, as the use of organochlorides decreased due to their ecotoxicological effects, they have been gradually replaced by second-generation less persistent pesticides such as organophosphates, carbamates, and pyrethroids which are now used globally [19,20]. Further, the newly developed neonicotinoids pesticides are currently used worldwide as they are less likely to accumulate in the environment, and because they are recognized as less toxic to mammals since their toxicity is insect-selective [21]. However, terrestrial mammal, bird, reptile, and amphibian exposure to these chemicals and the many toxic effects of organophosphates, carbamates, pyrethroids, and neonicotinoids are concerning [19,20,22–25]. Besides pesticides, anticoagulant rodenticides have been investigated as environmental pollutants in faunal populations. Secondary exposure to rodenticides has been reported in raptors, rodent-eating carnivorans, and other mammals [1,26–29]. Thus, animals are currently exposed to a wide variety of organic chemicals during their daily lives, and a toxicological evaluation of the various ranges of chemicals in fauna is crucial.

Xenobiotic metabolism as a defense mechanism in mammals

Processing of xenobiotics after exposure includes four biological processes: absorption, distribution, metabolism, and excretion (ADME). These processes are important for determining xenobiotics chemical bioactivity and toxicity. Xenobiotics mostly lose their biological activity, particularly during metabolic reactions, and are converted into more water-soluble forms. Thus, metabolic reactions are important for evaluating the biological or toxicological effect of xenobiotics on animals [30,31] as they function as a defense mechanism for a wide variety of xenobiotics.

Generally, xenobiotic metabolism consists of three phases: phase I, II, and III (**Figure 1-1**). Phase I and II reactions are enzymatic reactions catalyzed by xenobiotic metabolism enzymes (XMEs). Phase I reactions include oxidation, reduction, hydration, or hydrolysis, and are conducted mainly by cytochrome P450 (CYP) but also by other enzymes such as esterase, Flavine-containing monooxygenase (FMO), alcohol dehydrogenase (ADH), and aldehyde dehydrogenase (ALDH). Phase II enzymes catalyze conjugation reactions including glucuronidation by UDP-glucuronosyltransferase (UGT), sulfation by

sulfotransferase (SULT), glutathione conjugation by glutathione-S-transferase (GST), acetylation by N-acetyltransferase (NAT), and methylation by methyltransferases such as catechol-*O*-methyltransferase (COMT). The phase III reaction is the process of exporting the chemicals or their metabolites out of cells and is catalyzed by ATP binding cassettes (ABC), including the multidrug resistance protein (MRP) family and the solute carrier (SLC) transporters. The former two reactions catalyze the transformation of xenobiotics and are usually considered essential “detoxification” reactions in animals [31–33].

Among XMEs, there are huge inter-species and intra-species differences which may explain toxicological effects in “poor-metabolizer” individuals and species [34–37]. For example, dogs and other Canidae genetically lack NAT enzymes, so their capacity for metabolizing NAT substrates, such as tolbutamide and several sulfonamides, differs [34,38]. In the case of cats, a genetic deficiency of one isoform in the UGT gene, UGT1A6 [39] and the alternative metabolic pathway may explain the high toxicity of acetaminophen to cats [40,41]. Further, various genetic variation and expression differences in XMEs in the human population may cause the notable differences in metabolism and sensitivity to xenobiotics [42,43]. Therefore, it is not easy to extrapolate experimental animal pharmacokinetics data to wild fauna, and, thus, XMEs are crucial for evaluating the effect of xenobiotics on wildlife.

Although XMEs, as defense mechanisms of animals, are of great importance, XMEs in mammals are poorly understood. Several approaches have been conducted to identify the enzymatic features of wild animal XMEs, e.g. in vitro analysis using hepatic microsomes, genetic cloning of XMEs genes, and in vivo exposure testing [44,45]. Nevertheless, these analyses cannot cover a comprehensive selection of animals, and a systematic analysis of XMEs in mammals is necessary.

Carnivora as a target species for XMEs analysis

Among various mammals, the order Carnivora includes a great diversity of families such as Canids (e.g. dogs and foxes), Ursids (bears), Mustelids (e.g. stoats and badgers), Pinnipeds (e.g. seals and walruses), Felids (e.g. small and large cats), Hyaenids (hyaenas), Herpestids (meerkat and mongooses), and Viverrids (e.g. civets and genets) [46–48] (**Figure 1-2**). Carnivora includes several animals which are highly carnivorous such as Felids, yet there is great diversity in the foraging habits among species of this group. Due to their diet,

almost all Carnivora are recognized as apex predators in their respective food chains. Their high trophic position in the food chain often leads to marked effects of biomagnification and bioaccumulation of numerous environmental pollutants and studies have identified significant concentrations of POPs in several Carnivorans [1,13,16].

Further, various species in this taxon are considered umbrella species which are one or a few key species, usually of large-bodied animals at higher trophic levels (such as large felids and bears) that are used as surrogates for the conservation of entire biodiversity pools [49,50]. Furthermore, as keystone species, carnivora are important for biodiversity and ecosystems. Keystone species are generally defined as those that have a specific amount of impact on the ecosystem relative to their abundance [51]. As most keystone species are top-predator species, they can initiate trophic cascades, help control the populations of prey species, and facilitate the availability of resources essential to other species (such as carrion or safe breeding sites) [52,53]. Thus, these species are vital for maintaining an ecological structure. Carnivora species are a highly threatened order, with 84 of 297 species classified as vulnerable, endangered, critically endangered, or extinct on the IUCN red list. Further, almost half of Carnivora species (140 out of 297 species) are identified as having “Decreasing” population status (**Figure 1-3**) in the IUCN classification.

Moreover, a number of the Carnivora are recognized as “flagship” species, which are described as “popular, charismatic species that serve as symbols and rallying points to stimulate conservation awareness and action” [54] (e.g. giant panda, lion, and bears). Indeed, Carnivora species are more often used on the covers of US conservation and nature magazines than other mammals [55]. Therefore, from a social aspect, this taxon is valuable for promoting conservation activities related to zoological medicine.

Aim of this study

Since XMEs are important as a defense mechanisms against numerous chemicals, and Carnivora is an important group from an ecological and toxicological perspective, an analysis of XMEs in this taxon is crucial. In this dissertation, I aim to identify the XMEs across Carnivora species to comprehensively identify and characterize XMEs in the Carnivora group.

In *Chapter 2*, I investigate the CYP evolutionary history in Carnivora to reveal genetic variation of these enzymes. In *Chapter 3*, I further demonstrate UGT

genetic analysis as phase II metabolic enzymes. In *Chapter 4,* SULT enzymatic and genetic features are identified to provide a comprehensive understanding of the characteristics of faunal XEMs. In *Chapter 5*, I summarized all results, practical applications, and make recommendations for future research.

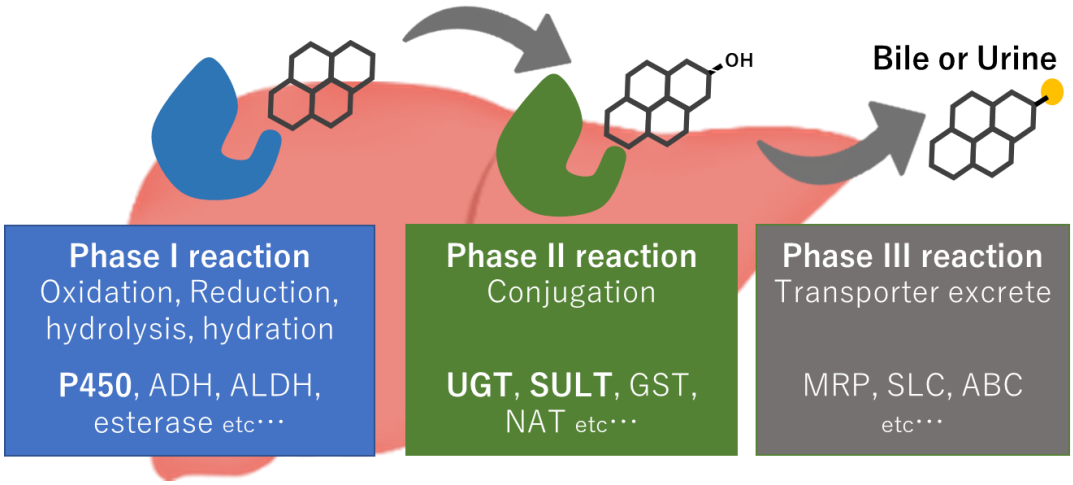

Figure 1-1. Scheme of xenobiotic metabolism in the liver. Xenobiotic metabolism reaction consists of three Phases. XMEs catalyzing each reaction are also noted. Enzymes analyzed in this thesis are shown as bold characters.

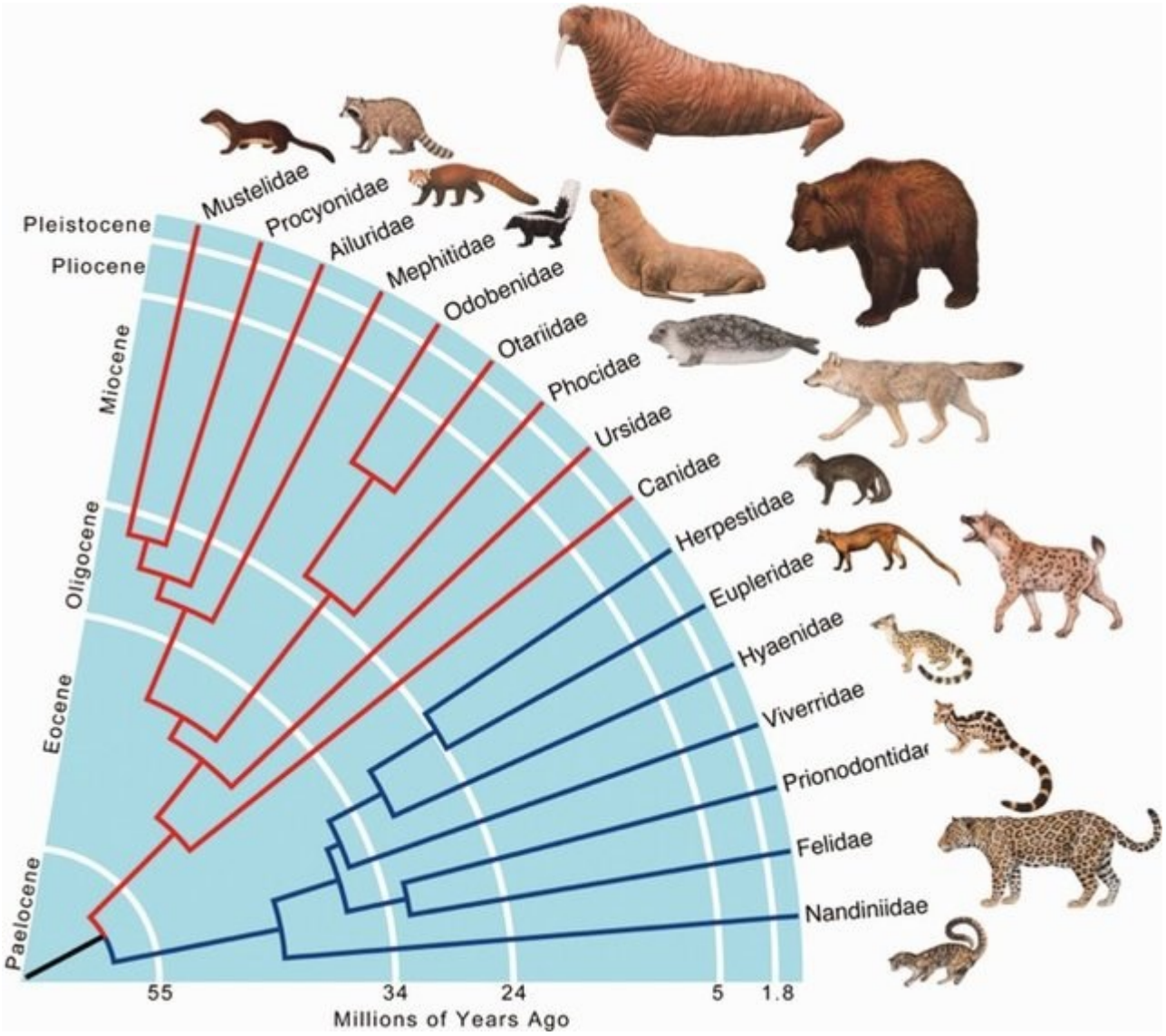

Figure 1-2. Carnivora phylogeny. Red lines show phylogeny of Caniformia (dog-like), and blue represents Feliformia phylogeny (cat-like). Figure is cited from [56].

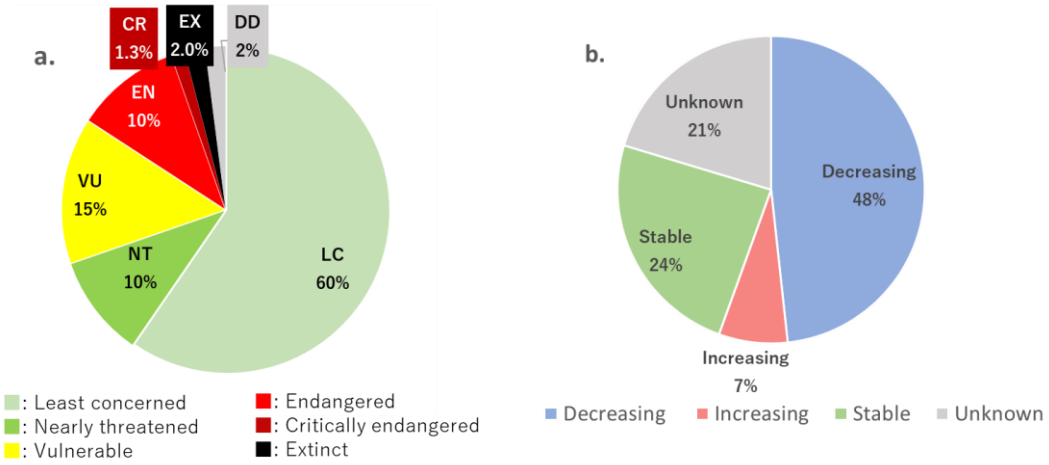

Figure 1-3. a. Carnivora species classification by IUCN red list categories. Among 297 species, 43 are in VU (vulnerable), 31 are in EN (Endangered), 4 are CE (Critically endangered), and 6 are EX (Extinct in the wild) by IUCN Red List categories [57]. **b. Carnivora population features**. Almost half of them are recognized as “Decreasing” status [57].

Chapter 2

Specific duplication and loss of Cytochrome P450 families 1-3

Introduction

Cytochrome P450s (CYP) catalyze major xenobiotics metabolism for a wide range of chemicals, such as drugs, phytochemicals, and environmental pollutants, and are considered the most important enzymes for detoxification [58]. The CYP genes form a superfamily and are divided into gene families based on >40% sequence similarity, but into subfamilies when sequence similarities are >55%. Among various CYP families in mammals, the CYP1-3 families are considered one of the main families that catalyze xenobiotic metabolism [33,59,60], although other CYP families are generally considered important for biosynthesis of numerous endogenous chemicals such as steroids, bile acids, cholesterols, eicosanoids, fatty acids, etc. [61,62].

These CYP genes are considered the fastest-evolving gene systems [63–65] and through gene duplication and loss events, xenobiotic-metabolizing CYPs, CYP1-3 genes, have evolved, duplicated or been lost, and diversified. Such an evolutionary history is essential for characterizing isoform-specific substrate-specificity and further characterizing species-specific metabolism in animals [66].

Reports suggest that CYP genetic duplication or loss affects their xenobiotic metabolism capacity in several animals and that these evolutionary consequences might be due to the need of some insects, birds, and herbivorous mammals to manage constant exposure to phytochemicals [67–70]. This “plant-arms race” concept may explain the necessity of CYP duplication events in herbivorous species: it hones detoxification systems in response to phytochemicals or toxins originating in plants. However, a detailed analysis of CYP duplication events in species of the Carnivora order is not available.

As mentioned in *Chapter 1*, Several reports suggest their genetic loss of certain xenobiotic metabolism enzymes: UDP-glucuronosyltransferase (UGT) in felids and pinnipeds, highly suggested the strong relationship with high-carnivory and genetic loss of xenobiotic metabolism enzymes [71–73]. However, there have been few reports analyzing CYP genetic loss and duplication event in this taxon, arouse us to investigate their evolutional history in comprehensive carnivora species and the relations with food habitat and their genetic duplication/loss events.

Recent innovations in next generation sequencing (NGS) systems have enable us to manage large amounts of genetic data from a wide range of wild mammals, and I can also utilize these high-quality genetic assembly data freely through an online database [74]. These whole genomics data enabled us to comprehensively analyze genetic duplication and loss events in Carnivora, and further to compare phylogenetic relationships of each gene to provide an understanding of the CYP evolutionary features in this taxon.

Firstly, I investigated and compared the synteny of CYP1-3 gene isoforms’ loci in Carnivora species. I then conducted a phylogenetic analysis to further detected specific gene duplication or loss events in each studied species to reveal the evolutionary features of CYP genes in Carnivora.

Materials and methods

Phylogenetic analyses were performed on the CYP genes of human (*Homo sapiens*), dog (*Canis lupus familiaris*), red fox (*Vulpes vulpes*), domestic ferret (*Mustela putorius furo*), ermine (Mustela erminea), mink (*Neovison vison*), badger (Meles meles), North American river otter (*Lontra canadensis*), Eurasian river otter (*Lutra lutra*), sea otter (*Enhydra lutris kenyoni*), polar bear (*Ursus maritimus*), giant panda (*Ailuropoda melanoleuca*), black bear (*Ursus americanus*), brown bear (*Ursus arctos*), meerkat (*Suricata suricatta*), striped hyena (*Hyaena hyaena*),

domestic cat (*Felis catus*), Amur tiger (*Panthera tigris*), cheetah (*Acinonyx jubatus*), puma (*Puma concolor*), Canada lynx (*Lynx canadensis*), leopard (*Panthera pardus*), lion (*Panthera leo*), leopard cat (*Prionailurus bengalensis*), fishing cat (*Prionailurus viverrinus*), Weddell seal (*Leptonychotes weddellii*), harbor seal (*Phoca vitulina*), gray seal (*Halichoerus grypus*), Hawaiian monk seal (*Neomonachus schauinslandi*), northern elephant seal (*Mirounga angustirostris*), southern elephant seal (*Mirounga leonine*), northern fur seal (*Callorhinus ursinus*), Steller's sea lion (*Eumetopias jubatus*), California sea lion (*Zalophus californianus*), and Pacific walrus (*Odobenus rosmarus divergens*). Sequences were retrieved using National Center for Biotechnology Information (NCBI) BLAST searches using the following query sequences: human and dog CYP1A1, 1A2, and 1B1 for CYP1 searching, human CYP2A6, 2B6 2D6, 2E1, 2F1, 2J2, 2S1, 2U1, 2W1, 2S1, rat 2T1 and 2G1, and dog 2C21, 2C41, and 2C23 were used for CYP2 investigation, and human CYP3A4, dog CYP3A12 and 3A26, and cat CYP3A131 and 132 were used for CYP3. These isoform queries were sufficiently comprehensive for detecting target genes and hitting other additional subfamily isoforms in Carnivora (e.g. the CYP2C6 blast search also detected CYP2Es and other subfamily genes). BLAST searches were conducted on the database Nucleotide collection (nr/nt) for each species using Blastn (optimized for similar sequences). The gene sequences used are listed in the **Supplementary data,** and the protein coding region of each isozyme was analyzed. The deduced amino acid sequences were aligned using MUSCLE (Multiple Sequence Comparison by Log-Expectation) and were used for model selection (models showing minimal sets of BIC was chosen) and construction of maximum likelihood trees (bootstrapping = 100) using MEGA X (Molecular Evolutionary Genetics Analysis) [75]. The aligned sequence lengths analyzed were 1965 bp in CYP3As and 1581 bp in CYP2Cs. The JTT+G model was used. All positions containing gaps and missing data were eliminated, and the total lengths of protein-coding sequence alignments were used for phylogenetic analysis. Foraging habits of each analyzed species are also listed in the **Table 2-1.**

Synteny analysis of CYP genes

Sequence data from genome projects are freely available. NCBI's genome data viewer (https://www.ncbi.nlm.nih.gov/genome/gdv/) or JBrowse [76] were used to visualize the chromosomal synteny maps for each species. The latest genome

assemblies were used and listed in **Table 2-2.** UCSC (University of California, Santa Cruz) BLAT (a BLAST-like alignment tool) (http://genome.ucsc.edu/index.html) was used for additional confirmation of missing genes. The Masked palm civet CYP genes were also retrieved and used to fill the gap for Feliformia species from recently assembled and annotated chromosome-level genomic data [77]

Results

CYP number counts and isoforms in CYP1As and 2ABGFSs clusters

Gene number counts for CYP 1-3 genes are shown in **Figure 2-1.** I found CYP gene coding loci where multiple CYPs were coded as a "cluster" of the CYP genes. CYP1As, CYP2ABGFSs, CYP2Cs, and CYP3As in each Carnivoran consist of a gene cluster**.** Several CYP gene clusters were conserved among Carnivorans. The CYP2ABFGSTs cluster coded CYP2As, 2Bs, 2Fs, 2Gs, and 2Ss annotated genes and was between AXL Receptor Tyrosine Kinase (AXL) and Egl-9 family hypoxia inducible factor 2 (EGNL2), and the CYP1As cluster was between C-terminal Src kinase (CSK) and Enhancer of mRNA decapping 3 (EDC3) when CYP1A1 and 1A2 orthologs in analyzed Carnivora were coded in this cluster. I further found specific gene duplication in brown bear CYP2As in the CYP2ABFGSTs cluster (**Fig. 2-1**).

CYP isoforms in CYP2Cs and CYP 2CEs cluster

Synteny of CYP2C coding loci are shown in **Figure 2-2**. The CYP2Cs coding loci also consisted of gene clusters, labeled the CYP2Cs clusters, and were highly conserved between *Helicase, lymphoid specific* (HELLS) and the *PDZ and LIM domain 1* (PDLIM1) among carnivorans, humans, and rodents. Multiple CYP2Cs were coded in the Carnivoran cluster. In almost all Mustelidae (except badger), Felidae (except the domestic cat), Pinnipedian, Canidae, and meerkat the analyzed genome had two CYP2Cs annotated genes in this cluster, whereas CYP2Cs in badger had three isoforms (with one isoform in another un-scaffolded contig), and the domestic cat had one intact isoform and one possible dysfunctional gene. The striped hyena genome had three isoforms in this cluster, whereas the meerkat had two. Some species genomes had CYP2Cs in several un-scaffolded contigs, and I did not find completely-connected cluster loci for the Pacific walrus genome although I found two possible isoforms in different contigs.

In contrast, within Ursidae I found huge species differences. The giant panda genome contained one possible CYP2C in this cluster and additional un-scaffolded isoforms in the contig NW_023254381.1. In contrast, the polar bear genome contained two possible CYP2Cs in this cluster with a partial additional isoform (CYP2C41-like) in the un-scaffolded contig NW_024425153.1. However, in the brown bear genome, I found three annotated CYP2Cs in this cluster. Moreover, the black bear genome had five possible isoforms and three partial

isoforms, even though this cluster seemed to be on two separated contigs, and the partial isoforms on two other contigs (**Fig. 2-2**).

I further found other specific CYP2Cs loci coding CYP2Cs and 2Es in Carnivora between *Synaptonemal complex central element protein 1* (SYCE1) and *Scavenger receptor family member expressed on T Cells* 1 (SCART1): which were labeled the CYP2CEs cluster. Only Canids, ursids, and Pinnipeds had CYP2Cs in this cluster whereas other Carnivoran genomes (Feliformia (Felidae, striped hyena, and meerkat) and Mustelidae) had only CYP2Es in this cluster.

Other CYP2 subfamily genes such as CYP2Ds, 2Js, 2Rs, 2Us, and 2Ws did not show any duplication in Carnivora coded as isolated genes, although coding loci were highly conserved among all analyzed Carnivorans and among other mammals (data not shown). Nevertheless CYP2J, 2R, 2S, 2T, 2U, and 2Ws are generally known as biosynthesis-type or unknown substrate isoforms, and I did not include these genes in the analyses.

Synteny analysis of CYP3As cluster

I also analyzed the CYP3As gene cluster shown in **Figure 2-3,** and this cluster, which was also conserved among carnivorans, is between *Zinc finger and SCAN domain containing 25* (ZSCAN25) and *Olfactory receptor family 2 subfamily AE member 1* (OR2AE1) or *Tripartite motif containing 4* (TRIM4). All Felidae analyzed had only two possible isoforms of CYP3As, and no species-specific differences were observed. However, the Mustelidae genome had four or three possible isoforms in the CYP3As cluster and the ermine and Canadian river otter had one pseudogene annotated gene in this cluster. In Ursidae, the brown and black bear genomes also contained four annotated CYP3As, whereas the polar bear and black bear genomes had three isoforms. The giant panda genome contained only one isoform annotated as “LOW-QUALITY PRTOTEIN” coding gene in this cluster, with six other very short partial un-scaffolded isoforms (CDS length less than 515 bp) observed. Canids also have multiple CYP3As in this cluster, and the dog genome had four isoforms in this cluster (chromosome 6: NC_051810.1) with several intact and partial isoforms. Recently, two dog CYP3As were characterized and renamed, and the NCBI annotated name was different to the CYP nomenclature in dog CYP3As. The NCBI naming system was followed and genes were renamed in this paper (CYP3A4; Gene ID: 479740 as CYP3A98 and CYP3A12-like; LOC119875773 as CYP3A99) [78]. Red fox CYP3As were also on three un-scaffolded contigs (NW_020356965.1,

NW_020356599.1, and NW_020356653.1) with two isoforms and three partial isoforms. However, in the Arctic fox genome, CYP3As were not coded as a cluster and these genes were coded on two different loci on same the chromosome (chromosome 3: NC_054826.1 with two intact and one short isoform). In several Pinnipedia genomes, CYP3As were also located on several un-scaffolded contigs, suggesting much higher quality assemblies are essential for clear analysis. Three Otariidae or Odobenidae genomes (from the Pacific walrus, northern fur seal, and Stellar sea lion) had two intact or partial CYP3As and one annotated pseudogene of CYP3As, whereas four isoforms were observed in the California sea lion genome (**Figure 2-6**). Phocidae genomes also have scattered genes of CYP3As and I could not find a clear CYP3A cluster or isoform, with 1-2 intact CYP3As and several partial genes in each genome.

Phylogeny of CYP2Cs in Carnivorans

I performed a phylogenetic analysis of CYP2Cs in carnivorans, and I revealed Carnivoran CYP2Cs were divided into three clades, namely CYP2C41s, CYP2C21s, and CYP2C23s (**Figure 2-4**). Based on the phylogeny, the CYP2C23s clade was located close to CYP2Es clades. Each clade contained orthologous Carnivoran genes to CYP2C41 and 2C21 in dogs and CYP2C23 in rats, respectively. Almost all CYP phylogenies in the CYP3C41s, 2C21s, and 2C23s clades followed their organisms' phylogeny order. I also found specific duplication of the CYP2C21s clade in Ursidae, suggesting these duplication events occurred after the divergence of Ursidae.

Phylogeny of CYP3As in Carnivorans

I conducted similar phylogenetic analysis on CYP3As that revealed a Caniformia-clade and a Feliformia-unique clade in CYP3As (**Figure 2-5**), suggesting the CYP3As evolutionary history between Feliformia and Caniformia was completely different. Based on the phylogeny, the Caniformia-clade was further subdivided into three clades which were labeled Caniformia CYP3As clade 1 to clade 3. For clade 1, almost all Caniformia species possessed these genes, and Mustelidae had two each specifically duplicated clades (1-1 and 1-2), suggesting canid-specific and Mustelid-specific duplication of genes in this clade. Canidae also showed lineage specific duplication in this clade. Ursidae, Mustelidae, and some Pinniped genomes possessed CYP3As in clade 2, whereas Canidae and Feliformia did not have isoforms in this clade. In clade 3,

however, Canidae, Mustelidae, and Ursidae have these genes and canids have a family-specific duplication of this clade similar to clade 1. Although some pinnipeds have multiple CYP3As, some with partial genes were removed for phylogenetic analysis to ensure clear results were produced, so there could be some pinniped CYP3As that might have been classified into clade 3.

For the CYP3As Feliformia-clade, Felidae CYP3As were divided into two clades named CYP3A131s and CYP3A132s as per the domestic cat CYP3A131 and CYP3A132 [79]. In each clade, specific clades for Felidae were further established and only one isoform from each respective Felidae species was contained in these clades. However, other Feliformia CYP3As genes were not classified into these CYP3A131s and CYP3A132s clades, suggesting unique CYP3As loss or duplication events occurred in each species through the evolutionary history of Feliformia.

Discussion

CYPs duplication and loss in mammals, and relationships with food habitats

Several reports have revealed CYP gene duplication events in variety of mammals, especially herbivorous mammals. Recently, a koala genome project revealed that a huge expansion of CYP2Cs was possibly an adaptation to a diet of eucalypts [67]. Further, among woodrat genomes, especially juniper-eating species, several reports have found higher gene copy numbers of CYP2As, 2Bs, and 3As compared to other rodents [68,80,81], and studies have also suggested that the woodrat's CYP2Bs gene expansion might contribute to their high metabolic capacity for terpenes from juniper plants. In this study, I found specific duplication of CYP2Cs and CYP2As in the brown bear, CYP2Cs in the black bear, CYP2Cs and 3As in the badger, and CYP3As in the dog. All these animals are omnivorous and their foraging habits include a wide variety of food types, indicating that these species have a "generalist" diet [82–86]. Therefore, these gene expansions might be the consequences of a need for detoxification of a wide variety of plant-secondary metabolites in their daily diets.

However, in other omnivorous or herbivorous animals, (e.g. the red fox, Arctic fox, and giant panda), I did not find any specific duplication of CYPs. In the red fox genome, CYP3As clusters were separated because of assembly quality, which suggested these regions require re-assembling or target re-sequencing. Further, I found separated clusters of CYP3As for the Arctic fox, suggesting these

loci in Arctic fox genome are instable. Hence, further in-depth genomic analysis is required to clarify whether the CYP3A expansion is limited to dogs or is also applicable to other Canidae species.

Notably, I assumed giant panda genomes would show expansion of CYPs in response to various plant secondary metabolites in their exclusively bamboo diets. However, our result suggested that there was no expansion of any CYPs, or even a contraction trend of CYP3As in the giant panda. These trends were also discovered by our analysis of UGTs genomics for this species (*Chapter 3*). These results strongly suggested that giant panda do not rely on an “enzymatic-strategy” to deal with their daily toxin intake. Their unbalanced “specialist” bamboo-exclusive diet might be the reason they have not evolved expanded CYP or other xenobiotic metabolism enzymes, and gut microbiota is an alternative strategy they might use instead [87–89].

CYP2Cs in Carnivora

From the phylogenetic analysis of this study, I identified that Carnivora CYP2Cs are divided into 3 clades, which were possibly orthologs of CYP2C21s, CYP2C41 in the dog, and CYP2C23s in the rat [90–92]. These features strongly suggest that the substrate specificity of dog CYP2Cs is similar to that of CYP2C21 and CYP2C41 in other Carnivora. Previous studies have revealed that dog CYP2C21 showed substrate specificity for diclofenac, midazolam, and the 4-methyl-N-methyl analog of sulfaphenazole, O-desmethyltramadol, testosterone (16-alpha OH), and (S)-Mephenytoin [91,93–95]. CYP2C41 also showed similar substrate specificity to diclofenac and midazolam albeit with lower activity. However, comprehensive and systematic analysis of recombinant CYP2C21 and CYP2C41 in the dog is necessary for clarification of specific substrate-specificity. Our results together with this substrate specificity suggest that Carnivora CYP2C21s and 2C41s might also show similar substrate specificity. Our analysis revealed brown bear and black bear also showed gene expansion in CYP2C21 and 2C41s. Since data regarding substrate specificity of CYP2Cs in Carnivora is limited, I need to estimate Carnivora CYP2Cs in Ursidae from other mammals. Human CYP2Cs showed metabolism of a wide variety of chemicals including endogenous eicosanoids and fatty acid [96], xenobiotic drugs such as antimalarials, oral antidiabetics, most NSAIDs, most proton pump inhibitors and warfarin [32,59], and some terpenoids [97–99]. Interestingly, brown bears, black bears, and even

badger are known to consume pine nuts [85,100,101], which are from conifer trees that contain terpenoids [102,103]. Further study investigating recombinant CYP2Cs is essential to confirm whether these expanded CYP2Cs in the Ursid and badger are able to metabolize the terpenoids in their daily diets.
Among Canids, CYP2C41s show polymorphism as a complete loss in several breeds [104], suggesting some dogs have contracted CYP2Cs. This further suggests that other species might also show polymorphism or copy number variants within species, providing further justification for the importance of genomic analyses that use several individuals to conclude isoform numbers in each species.

CYP2C23s orthologous genes in Carnivora

From the phylogenetic analysis, I discovered a possible orthologue to rat CYP2C23 and mouse CYP2C44 in Carnivora. The coding locus of rat CYP2C23 and mouse CYP2C44 (between Erlin1 and Cpn1) was neither in the CYP2Cs cluster, nor in a similar region to that of Carnivorans. These isoforms have been cloned and characterized as arachidonic acid or eicosanoid metabolizing CYPs and are closely related to the endogenous biosynthesis of these animals [90,105,106], although humans have pseudogenes of these isoforms. I found possible orthologues to rodents CYP2C23s in Carnivora, which strongly suggests these isoforms in Carnivora also have similar eicosanoid metabolism roles. I also found specific deletion of CYP2C23s in Feliformia and Mustelidae. This could indicate that eicosanoid metabolism in these animals is different to other carnivorans. A further Blast analysis indicated possible CYP2C23s orthologues in cattle, horse, and pangolin in loci between Erlin1 and Cpn1 (data not shown), suggesting the CYP2C23 gene translocated after Carnivora divergence.

CYP3As in mammals

Human CYP3As catalyzes a wide range of prescribed drugs and is considered one of the most important subfamilies for drug metabolism in humans [59,107], yet they also catalyze metabolism of endogenous chemicals such as steroids, cholesterols, and bile acids [59,108]. Canine CYP3As (e.g. CYP3A12, 3A26, 3A98, and 3A99) have been cloned and characterized, showing similar substrate specificity patterns as human CYP3As [91,109,110]; however, with different expression patterns. In contrast, CYP3A12 and 3A26 are liver specific [109,111], while CYP3A98 is expressed in the intestine and 3A99 in the liver and intestine

[78]. These features and the phylogenetic result indicate the CYP3As clade 3 could be intestinal-specific isoforms whereas the CYP3As clade 1 could be liver-specific orthologs in Carnivora. However, the CYP3As clade 2 expression patterns remain unclear. This tissue specific expression of the CYP3As, especially in the liver and intestine, has also been observed in evolutionarily distant species, like humans.

Among Feline CYP3As, similar expression patterns have been characterized, which means CYP3A132 is mainly expressed in the liver, whereas CYP131 is mainly expressed in the intestine with lower levels in the liver [79,112,113]. These features, together with our result, further indicate that the Feliformia CYP3A132 may be major CYPs in the liver, whereas CYP3A131 may be intestine specific in Feliformia. In other Feliformia, such as the striped hyena, meerkat, and masked palm civet, however, I found other clades of CYP3As. Unfortunately, Feliformia genomic data is limited and I could not clearly demonstrate the evolutionary history of CYP3As in this taxon. Further studies on a greater variety of Feliformia species' genomics and expression pattern are necessary.

Similar to CYP2Cs, I found CYP3As expansion in omnivorous species: the dog, badger, brown bear, and black bear. CYP3As catalyze a wide range of xenobiotics including some pyrrolizidine alkaloids [114–116] found mainly in the families Asteraceae, Boraginaceae, and Fabaceae. Thus, the omnivorous diets of bears and badgers might be an evolutionary driving force enabling these species to cope with accidental intake of these toxins.

Our phylogenetic analysis indicated that diversification of CYP3As in Caniformia and Feliformia was completely different, suggesting that substrate specificity of CYP3As among these taxa is different. However, the phylogenetically distant isoform human CYP3A4 showed similar substrate specificity to CYP3As in dogs and cats (albeit with several differences) [78,79,95], indicating substrate specificity in Carnivora need not range widely to cope with a variety of chemicals. However, further systemic analysis CYP3As function in Carnivora is required.

Other CYP isoforms

In our analysis, CYP1-3 families did not show strong differences. However, in domestic cat, specific duplication and loss events were observed. For instance, CYP2C21 has been reported as a pseudogene [117], and CYP2E1, which mainly catalyzes acetaminophen or alcohol, has been specifically duplicated in this species [118]. In our study, I only identified a similar phenomenon of CYP2E

duplication in the fishing cat (*Prionailurus bengalensis*) and I did not record CYP2C21 loss in any other Felidae species, suggesting that these duplication and loss events might be specific to cats or Felidae species closely related to cats. Expression of CYP2C has also been limited in cats, whereas CYP2Es are dominant in the liver compared to other isoforms [113,119], which makes it difficult to clarify which isoforms are important for xenobiotic metabolism in Felidae. Thus, a functional analysis is essential for further discussion.

Short conclusion

In this study I comprehensively analyzed the evolutionary features of CYP1-3 families in Carnivora. I found specific expansion of CYP2C and 3As in omnivorous Carnivora such as the badger, brown bear, black bear and dog genomes. Our phylogenetic analysis further revealed possible orthologs of CYP2C21s, 2C41s, and 2C23s in Carnivora. Furthermore, I found the evolution of CYP3As was completely different in Caniformia and Feliformia, and within each taxon I detected specific CYP3As duplication events. These studies provide fundamental evolutionary and genetic information for extrapolating the pharmacokinetics or toxicokinetic of experimental animals to that of wild Carnivora, which include a wide variety of top-predator, key-stone, and rare species threatened with extinction.

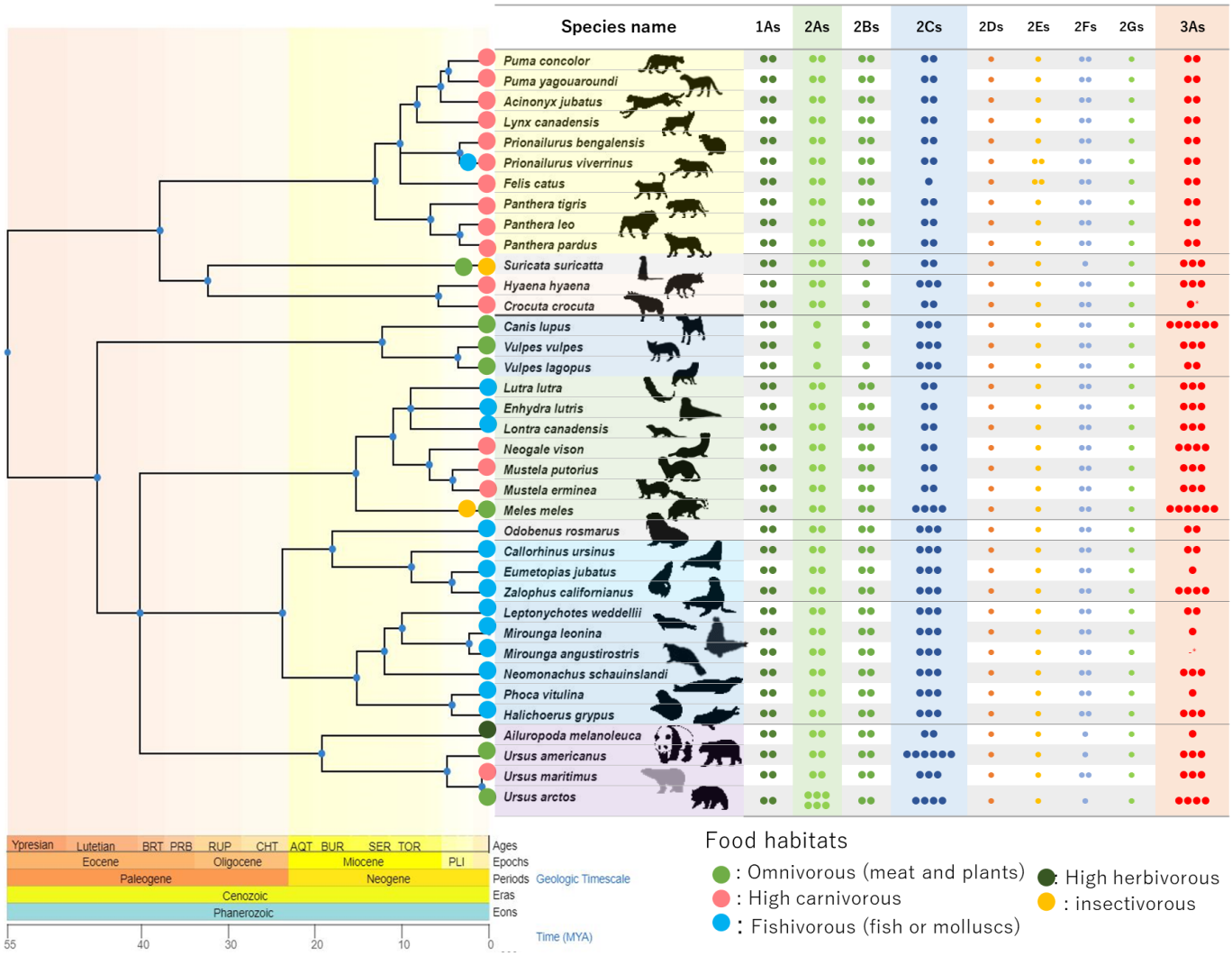

Figure 2-1. Isoform numbers of CYP1-3 families among Carnivora.

Gene numbers for CYP1As, 2A, 2B, 2C, 2D, 2E, 2F, 2G and 3As are shown by number of small filled circles. Large filled circles next to the scientific name of each species are colored by known diet (**Table 2-1**). Isoforms coding “low quality” or partial genes were not counted in this case. The phylogenetic tree was created with TimeTree 5 [120].

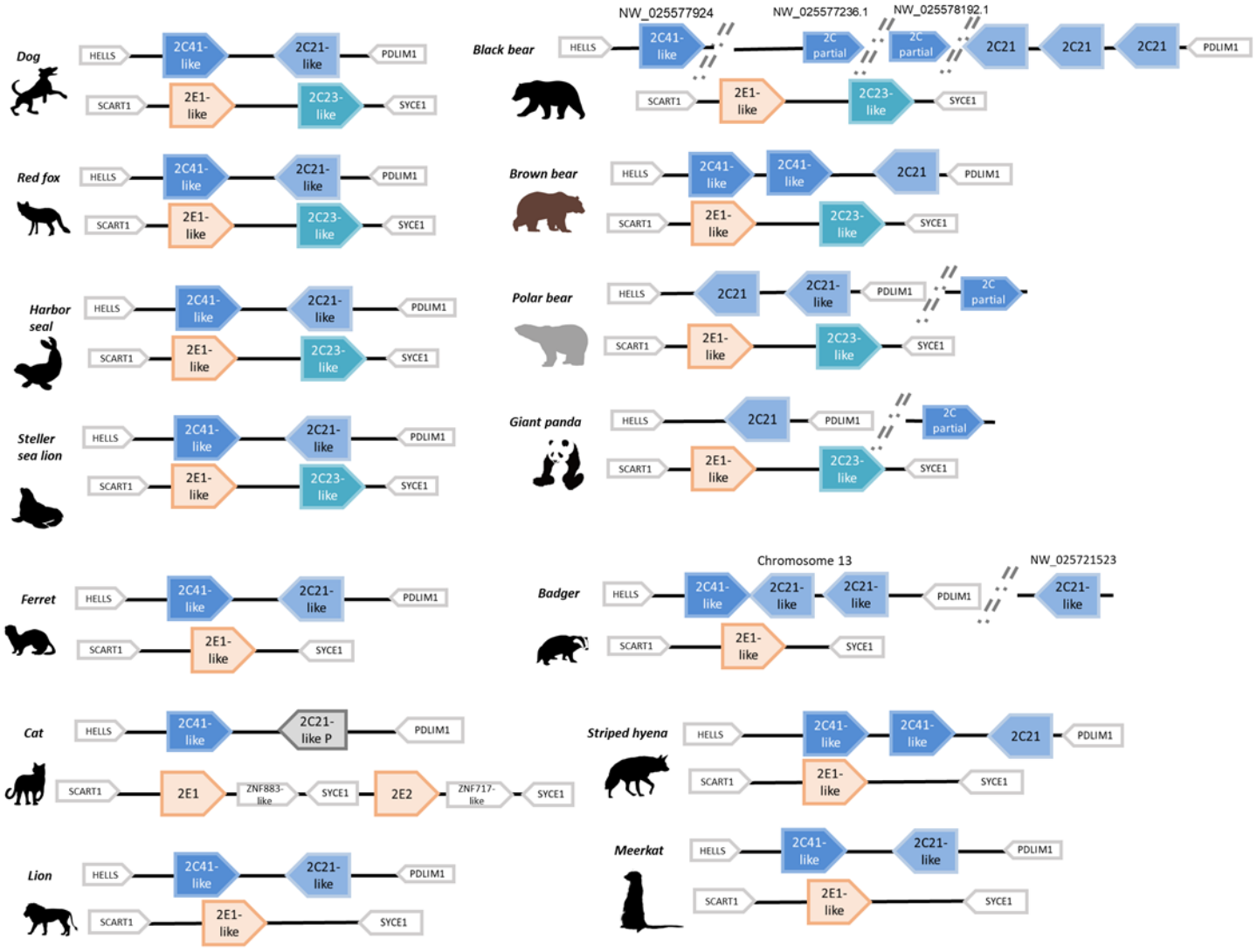

Figure 2-2. Synteny of CYP2Cs and CYP2CEs clusters in Carnivora.

Synteny of CYP2C cluster and CYP2CE cluster among Carnivora are shown. Representative species for each family were selected. Phylogenetic analysis supported classification were applied for each isoform and colored based on each clade. CYP2C41s are shown as blue, CYP2C21s as pale blue, CYP2C23s as brilliant blue, and CYP2Es as pale orange. Letters on each locus shows coding contigs if separately coded. Pseudogenes are shown as black or gray blocks.

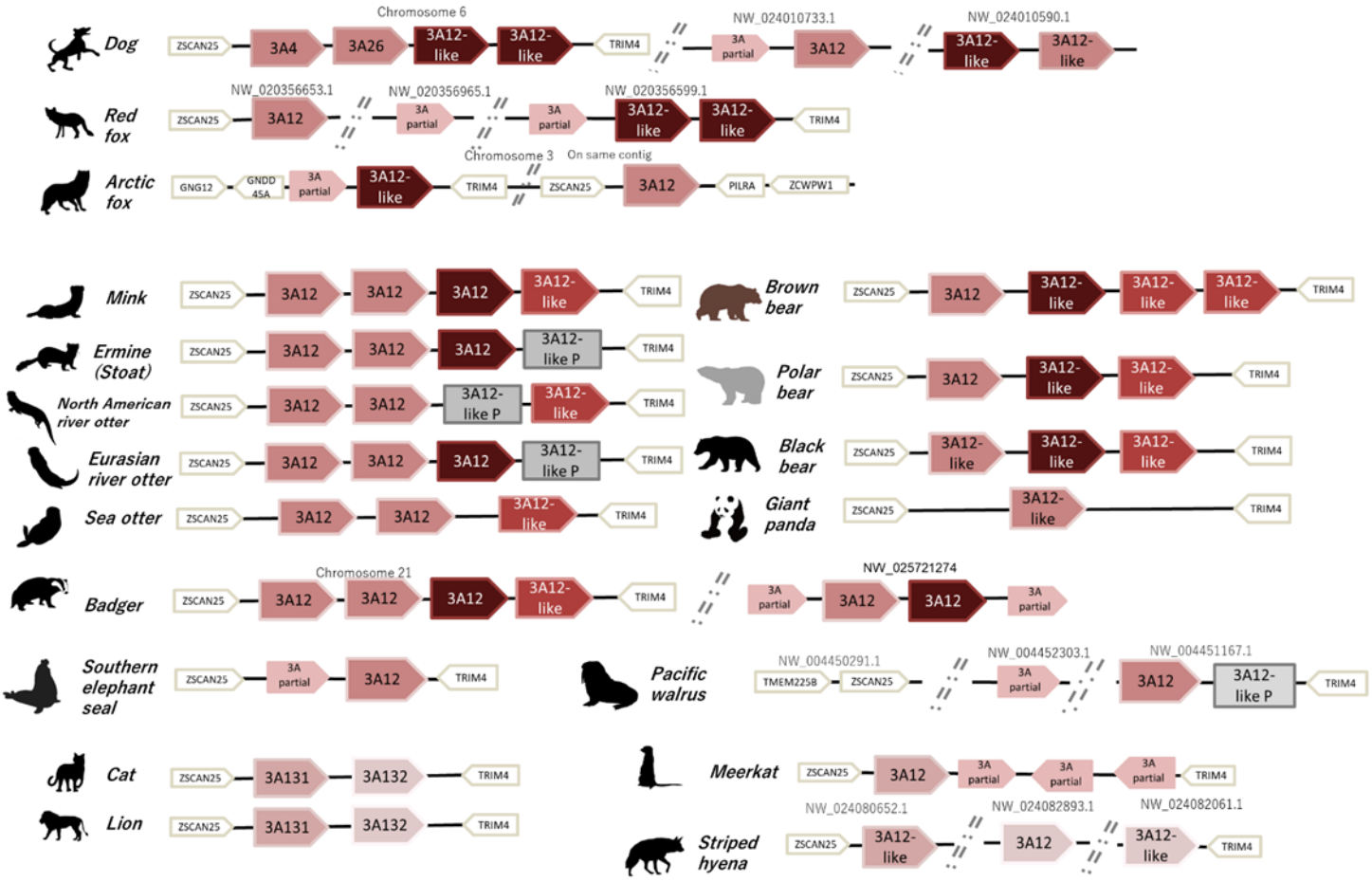

Figure 2-3. Synteny of CYP3As cluster in Carnivora.

Synteny of CYP3A cluster among Carnivora is shown. Representative species for each family were selected. Phylogenetic analysis supported classification were applied for each isoform and colored based on each clade. Caniformia CYP3As in clade 1, clade 2 and clade 3, CYP131s and CYP132s in Felidae, are differently colored. Letters on each locus shows coding contigs if separately coded. Pseudogenes are shown as gray blocks.

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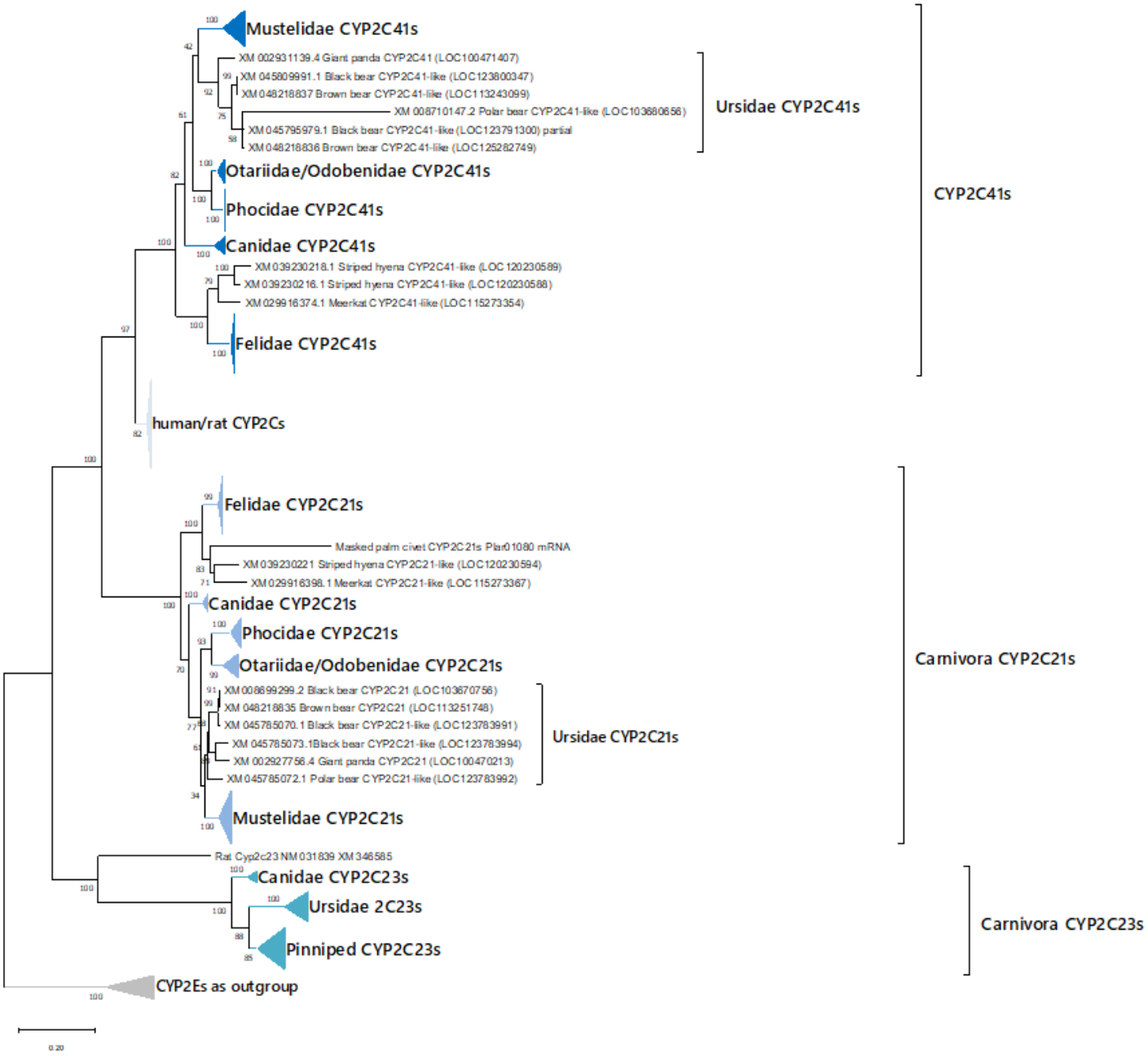

Figure 2-4. Phylogenetic tree of CYP2Cs

Phylogenetic tree of CYP2Cs sequences in human, and carnivorans. Gene sequences of protein-coding regions for each isozyme were analyzed. The numbers next to the branches indicate the number of occurrences per 100 bootstrap replicates. Genes and clades are tentatively labeled with carnivoran CYPs examined in this article. Clades of CYP2C41s, CYP2C21s and Carnivora 2C23s are shown as differently colored triangles. CYP2Es are shown as an outgroup.

Figure 2-5. Phylogenetic tree of CYP2Cs

Phylogenetic tree of CYP3As sequences in carnivorans. Gene sequences of protein-coding regions for each isozyme were analyzed. The numbers next to the branches indicate the number of occurrences per 100 bootstrap replicates. Genes and clades are tentatively labeled with carnivoran CYPs examined in this article. Canifromia clade 1, clade 2 clade 3 and Felidae CYP3A131s and 3A131s clades are shown with differently colored triangles. CYP3Cs are shown as out group.

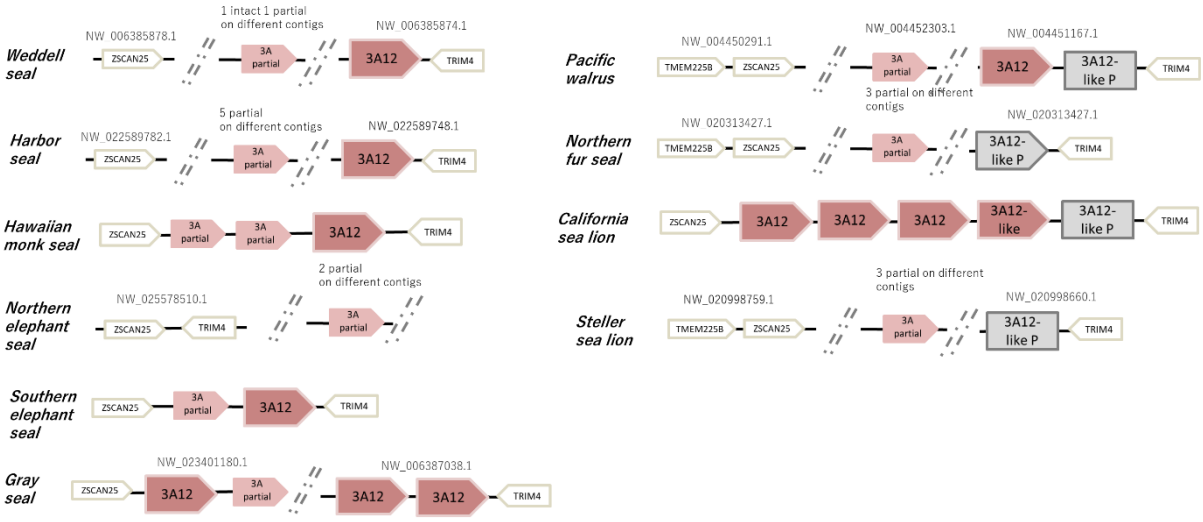

Figure 2-6. Synteny of CYP3As in pinnipeds. Pinniped CYP3A clusters are scattered on several un-scaffolded contigs and difficult to analyze.

Species	Food habitats	References
Acinonyx jubatus	Carnivore	[121,122]
Ailuropoda melanoleuca	Herbivore	[88,123]
Callorhinus ursinus	Fishivore	[124–127]
Canis lupus familiaris	Omnivore	[128–130]
Crocuta crocuta	Insectivore, Omnivore	[131,132]
Enhydra lutris kenyoni	Fishivore	[127,133]
Eumetopias jubatus	Fishivore	[127,134]
Felis catus	Carnivora	[86,135]
Halichoerus grypus	Fishivore	[136]
Hyaena hyaena	Carnivore	[137,138]
Leptonychotes weddellii	Fishivore	[127,139]
Lontra canadensis	Fishivore	[140,141]
Lutra lutra	Fishivore	[142]
Lynx canadensis	Carnivora	[143,144]
Meles meles	Insectivore, Omnivore	[85,145]
Mirounga angustirostris	Fishivore	[126,146]
Mirounga leonina	Fishivore	[147,148]
Mustela erminea	Carnivora	[149,150]
Mustela putorius furo	Carnivora	[151,152]
Neogale vison	Carnivora	[18,153,154]
Neomonachus schauinslandi	Fishivore	[127,155]
Odobenus rosmarus divergens	Fishivore	[156,157]
Panthera leo	Carnivora	[158,159]
Panthera pardus	Carnivora	[160,161]
Panthera tigris	Carnivora	[162,163]
Phoca vitulina	Fishivore	[127,164]
Prionailurus bengalensis	Carnivora	[165,166]
Prionailurus viverrinus	Carnivora	[165]
Puma concolor	Carnivora	[167,168]
Puma yagouaroundi	Carnivora	[169,170]
Ursus americanus	Omnivore	[83,171–173]
Ursus arctos	Omnivore	[82,174,175]

Ursus maritimus	Carnivore	[175,176]
Vulpes lagopus	Omnivore	[177,178]
Vulpes vulpes	Omnivore	[179,180]
Zalophus californianus	Fishivore	[181,182]

Table 2-1. Food habitats of Carnivora species.

I referred the food habitat of Carnivorans from literatures listed above and used for Figure 2-1, and also Figure 3-1 in **Chapter 3**.

Assembly Accession	Organism Name	Annotation Name
GCF_003709585.1	*Acinonyx jubatus*	NCBI Annotation Release 101
GCF_002007445.2	*Ailuropoda melanoleuca*	NCBI eukaryotic genome annotation pipeline
GCF_003265705.1	*Callorhinus ursinus*	NCBI Annotation Release 100
GCF_003254725.2	*Canis lupus dingo*	NCBI Annotation Release 102
GCF_014441545.1	*Canis lupus familiaris*	NCBI Annotation Release 106
GCA_008692635.1	*Crocuta crocuta*	Annotation submitted by BGI
GCF_002288905.1	*Enhydra lutris kenyoni*	NCBI Annotation Release 100
GCF_004028035.1	*Eumetopias jubatus*	NCBI Annotation Release 100
GCF_018350175.1	*Felis catus*	NCBI Annotation Release 105
GCF_012393455.1	*Halichoerus grypus*	NCBI Annotation Release 100
GCF_003009895.1	*Hyaena hyaena*	NCBI Annotation Release 100
GCF_018350155.1	*Leopardus geoffroyi*	NCBI Annotation Release 100
GCF_000349705.1	*Leptonychotes weddellii*	NCBI Annotation Release 101
GCF_010015895.1	*Lontra canadensis*	NCBI Annotation Release 100
GCF_902655055.1	*Lutra lutra*	NCBI Annotation Release 100
GCF_007474595.2	*Lynx canadensis*	NCBI Annotation Release 102
GCF_022079265.1	*Lynx rufus*	NCBI Annotation Release 100
GCF_922984935.1	*Meles meles*	NCBI Annotation Release 100
GCF_021288785.1	*Mirounga angustirostris*	NCBI Annotation Release 100
GCF_011800145.1	*Mirounga leonina*	NCBI Annotation Release 100
GCF_009829155.1	*Mustela erminea*	NCBI Annotation Release 100
GCF_011764305.1	*Mustela putorius furo*	NCBI Annotation Release 102
GCF_020171115.1	*Neogale vison*	NCBI Annotation Release 100
GCF_002201575.2	*Neomonachus schauinslandi*	NCBI Annotation Release 101
GCF_000321225.1	*Odobenus rosmarus divergens*	NCBI Annotation Release 101
GCF_018350215.1	*Panthera leo*	NCBI Annotation Release 100
GCF_001857705.1	*Panthera pardus*	NCBI Annotation Release 100

GCF_018350195.1	*Panthera tigris*	NCBI eukaryotic genome annotation pipeline
GCF_023721935.1	*Panthera uncia*	NCBI Annotation Release 100
GCF_004348235.1	*Phoca vitulina*	NCBI Annotation Release 100
GCF_016509475.1	*Prionailurus bengalensis*	NCBI Annotation Release 100
GCF_022837055.1	*Prionailurus viverrinus*	NCBI Annotation Release 100
GCF_003327715.1	*Puma concolor*	NCBI Annotation Release 100
GCF_014898765.1	*Puma yagouaroundi*	NCBI Annotation Release 100
GCF_006229205.1	*Suricata suricatta*	NCBI Annotation Release 100
GCF_020975775.1	*Ursus americanus*	NCBI Annotation Release 100
GCF_023065955.1	*Ursus arctos*	NCBI eukaryotic genome annotation pipeline
GCF_017311325.1	*Ursus maritimus*	NCBI Annotation Release 101
GCF_018345385.1	*Vulpes lagopus*	NCBI Annotation Release 100
GCF_003160815.1	*Vulpes vulpes*	NCBI Annotation Release 100
GCF_009762305.2	*Zalophus californianus*	NCBI Annotation Release 101

Table 2-2. Assemble and annotation information.

Assembly accession number, species scientific names, and annotation information used in this study are shown. These annotations and assembles were also used in *Chapter 3* and *4.*

Chapter 3

Duplication and loss of UDP-glucuronosyltransferase

1. Introduction

In Chapter 1, I described the CYP genomics in Carnivora. However, various chemicals, especially most of carcinogens, show metabolic activation after CYP-mediated metabolism, phase II reactions after CYP metabolism could be much important for detoxification.

UDP-glucuronosyltransferases (UGTs) are a superfamily of enzymes which catalyze the glucuronide conjugation reaction to both endogenous (e.g., bilirubin, bile acids, several hormones, neurotransmitters) [183–186] and exogenous chemicals (e.g., prescribed drugs, veterinary drugs, plant-derived chemicals, and environmental pollutants) [187–191]. Using UDP-glucuronide as a donor, UGTs transfer a glucuronide moiety into substrate substances to increase hydrophilicity and generally drive deactivation for excretion through bile or urine.

Hence, UGTs are generally considered among the major detoxification enzymes for mammals.

The mammalian UGTs superfamily consists of two families (1 and 2) and is subdivided into the 1A, 2A, and 2B subfamilies based on amino acid sequence levels [192,193]. A vast variety of mammal UGTs has diverged in each species through gene duplication and loss events [66,194]. For instance, in humans there are 19 isoforms (9 in UGT1 and 10 in UGT2) showing different substrate specificities in enriching the metabolism with a wide range of chemicals.

Recent studies on UGTs suggested strong genetic differences among Carnivora. Shrestha et al. (2011) [16] and Kakehi et al. (2015) [17] found a genetic dysfunction (pseudogene) in the major phenol-metabolizing enzyme UGT1A6 in Felidae, brown hyena (*Parahyaena brunnea*) and Otariidae. The carnivora-specific isoform UGT2B31 pseudogene also appeared to be present in all Felidae, indicating a limited capacity for glucuronidation [71]. Very limited numbers of UGT1A/2B isoforms were observed in these species, suggesting contraction of these genes during evolution [71,72].

These reports strongly suggest the presence of a large genetic diversity in UGT even within Carnivora, however no further Carnivora species have yet been examined in this regard. Recent improvements in WGS (whole genome sequencing) techniques allow us to utilize a large volume of genomic data from a variety of wild carnivorans [195]. These data enable the comprehensive investigation of evolutionary history, including gene duplication/loss events, and sequence comparisons of each isoform. In the present study, I utilized genomic data from a large variety of Carnivora species to evaluate the genetic synteny of each UGT. I also conducted phylogenetic analysis of each subfamily to analyze evolutionary inter-species differences in this enzyme superfamily.

2. Materials and methods

2-1. *Data retrieval for UGT phylogenetic analysis*

Phylogenetic analyses were performed on the UGT genes same species in *Chapter 1*. Human (*Homo sapiens*), rat (*Rattus norvegicus*), mouse (*Mus musculus*), dog (*Canis lupus familiaris*), red fox (*Vulpes vulpes*), domestic ferret (*Mustela putorius furo*), ermine (*Mustela erminea*), mink (*Neovison vison*), Badger (*Meles meles*), North american river otter (*Lontra canadensis)*, Eurasian river otter (*Lutra lutra*), sea otter (*Enhydra lutris kenyoni*), polar bear (*Ursus maritimus*), giant panda (*Ailuropoda melanoleuca*), black bear (*Ursus americanus*), brown bear (*Ursus arctos*), meerkat (*Suricata suricatta*), striped hyena (*Hyaena hyaena*), Domestic cat (*Felis catus)*, Amur tiger (*Panthera tigris*), cheetah (*Acinonyx jubatus*), puma (*Puma concolor*), Canada lynx (*Lynx canadensis*), leopard (*Panthera pardus*), Lion (*Panthera leo*), Leopard cat (Prionailurus bengalensis), Weddell seal (*Leptonychotes weddellii*), harbor seal (*Phoca vitulina*), gray seal (*Halichoerus grypus*), Hawaiian monk seal (*Neomonachus schauinslandi*), northern elephant seal (*Mirounga angustirostris*), southern elephant seal (*Mirounga leonine*), northern fur seal (*Callorhinus ursinus*),

Steller's sea lion (*Eumetopias jubatus*), California sea lion (*Zalophus californianus*), and Pacific walrus (*Odobenus rosmarus divergens*). Sequences were retrieved by National Center for Biotechnology Information (NCBI) BLAST searches, using the following query sequences: for UGT1, human and dog UGT1A1, UGT1A6, UGT1A2, and UGT1A7; for UGT2, dog UGT2B31, human UGT2B8, cat UGT2E1, dog UGT2A1, and human UGT2A1. BLAST searches were conducted in the Nucleotide collection database (nr/nt) for each species using Blastn (optimized for somewhat similar sequences). This blast search for UGT1As were comprehensive enough to detect UGT1 and 2s in Carnivora, and UGT2Bs search also covered UGT1As in Carnivora. For UGT1As, only 1st exons for each isoform were analyzed, and for UGT2Bs, all protein coding sequences were used. The deduced amino acid sequences were aligned using MUSCLE (Multiple Sequence Comparison by Log-Expectation) and were used for model selection (minimal BIC) and construction of maximum likelihood trees (bootstrapping = 100) using MEGA X (Molecular Evolutionary Genetics Analysis) [75]. The JTT+G+I model was used. All positions containing gaps and missing data were eliminated, and alignment of the total length of the protein-coding sequence (1365 for 1st exon of UGT1As and 1689 bp for UGT2s) was used for phylogenetic analysis. The results of phylogenetic analyses for human, mouse, rat, and dog UGT1A, UGT2A and UGT2B genes were referenced to published phylogenic analyses [192,193] for verification. Lists of the food habitats of each Carnivora were referenced from publications and listed in **Table 2-1.**

2-2. Synteny analysis of UGT genes

Sequence data from genome projects are freely available. NCBI's genome data viewer (https://www.ncbi.nlm.nih.gov/genome/gdv/) or JBrowse [76] were used to visualize the chromosomal synteny maps for each species. The latest genome assemblies were used and listed in **Table 2-2.** UCSC (University of California, Santa Cruz) BLAT (a BLAST-like alignment tool) (http://genome.ucsc.edu/index.html) was used for additional confirmation of missing genes. The Masked palm civet CYP genes were also retrieved and used to fill the gap for Feliformia species from recently assembled and annotated chromosome-level genomic data [77]

3. Results

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

3-1-1. *UGT1A coding loci and isoform number in mammals*

UGT1A and UGT2A/B coding loci in rodents, humans, and carnivorans were analyzed and compared. UGT1A coding loci were highly conserved among Mammalia, in accordance with previous reports [39,194], and almost all isozymes were coded between MROH2A (maestro heat like repeat family member 2A) and USP40 (ubiquitin specific peptidase 40). Generally, UGT1As are coded by a common four exons (exons 2–5) and a unique alternative exon (exon 1), for which each gene product is spliced and named (UGT1A1-12). In this analysis, these features were also detected in all Carnivora analyzed (Figure 1). DNAJB3 (DnaJ Heat Shock Protein Family (Hsp40) Member B3) was located between exon 1 of UGT1A1 and UGT1A2, as in previous findings [39,72,192,193]. I also compared our isoform numbers with those annotated in published genomic data and found strong variation among Carnivora. In Canidae, more than 9 isoforms were detected (12 for dog, 10 for red fox and 9 for Arctic fox), followed by Ursidae (4-9 isoforms), Mustelidae (2-4 isoforms), pinnipeds (Odobenidae, Otariidae and Phocidae) (1-3 isoforms) and Felidae (2) (**Figure 3-3**). I also compared length of coding locus as a means of tracking genetic duplication/loss events in these conserved regions (MROH2A to USP40) (**Figure 3-3**). However there were substantial differences even within Ursidae. Polar bear and giant panda tended to have a limited number of isoforms and length of conserved region (polar bear: 4 isoforms, 98 kb; giant panda: 4 isoforms, 85.4 kb), while black and brown bear had relatively longer regions and number of isoforms (brown bear: 7 isoforms, 128.6 kb; black bear: 9 isoforms, 156.1 kb).

3-1-2. *UGT2A/B coding loci and isoforms number in mammals*

Similar to UGT1As, UGT2 coding loci were also conserved between SULT1B1 (sulfotransferase family 1B member 1) and YTHDC1 (YTH domain-containing protein 1) in all carnivora analyzed (Figure 2). This agrees with previous reports [71,192,193]. I again counted and compared the isoform number for UGT2A/2Bs and the respective lengths of coding loci (YTHDC1 to SULT1B1) (**Figure 3-2)**. Similar to UGT1As, Canidae had a higher number of UGT2B isoforms (3-4 isoforms) while pinnipeds and Felidae had very limited numbers (0-1 isoforms) (**Figure 3-3**). Mustelidae had comparatively moderate numbers of UGT2Bs (1-4 isoforms) but with interspecies variation within the family. Blast analysis using UGT2B31 in dog as a query further revealed possible other isoforms in UGT2Bs

in brown and black bear. I found 9 further possible isoforms (8 complete and 1 partial) on un-scaffolded contigs (NW_025929643 and NW_025929709) in brown bear and 6 other partial isoforms in black bear. Including these un-scaffolded isoforms, brown bear had 11 UGT2B isoforms, which was highest number in the analyzed carnivora. Since some annotations contained incomplete UGT2B sequences, I attempted to avoid mis-estimation of gene counts by excluding shorter UGTs (< 1500bp) in **Figure 3-3** (1590 bp for UGT2B7 in human) and distinguishing them as black boxes in **Figure 3-2**.

3-2-1. *Phylogenetic analysis and sequence comparison of UGT1As in Carnivora*

UGT1A phylogenetic analysis in this study revealed four possible clades for this subfamily in mammals: UGT1A1, UGT1A2-5, UGT1A6, and UGT1A7-12 (**Figure 3-2**). Almost all Carnivora had only a single orthologues gene of UGT1A1 and UGT1A6, suggesting strong conservation of these genes. However, I found that Canidae and brown/black bear showed specific expansions of gene isoform numbers: in canids, clade UGT1A2-5, and in both ursids and canids, clade UGT1A7-12 (**Figure 3-4**). Other Ursidae analyzed in this study (giant panda and polar bear) had no such specific duplication in these clades, and no other specific duplications in Carnivora UGT1As were observed in this study. In contrast, other carnivora families were found to have undergone genetic loss or contraction in the UGT1A7-12 clade. No species in Felidae and Phocidae had any annotated genes in this clade, suggesting complete loss of UGT1A7-12, and some species in Otariidae and Mustelidae (northern fur seal, california sea lion, ermine, river otter and mink) had only one annotated isoform.

3-2-2. *Phylogenetic analysis of UGT2Bs in Carnivora*

The phylogeny of the UGT2 family was also analyzed (**Figure 3-5**). I found three clades of UGT2s: UGT2As, UGT2Bs and UGT2Es. Almost all UGT2E1s in Carnivora were registered as UGT2C1-like or UGT2A3-like in the NCBI database, in the cat, this isoform was recently renamed to UGT2E1 by the UGT nomenclature committee. I therefore renamed these isoforms to UGT2E1s based on their phylogeny, following the committee's suggestions [192,193]. Almost all Carnivora except for Felids had paralogues of UGT2B31s within the same clade, which agrees with previously reported results [71]. I also observed some specific duplications and losses. Canidae, some Ursidae and some Mustelidae had possible multiples of functional UGT2Bs, and these isoform duplications were

clustered into species-specific clades, implying that the duplications were each species-specific. I also demonstrated the existence of annotated UGT2Es isoforms in all Felidae and Ursidae, and found isoforms annotated as “low-quality” UGT2C1-like in dog and red fox but in no other species. No other specific duplication or loss events were observed in the UGT2 family.

3-3. *Sequence comparison of UGT1As and 2Bs in Carnivora*

Similar to the findings of previous studies, I also observed that all analyzed Felidae and Otariidae possessed UGT1A6 pseudogenes. I also found additional UGT1A6Ps in some species. Annotated UGT1A6 in sea otter contained s nonsense mutation and was registered as a pseudogene in the NCBI database.

I further compared the sequence of candidate UGT1A7-12 isoforms in northern fur seal, California sea lion, ermine, river otter and mink, as these species only have a single isoform in this clade. I determined a specific common stop codon (TAG: 526-528 bp) in UGT1A7-12 in the two pinnipeds (**Figure 3-6**), indicating a dysfunction of these genes in the analyzed Otariidae. No possible nonsense mutations in UGT1A7-12 were observed in the three mustelids.

I also compared the sequences of UGT2Bs, and found no dysfunctional mutation in any analyzed species except for UGT2B31 in all felids.

4. Discussion

Relations between diet and UGT2Bs expansion

The generally accepted “animal-plant warfare” hypothesis considers the evolution of the xenobiotic metabolism as one of major defense mechanisms in animals against daily exposure to xenobiotics; in this regard, plant secondary metabolites are likely among the major sources of evolutionary pressure [66,196–198]. Several studies have shown that herbivorous mammals and birds have experienced a huge expansion of UGT families [45,68,199,200]. In placental mammals, Kawai et al. (2021) [30] recently demonstrated a relationship of herbivorous diet with a large number of UGT2B genes, but less with UGT1A genes. This strongly suggests that UGT2Bs might be important for the daily metabolism of plant secondary metabolites. Moreover, a recent genomic analysis in woodrats (highly herbivorous) indicated significant duplication of UGT2Bs, in contrast to closely-related omnivorous rats and deer mice [68]. Similarly, sika deer genomic analysis [199] suggested that genes in the UGT2B subfamily have a strong correlation with the adaptation of the species to a high-tannin diet. These reports underline the evolutionary importance of UGT2Bs in herbivorous adaptation. The present study demonstrated the expansion of UGT2Bs in Canidae (red fox and dog), brown bear and some Mustelidae (badger, mink and ermine) (**Figure 3-3**). The canid and brown bear UGT2B expansion might be explained by the omnivorous diet of these species [149,202]. The brown bear showed a unique UGT2B expansion and the largest observed number of UGT2B isoforms; this was not the case for the closely-related polar bear. Although data for such an inference are limited, black bear also showed a similar possibility for multiple functional UGT2Bs. These two species have a very generalist diet including much plant matter such as green vegetation, fruits, cereals and hard masts (e.g., nuts and acorns) [83,171,203–205]. It is likely that the observed UGT2B duplication is the result of adaptation to plant-based food items in the diet of these species.

In this analysis, results for the giant panda, a strictly herbivorous species, contradicted the “plant-animal warfare” assumption. UGT1As in this species indicated slightly contracted evolution compared to other omnivorous canids and ursids. The UGT2B gene family was lost completely. The cause for this might be the species’ exclusive bamboo diet, which may have led to the evolution of settled isoforms to deal with bamboo phytochemicals such as flavonoids [206]. Some reports have supported this interpretation, showing that specialists tend to have

a wider variety of phytochemical metabolites than generalists [80,207,208]. Seasonal fluctuations in gut microbiota have been suggested as an alternative strategy to provide a pathway for the metabolism of bamboo-related chemicals [87,89,209].

Interestingly, among canids there were some differences in UGT2B evolution even in closely related species such as red fox and arctic fox. I detected 4 annotated isoforms annotated in arctic fox, with 3 having a limited length of the protein coding loci (XM_041724971.1: 1316 bp, XM_041724972.1: 842bp, XM_041726888.1: 183 bp). This information was automatically annotated based on NCBI annotation pipeline using RNA-seq data from several tissues. Further investigation is required to determine whether this species has a limited number of UGT2Bs, but the reduced length of 2B coding loci in this species in comparison to dog and red fox suggests so. The dietary difference between arctic fox (an obligate carnivore) and red fox (a mesocarnivore) also might explain UGT2B contraction in the former [149,177,210,211], although a detailed examination is still limited.

Badgers also have an omnivorous diet, while American mink and ermine (both in the subfamily Mustelinae) are both highly carnivorous [85]. The UGT2B expansion observed in the latter two species does not agree with the patterns discussed above. This might suggest the presence of a possible opportunistic omnivorous diet in a common ancestor of Mustelinae, and UGT2B duplication and the current number of pseudogenes in these species could be the evolutionary footprint of that ancestor's diet. Further study of other mustelids is required to clarify UGT2B evolution and the functional importance of UGT2B isoforms in this highly carnivorous family.

UGT1A evolution and adaptation to species-specific diets

In addition to UGT2Bs, UGT1As also showed significant expansion in Canidae and some Ursidae (brown bear and black bear) in this study. This also appears to be related to diet, as in UGT2Bs. A variable preference for plant food sources in Carnivora might partially explain the different UGT evolutional patterns and may have been a cause of the UGT1A expansion in adapting to species-specific plant diets. I also showed that the duplication of UGT1A2-5 in Canidae and UGT1A7-12 in Canidae and two bear species are family- or species-specific

features, indicative of evolutionary events at these taxon levels. A previous study on avian UGTs suggested a correlation of herbivore diet and UGT1A numbers [200]. While the genetic expansion of avian UGT1As was observed especially in isoform clades relevant to mammalian UGT1A2-5, I observed expansion of both UGT1A2-5 and UGT1A7-12. Further investigation into the substrate specificity of each isoform and the relationship to species-specific dietary plants is needed to support this hypothesis.

Recent genomic research in Hyaenidae also suggested UGT expansion in the aardwolf (*Proteles cristata*), an insectivorous species [212]. The authors discussed the possible role of this UGT expansion as a defense mechanism against termite toxins. However, they only detected expansion in orthologues gene of UGT2A1 (LOC480777), not in UGT2Bs. UGT2A1/2 has been known to specifically express in nasal epithelium and is regarded as playing significant roles in odor signal termination [213,214]; these enzymes are highly conserved among mammals. The observed UGT2A1 expansion in aardwolf and its connection with the detoxification of termite toxins is thus still unclear. Because genomic data availability is slightly limited, I could not extend our analysis to aardwolf and brown hyaena data.

The Canidae/Ursidae-specific UGT1A expansion found in this study and the possible insectivory-derived expansion of UGTs in aardwolf indicate the importance of further research with a more comprehensive coverage of species and a more detailed partitioning of dietary habits (frugivore, folivore, nectarivore, insectivore, and others).

UGT duplication/loss and relation to functional glucuronidation

The observed UGT1A and 2B duplication in Canidae found in this study strongly suggests a substantial capacity for and wide range of chemical acceptance for glucuronide conjugation in this clade. In a previous report I revealed a strong glucuronidation capacity of *in vitro* dog liver microsome towards both UGT1A substrates[72] and UGT2B substrates [71]. Soars et al. (2001) [129] also reported a much stronger glucuronidation capacity for a wide range of chemicals of *in vitro* dog liver microsome compared to humans. This reasonably coincides with our results of genetic duplication in dogs, and our findings further indicate that these high capacities for glucuronidation may be present not only in dogs but also in other Canidae species like foxes. Still, our phylogenetic analysis suggested the duplication events in Canidae seemed to be species-independent, and further in

vitro or in vivo analysis for foxes glucuronidation capacity is essential. I also observed strong contraction of UGT1As, especially the UGT1A6 pseudogene, UGT1A7-12 loss, and complete loss of UGT2Bs in all Felidae. These findings are in accordance with *in vitro* studies of limited capacity of UGTs for a wide range of chemicals in cats [41,215,216]. Similar features of *in vitro* limited glucuronidation were also observed in pinnipeds [71,72]. The present study adds further information on possible UGT1A7-12 loss in the entire Otariidae and Phocidae clades, although some Phocidae have intact UGT1A6 genes. Kakehi et al. [17] already revealed the limited number of UGT1A6-12 isoforms in Pacific walrus and further discussed the possible loss of UGT1A7-12 genes in pinnipeds as a cause of limited glucuronidation capacity *in vitro*. The present study's results strongly support this hypothesis. UGT1A1-5s are generally considered as bilirubin-like-associated isoforms, whereas UGT1A6-12 isoforms are thought to be phenol-like-associated [43,217]. The present study thus suggests that almost all species of pinnipeds may have a more limited capacity of glucuronidation for a wide range of exogenous phenols, than previously implied [72]. *In vitro* or *in vivo* activity of UGTs in Ursidae, Mustelidae, or any other Carnivora have not yet been studied, and further research is needed to understand the relationship between the genetics and function of UGTs in individual species. Genomic information for a wider range of Carnivora taxa (e.g., Ailuridae, Procyonidae, Mephitidae, Vivveridae, Nandiniidae, Prionodontidae, and Eupleridae) is also required to fill the gaps in the evolutionary history of UGT duplication/loss.

Short conclusion

This study for the first time revealed the evolutionary characteristics of UGT in several Carnivora species, providing a more comprehensive understanding of UGT duplication and loss event in this clade. Our results indicate that omnivorous species like canids and some bears might have been subjected to significant selective pressure on both the UGT1A and 2B subfamily. Furthermore, I found significant contraction of UGT genes in pinnipeds and Felidae, providing additional indications that limited genetic variation of UGTs in this group is much more comprehensive than previously assumed. Although genomic information for some species still requires improved annotation or assembly, our findings provide fundamental information for more accurate extrapolation of pharmacokinetic or toxicokinetic result from experimental animals to wild carnivorans which are daily exposed to numerous anthropogenic chemicals.

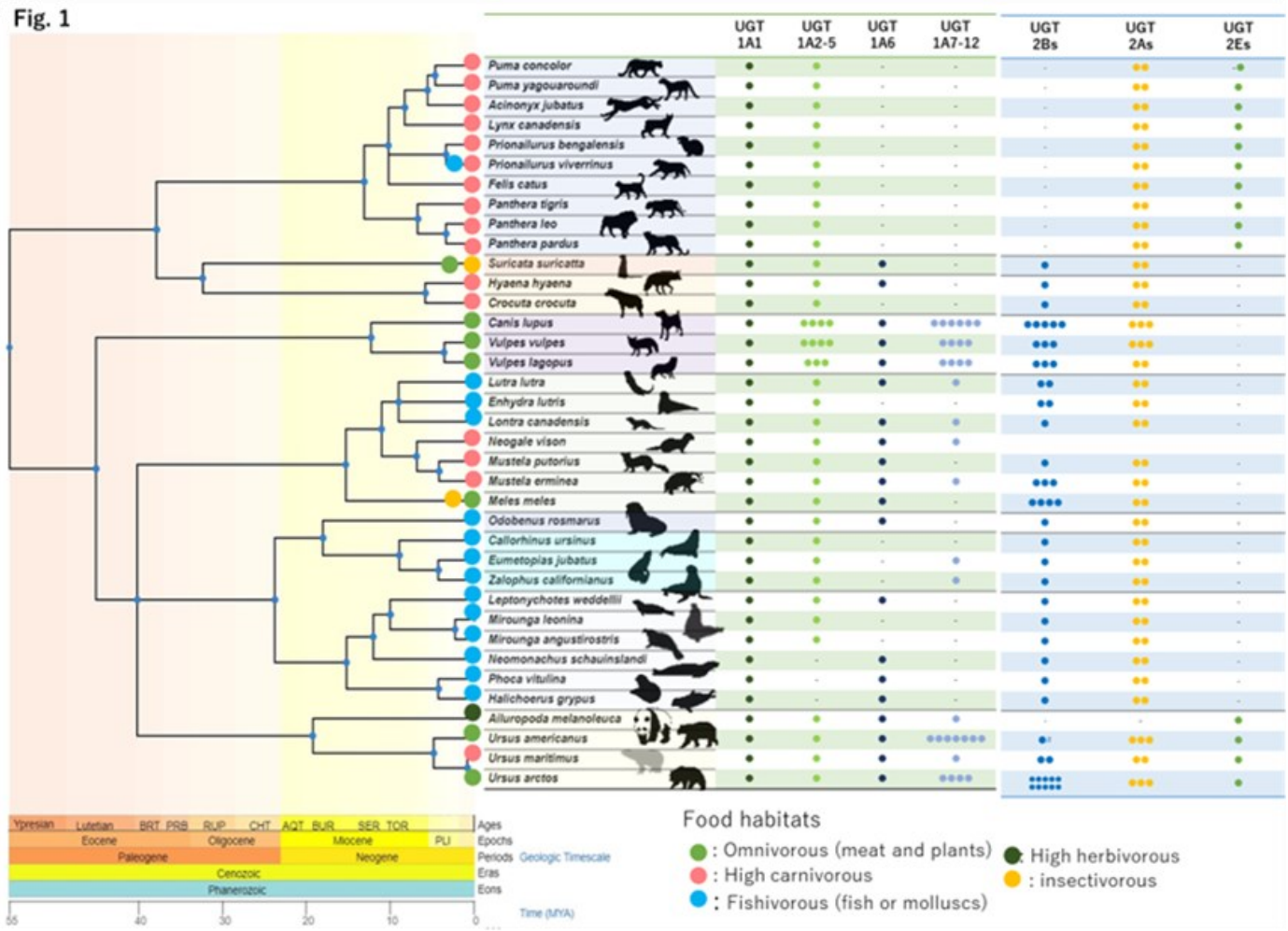

Figure 3-1. Gene numbers for UGT1A, 2A, 2B and 2C/E are shown by number of small filled circles. Large filled circles next to the scientific name of each species are colored by known diet. In brown bear (Ursus arctos) I detected 3 coding loci and all isoforms except “partial” or “low quality” were counted in this case. In black bear I omitted 6 other partially-coded genes. The phylogenetic tree was created with TimeTree 5 [58].

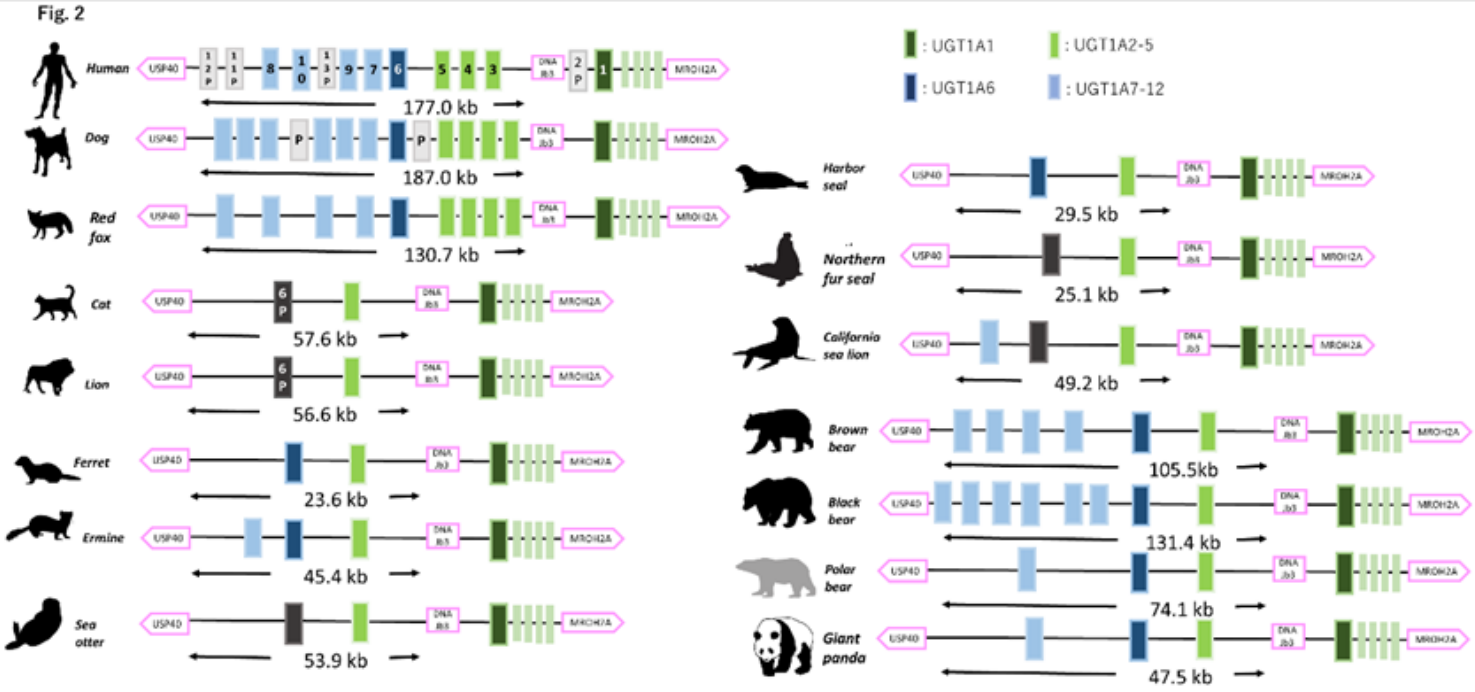

Figure 3-2. Synteny analysis of UGT1As in Carnivora.

Synteny of UGT1A coding loci among Carnivora is shown. Representative species for each family were selected. UGT1As are known to share common exons (2-5) among isoforms and are shown as pale green blocks. UGT1A1 is dark green, UGT1A2-5 is bright green, UGT1A6 is dark blue, and UGT1A7-12 is pale blue. Pseudogenes are shown as black or gray blocks. Lengths of coding loci from DNAJB3 to MROH2A are also shown as indicator for genetic loss in these loci.

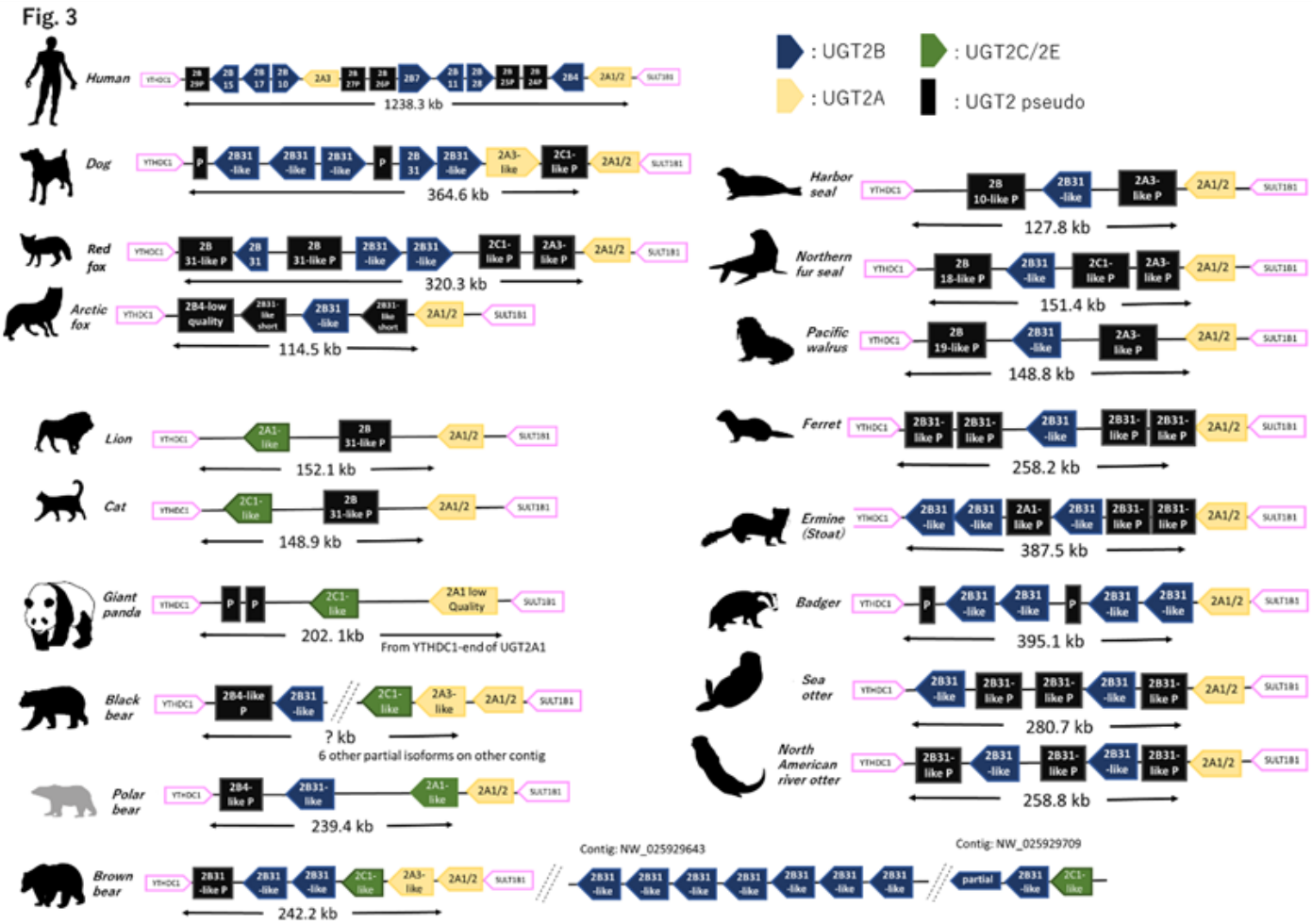

Figure 3-3. Synteny analysis of UGT2s in Carnivora.

Synteny of UGT2 coding loci among Carnivora is shown. Representative species for each family were selected. UGT2A1/2 are known to share common exons (2-5) among isoforms and are shown as yellow blocks with the same color in other UGT2As. UGT2Bs are navy, UGT2E/2Cs are green, and pseudogenes are black. Lengths of coding loci from UGT2As to YTHDC1 are also shown as indicator for genetic loss in these loci. Colors of each gene are based on phylogenetic analysis clades, and the names of each gene were based on the NCBA annotations.

Fig.4

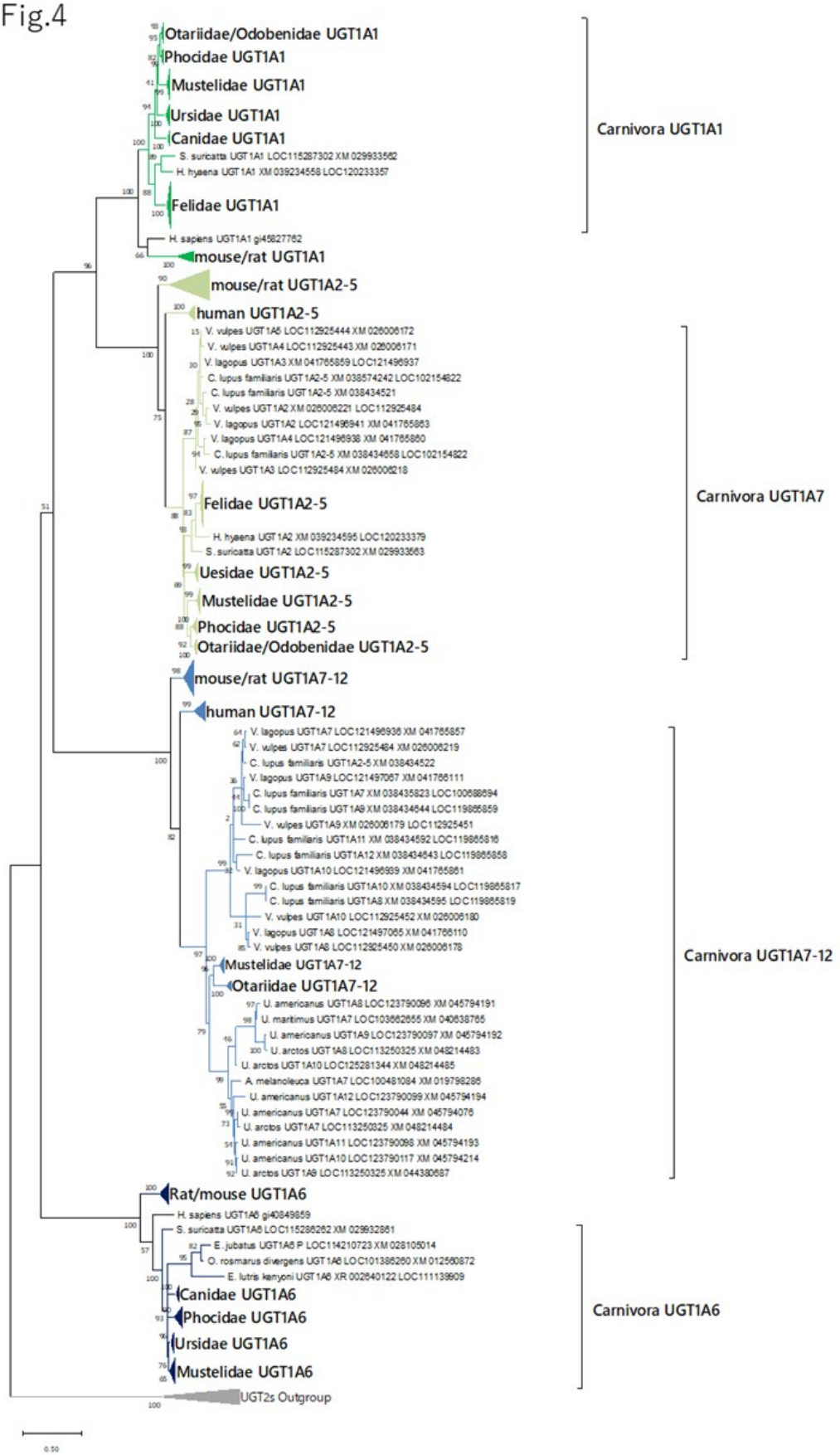
Otariidae/Odobenidae UGT1A1
Phocidae UGT1A1
Mustelidae UGT1A1
Ursidae UGT1A1
Canidae UGT1A1
S. suricatta UGT1A1 LOC115287302 XM 029933562
H. hyaena UGT1A1 XM 039234558 LOC120233357
Felidae UGT1A1
Carnivora UGT1A1
H. sapiens UGT1A1 gi45827762
mouse/rat UGT1A1
mouse/rat UGT1A2-5
human UGT1A2-5
V. vulpes UGT1A5 LOC112925444 XM 026006172
V. vulpes UGT1A4 LOC112925443 XM 026006171
V. lagopus UGT1A3 XM 041765859 LOC121496937
C. lupus familiaris UGT1A2-5 XM 038574242 LOC102154822
C. lupus familiaris UGT1A2-5 XM 038434521
V. vulpes UGT1A2 XM 026006221 LOC112925484
V. lagopus UGT1A2 LOC121496941 XM 041765863
V. lagopus UGT1A4 LOC121496938 XM 041765860
C. lupus familiaris UGT1A2-5 XM 038434658 LOC102154822
V. vulpes UGT1A3 LOC112925484 XM 026006218
Felidae UGT1A2-5
Carnivora UGT1A7
H. hyaena UGT1A2 XM 039234595 LOC120233379
S. suricatta UGT1A2 LOC115287302 XM 029933563
Uesidae UGT1A2-5
Mustelidae UGT1A2-5
Phocidae UGT1A2-5
Otariidae/Odobenidae UGT1A2-5
mouse/rat UGT1A7-12
human UGT1A7-12
V. lagopus UGT1A7 LOC121496936 XM 041765857
V. vulpes UGT1A7 LOC112925484 XM 026006219
C. lupus familiaris UGT1A2-5 XM 038434522
V. lagopus UGT1A9 LOC121497067 XM 041766111
C. lupus familiaris UGT1A7 XM 038435823 LOC100688694
C. lupus familiaris UGT1A9 XM 038434644 LOC119865859
V. vulpes UGT1A9 XM 026006179 LOC112925451
C. lupus familiaris UGT1A11 XM 038434592 LOC119865816
C. lupus familiaris UGT1A12 XM 038434643 LOC119865858
V. lagopus UGT1A10 LOC121496989 XM 041765861
C. lupus familiaris UGT1A10 XM 038434594 LOC119865817
C. lupus familiaris UGT1A8 XM 038434595 LOC119865819
V. vulpes UGT1A10 LOC112925452 XM 026006180
V. lagopus UGT1A8 LOC121497065 XM 041766110
V. vulpes UGT1A8 LOC112925450 XM 026006178
Mustelidae UGT1A7-12
Otariidae UGT1A7-12
Carnivora UGT1A7-12
U. americanus UGT1A8 LOC123790096 XM 045794191
U. maritimus UGT1A7 LOC103662655 XM 040638765
U. americanus UGT1A9 LOC123790097 XM 045794192
U. arctos UGT1A8 LOC113250325 XM 048214483
U. arctos UGT1A10 LOC125281344 XM 048214485
A. melanoleuca UGT1A7 LOC100481084 XM 019796286
U. americanus UGT1A12 LOC123790099 XM 045794194
U. americanus UGT1A7 LOC123790044 XM 045794076
U. arctos UGT1A7 LOC113250325 XM 048214484
U. americanus UGT1A11 LOC123790098 XM 045794193
U. americanus UGT1A10 LOC123790117 XM 045794214
U. arctos UGT1A8 LOC113250325 XM 044380687
Rat/mouse UGT1A6
H. sapiens UGT1A6 gi40849859
S. suricatta UGT1A6 LOC115286262 XM 029932861
E. jubatus UGT1A6 P LOC114210723 XM 028105014
O. rosmarus divergens UGT1A6 LOC101386260 XM 012560872
E. lutris kenyoni UGT1A6 XR 002640122 LOC111139909
Canidae UGT1A6
Phocidae UGT1A6
Ursidae UGT1A6
Mustelidae UGT1A6
Carnivora UGT1A6
UGT2s Outgroup
0.50

Figure 3-4. **Phylogenetic tree of UGT1As.**

Phylogenetic tree of UGT1A sequences in human, mouse, rat, and carnivorans. Gene sequences of protein-coding regions for each isozyme were analyzed. The numbers next to the branches indicate the number of occurrences per 100 bootstrap replicates. Genes and clades are tentatively labeled with carnivoran UGTs examined in this article. Clades of rodents, and human UGT1A1, UGT1A2-5, UGT1A6 and UGT1A7-12 in the phylogenetic tree are shown as triangles of the following colors: dark green for UGT1A1s, bright green for UGT1A2-5s, deep blue for UGT1A6s, and pale blue for UGT1A7-12s. UGT2As are shown as an outgroup.

Fig.5

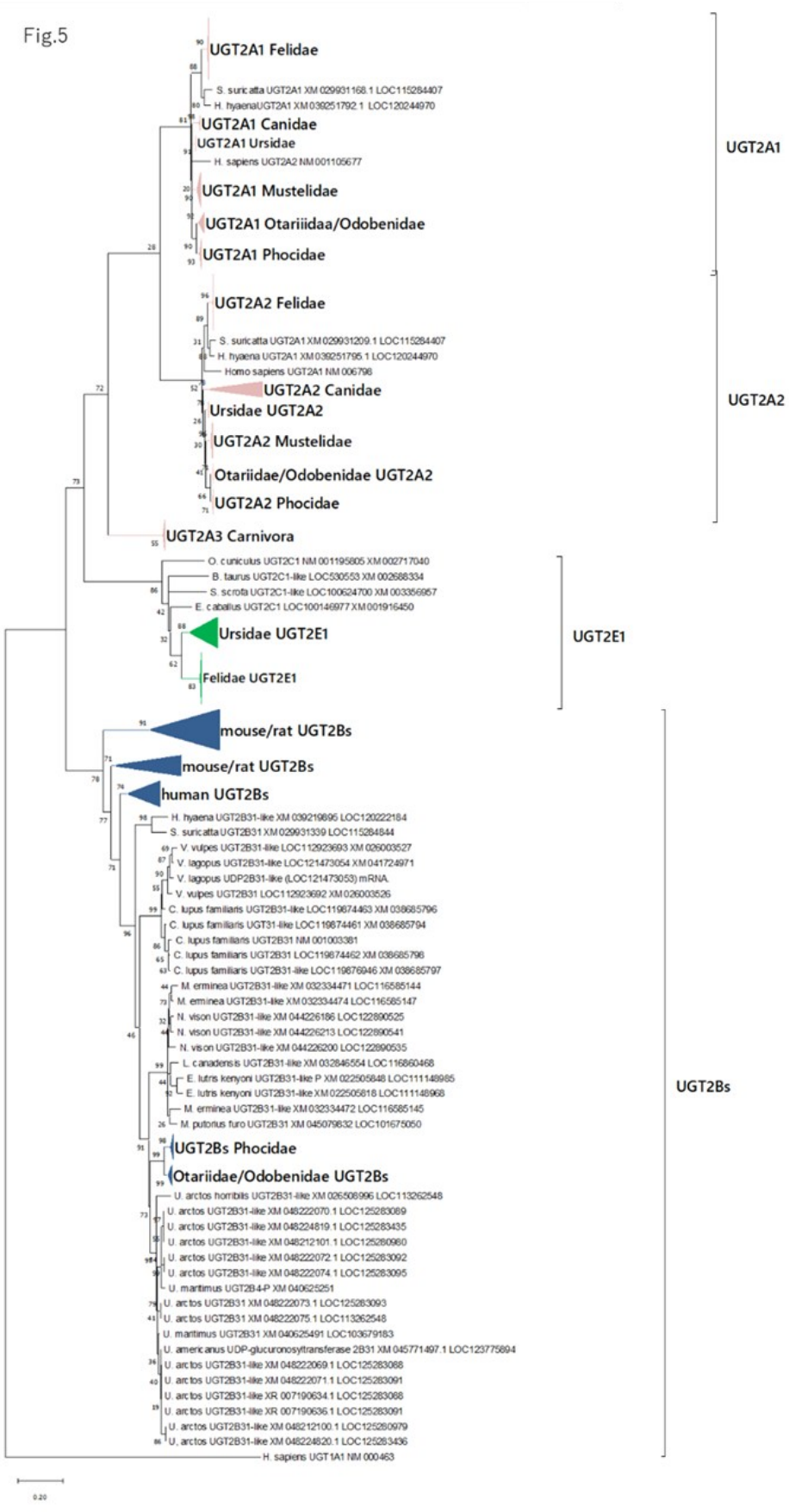
UGT2A1 Felidae
S. suricatta UGT2A1 XM 029931168.1 LOC115284407
H. hyaena UGT2A1 XM 039251792.1 LOC120244970
UGT2A1 Canidae
UGT2A1 Ursidae
H. sapiens UGT2A2 NM 001105677
UGT2A1 Mustelidae
UGT2A1 Otariiidaa/Odobenidae
UGT2A1 Phocidae
UGT2A1
UGT2A2 Felidae
S. suricatta UGT2A1 XM 029931209.1 LOC115284407
H. hyaena UGT2A1 XM 039251795.1 LOC120244970
Homo sapiens UGT2A1 NM 006798
UGT2A2 Canidae
Ursidae UGT2A2
UGT2A2 Mustelidae
Otariidae/Odobenidae UGT2A2
UGT2A2 Phocidae
UGT2A2
UGT2A3 Carnivora
O. cuniculus UGT2C1 NM 001195605 XM 002717040
B. taurus UGT2C1-like LOC530553 XM 002688334
S. scrota UGT2C1-like LOC100624700 XM 003356957
E. caballus UGT2C1 LOC100146977 XM 001916450
Ursidae UGT2E1
Felidae UGT2E1
UGT2E1
mouse/rat UGT2Bs
mouse/rat UGT2Bs
human UGT2Bs
H. hyaena UGT2B31-like XM 039219895 LOC120222184
S. suricatta UGT2B31 XM 029931339 LOC115284844
V. vulpes UGT2B31-like LOC112923693 XM 026003527
V. lagopus UGT2B31-like LOC121473054 XM 041724971
V. lagopus UDP2B31-like (LOC121473053) mRNA.
V. vulpes UGT2B31 LOC112923692 XM 026003526
C. lupus familiaris UGT2B31-like LOC119874463 XM 038685796
C. lupus familiaris UGT31-like LOC119874461 XM 038685794
C. lupus familiaris UGT2B31 NM 001003381
C. lupus familiaris UGT2B31 LOC119874462 XM 038685796
C. lupus familiaris UGT2B31-like LOC119876946 XM 038685797
M. erminea UGT2B31-like XM 032334471 LOC116585144
M. erminea UGT2B31-like XM 032334474 LOC116585147
N. vison UGT2B31-like XM 044226186 LOC122890525
N. vison UGT2B31-like XM 044226213 LOC122890541
N. vison UGT2B31-like XM 044226200 LOC122890535
L. canadensis UGT2B31-like XM 032846554 LOC116860468
E. lutris kenyoni UGT2B31-like P XM 022505848 LOC111148985
E. lutris kenyoni UGT2B31-like XM 022505818 LOC111148968
M. erminea UGT2B31-like XM 032334472 LOC116585145
M. putorius furo UGT2B31 XM 045079632 LOC101675050
UGT2Bs Phocidae
Otariidae/Odobenidae UGT2Bs
U. arctos horribilis UGT2B31-like XM 026506996 LOC113262548
U. arctos UGT2B31-like XM 048222070.1 LOC125283089
U. arctos UGT2B31-like XM 048224819.1 LOC125283435
U. arctos UGT2B31-like XM 048212101.1 LOC125280960
U. arctos UGT2B31-like XM 048222072.1 LOC125283092
U. arctos UGT2B31-like XM 048222074.1 LOC125283095
U. maritimus UGT2B4-P XM 040625251
U. arctos UGT2B31 XM 048222073.1 LOC125283093
U. arctos UGT2B31 XM 048222075.1 LOC113262548
U. maritimus UGT2B31 XM 040625491 LOC103679183
U. americanus UDP-glucuronosyltransferase 2B31 XM 045771497.1 LOC123775694
U. arctos UGT2B31-like XM 048222069.1 LOC125283088
U. arctos UGT2B31-like XM 048222071.1 LOC125283091
U. arctos UGT2B31-like XR 007190634.1 LOC125283088
U. arctos UGT2B31-like XR 007190636.1 LOC125283091
U. arctos UGT2B31-like XM 048212100.1 LOC125280979
U. arctos UGT2B31-like XM 048224820.1 LOC125283436
H. sapiens UGT1A1 NM 000463
UGT2Bs
0.20

Figure 3-5. **Phylogenetic tree of UGT2s.**

Phylogenetic tree of UGT2s sequences in human, mouse, rat, and carnivorans. Gene sequences of protein-coding regions for each isozyme were analyzed. The numbers next to the branches indicate the number of occurrences per 100 bootstrap replicates. Genes and clades are tentatively labeled with carnivoran UGTs examined in this article. Clades of rodents, and human UGT2As, UGT2Bs UGT2Es in the phylogenetic tree are shown as triangles of the following colors: navy for UGT2Bs, green for UGT2Es, and pink for UGT2As. UGT1As are shown as an outgroup.

Sequence nucleotide number in pinniuped UGT1A7-12s	574	575	576	577	578	579	580	581	582	583	584	585	586	587	588	589	590	591	592	593	594	595	596	597	598	598	598	598	598	598
Protein translated from codons in Human UGT1A9		I			L			C			H			Y			L			E			E			G			A	
H. sapiens UGT1A9 NM 021027	A	T	A	C	T	T	T	G	C	C	A	C	T	A	T	C	T	T	G	A	A	G	A	A	G	G	T	G	C	A
N. vison UGT1A7-11 XM 044241893 LOC122902436	G	T	A	T	T	T	T	G	T	C	A	T	T	A	T	C	T	T	G	A	A	G	A	A	G	G	A	G	C	A
C. lupus familiaris UGT1A7-12 XM 038434521	A	T	T	C	C	A	T	G	T	G	A	T	T	T	A	G	A	A	T	C	T	G	A	G	A	G	C	A	C	G
M. musculus Ugt1a9 NM 201644	G	T	A	T	T	T	T	G	T	G	A	C	T	A	T	C	T	T	G	A	A	G	A	G	G	G	T	G	C	C
C. ursinus UGT1A7-12 LOC112836383 XM 025888034	G	T	A	T	T	T	T	G	C	C	A	T	T	A	G	C	T	T	G	A	A	G	A	A	G	G	C	A	C	A
Z. californianus UGT1A7-12 LOC113919635 XM 035726859	G	T	A	T	T	T	T	G	C	C	A	T	T	A	G	C	T	T	G	A	A	G	A	A	G	G	C	A	C	A
Protein translated from codons in Z. californianus UGT1A7-12		V			F			C			H			*			L			E			E			G			T	

Figure 3-6. Sequences of UGT1A7-12 in Pinnipeds and other species.

Partial sequences of UGT1A7-12; UGT1A9 of human, Ugt1a9 in mouse, and other UGT1A7-12s in dog, mink, Steller sea lion and California sea lion are shown. Nunsense mutation at 586-588 aa were observed in two Otariidae.

Chapter 4

Enzymatic and genetic features of Sulfotransferases

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) [223–225].

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 [222,226–229]. Although SULT1 isoforms and their function have been well characterized in humans, rodents, and a few other experimental animal models [227,230–233], information is still limited in other mammalian species including wild mammals.

In *Chapter 2*, I reported specific loss of UGTs in Felidae and Pinnipedia, 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 [222,234]. 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.

Materials and methods

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).

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).

Measurements of in vitro SULT activity using carnivore liver cytosols

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).

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.

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.

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.

In silico genetic analysis of SULTs in carnivores

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 otter, sea otter, polar bear, giant panda, brown bear, meerkat, striped hyena, cat, Amur tiger, cheetah, puma, Canada lynx, leopard, Weddell seal, harbor seal, gray seal, Hawaiian monk seal, northern fur seal, southern elephant seal, Stellar sea lion, California sea lion, and Pacific walrus origins. Sequences were retrieved using National Center for Biotechnology Information (NCBI) BLAST searches using human and dog SULT1A1, 1B1, 1C1, 1C2, 1C3, 1E1 and SULT1D1 as the query sequence. BLAST searches have been conducted for database Nucleotide collection (nr/nt) for each species using Blastn (Optimize for somewhat similar sequences). The gene sequences used are listed in Supplementary Table S2, and the protein coding region of each isozyme was analyzed. The deduced amino acid sequences were aligned using MUSCLE (Multiple Sequence Comparison by Log-Expectation) and were used for model selection (model showing minimal set of BIC and AICc were chosen) and construction of maximum likelihood trees (bootstrapping = 100) using MEGA X (Molecular Evolutionary Genetics Analysis) [75]. The JTT+G model was used. All positions containing gaps and missing data were eliminated, and total 924 bp length of protein-coding sequence alignment are used for phylogenetic analysis. The results of phylogenetic analyses for human, mouse, rat, and dog SULT1 genes were examined in reference to the phylogenic analysis of published papers (C. Tsoi et al. 2001; Blanchard et al. 2004) to ensure that the analysis was conducted successfully.

Synteny analysis of SULT1 genes

Sequence data from genome projects are freely available. NCBI's genome data viewer (https://www.ncbi.nlm.nih.gov/genome/gdv/) or JBrowse [76] were used to visualize the chromosomal synteny maps for each species. The latest genome assemblies were used and listed in **Table 2-2**. UCSC (University of California, Santa Cruz) BLAT (a BLAST-like alignment tool)

(http://genome.ucsc.edu/index.html) was used for additional confirmation of missing genes.

Results

In silico genetic analysis of the SULT1 family in carnivores

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.

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).

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. I detected UGT activity or CYP450 concentration using same liver samples of these pinniped animals, and I detected certain amount of their activity to make sure their liver samples were not degraded.

Discussion

SULT1As are highly conserved in mammals

In this study, I 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 [237]. 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 [238]. 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.

SULT1Bs are also 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 [230,239]. 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.

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 [227,232]. Previous studies using immunoblots have found that canine SULT1D1 was highly expressed in the intestines and kidneys but lowly expressed in the liver. 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 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 primates (data not shown), which is consistent with SULT1A3 expression in these species. However, in pinnipeds, both SULT1A3 and 1D1 were missing from the genome, indicating that sulfation of catecholamine in these animals may be limited. Catecholamine sulfates are mainly found in the blood and may be precursors of active molecules that are later deconjugated by sulfatases in peripheral tissues. SULTs may be essential to regulate catecholamine function in other mammals. From our findings, pinnipeds might have completely different pathways to regulate neurotransmitter function and estrogen metabolism (this will be discussed later).

Physiological significance of 1E1 defects in pinnipeds

Surprisingly, one of the most important and well-characterized isoforms, SULT1E1, was completely absent in pinnipeds including Phocidae, Otariidae, and Odobenidae. In vitro enzymatic analysis also suggested remarkably limited SULT1E1 activity in the liver of harbor seals, northern fur seals, and Stellar sea lions. This is the first report of innate SULT1E1 deficiency in placental mammals. SULT1E1s are critically important for the metabolism of sulfate estrogens (estradiol and estrone) and have a very high affinity for a vast range of xenobiotics including some environmental pollutants, such as hydroxylated-polychlorinated biphenyls (OH-PCBs) and hydroxylated- polybrominated diphenyl ether (OH-PBDEs). A previous report by Tong et al. (2005) suggested that SULT1E1 ablation in mice caused severe thrombosis in the placenta, resulting in fetal loss in the knock out (KO) mice because of the excessive estrogen levels in the placenta. Moreover, Gershon et al. (2007) showed that excessive estrogen resulted in the low expression of COX-2, reduced cumulus expansion, and impaired ovulation in SULT1E1 KO mice. In addition, single nucleotide polymorphisms (SNP) in human SULT1E1 may be a risk factor for breast or endometrial cancer development [242]. 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 [221]. However, in chicken and turtle eggs, biosynthesis of estrogen sulfate was observed, suggesting the existence of estrogen-sulfotransferases in these species [243,244], 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.

This in vitro analysis has limitation and didn't completely reflect the SULT1E1 activity because I didn't investigate substrate specificity for other isoforms in Carnivorans and studies using recombinant SULTTs in carnivora is highly important for further discussion. Also in this in vitro analysis, I utilized environmental samples and I didn't conduct chemicals analysis to detect environmental pollutants in these specimens. Thus, some contaminants such as persistent organic pollutants (POPs) might have effect on SULT expression or activity [246]. Although this in vitro analysis had such limitation, I considered the result in this study suggested important species-differences of SULT activity in Carnivora.

SULT1Cs in carnivorans and genetic deficiency

Along with other SULT isoforms, SULT1Cs were highly conserved, with some differences between rodents, human, and carnivorans. Phylogenetic analysis revealed that SULT1C1, 1C2, and 1C4 in carnivorans were in the same clade of the phylogenetic tree as rodent SULT1C1, 1C2s and human SULT1C4, respectively. Rat SULT1C3 is considered to be an ortholog of mouse SULT1C1 and not an equivalent of human SULT1C3, suggesting that a comprehensive nomenclature system remains unestablished. In rats, several isoforms in the SULT1C2 clade were observed while mice, humans, and carnivorans had only one isoform in this clade. Human and rodent SULT1Cs are known to conjugate xenobiotics, such as p-nitrophenol, 1-naphthol, 2-ethylphenol, 2-n-propylphenol, and 2-sec-butylphenol [247]; they also conjugate procarcinogen hydroxyaryl amines, such as N-hydroxy-2-acetylaminofluorene, resulting in the metabolic activation of their carcinogenicity [229,248–250]. 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 [248,251], whereas rat SULT1Cs were still detected in adults and played important roles in xenobiotic metabolism into adulthood [252,253]. In Carnivora, only canine SULT1C4 has been cloned and characterized as a phenol-preferring SULT [254]. 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) [125], 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.

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 [234,255,256]. Previous reports have shown very limited function for UGTs in felines and pinnipeds, suggesting the compensatory activity of SULTs in these species [71,72]. 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 [257,258].

Short conclusion

This is the first comprehensive report of the genetic characteristics of SULT isoforms in wild, non-laboratory mammals. In this study, I 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.

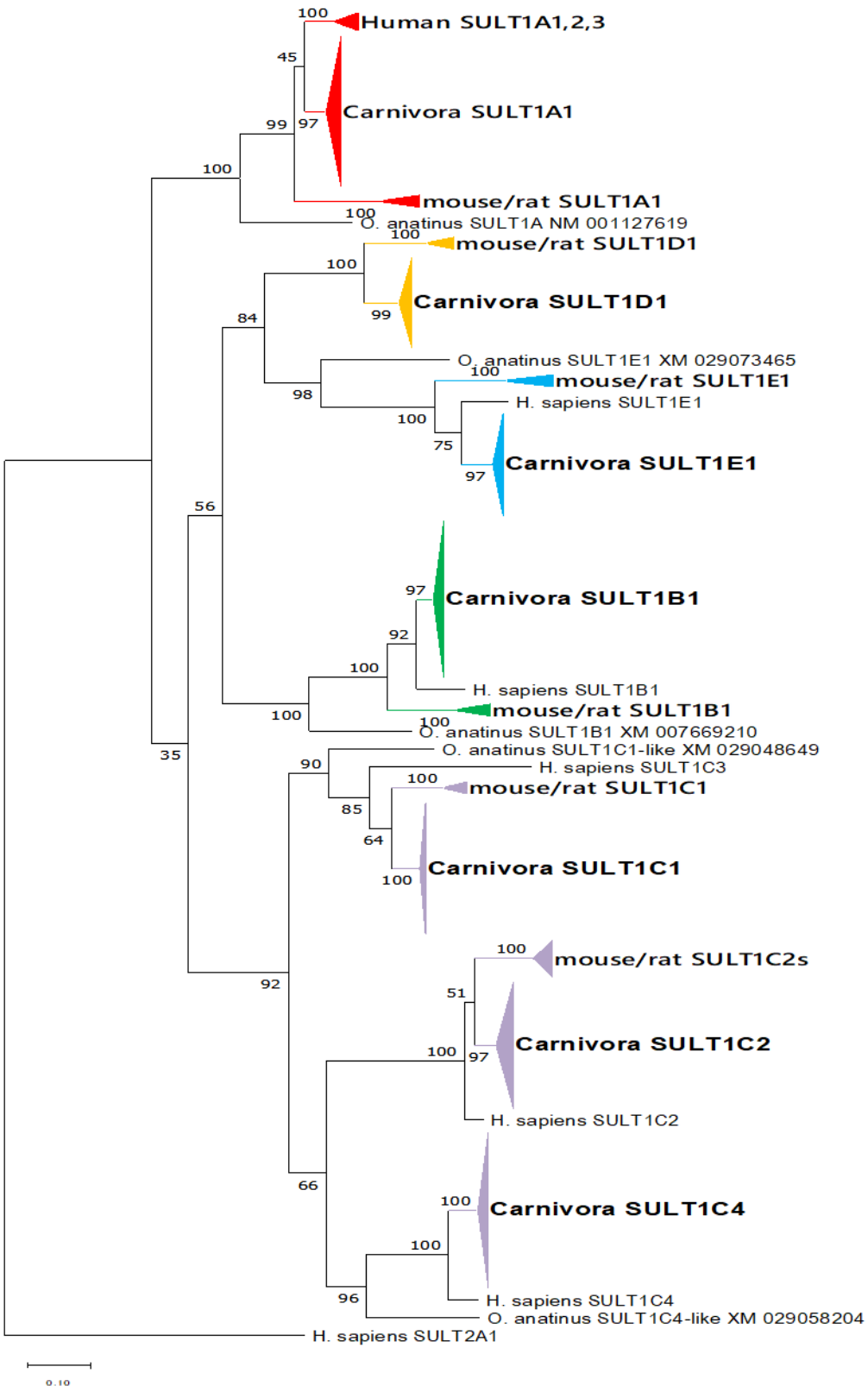

Figure 4-1. Phylogenetic tree of SULT1s in mammals including carnivorans.

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.

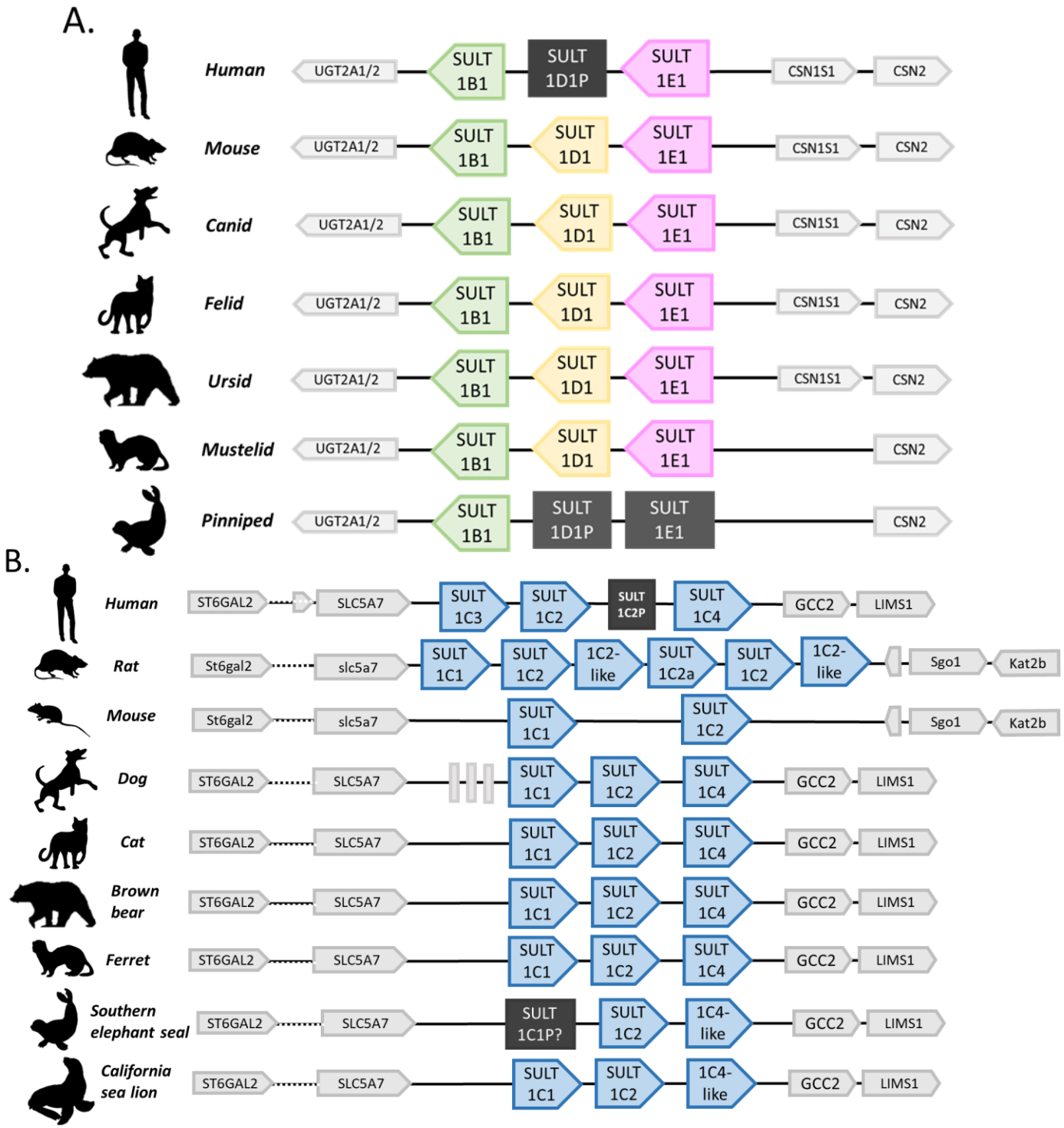

Figure 4-2. Genetic loci of SULTs in mammals.

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.

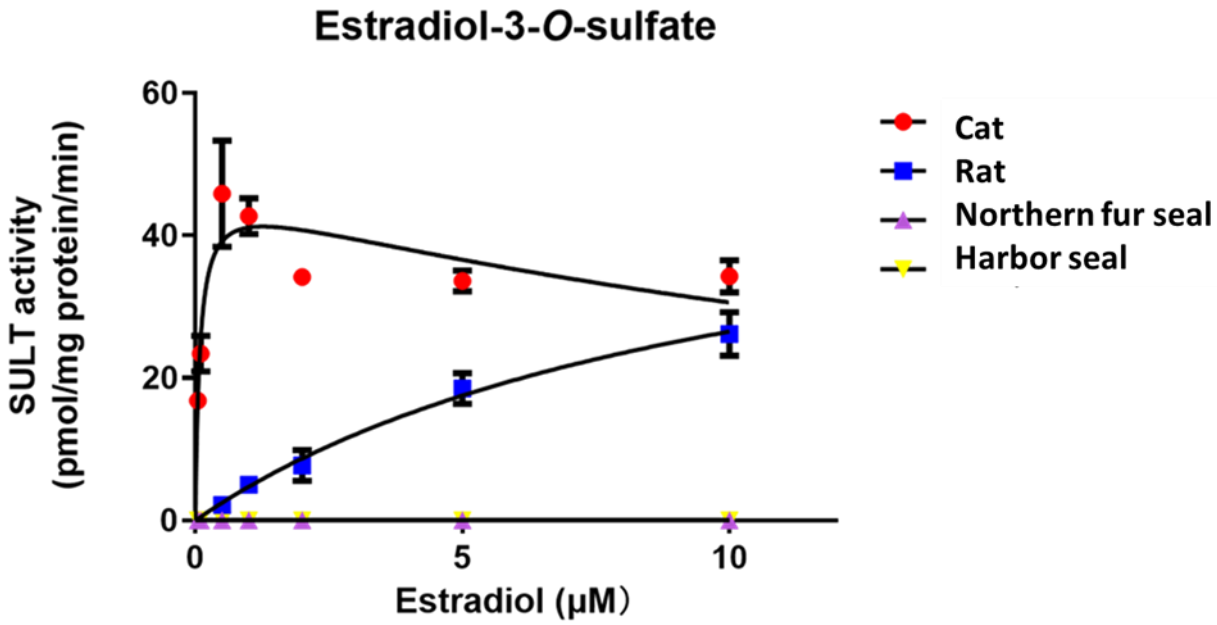

Figure 4-3. Michaelis-Menten plot for the in vitro SULT activity of estradiol.

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.

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

Figure 4-4a. continued.

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

Figure 4-4b. continued

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

Figure 4-4c. continued.

Figure 4-4a-c. 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.

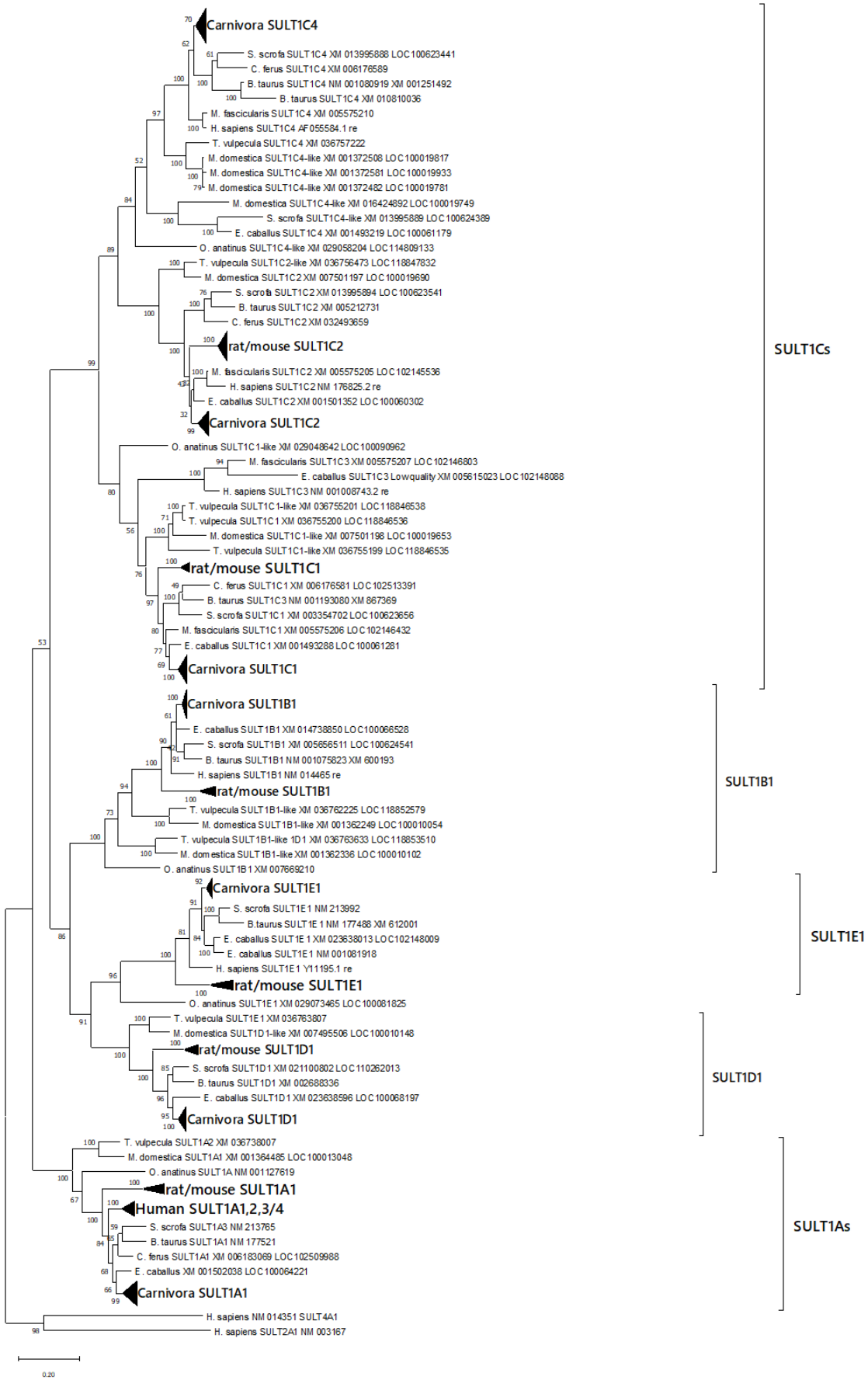
Carnivora SULT1C4
S. scrofa SULT1C4 XM 013995888 LOC100623441
C. ferus SULT1C4 XM 006176589
B. taurus SULT1C4 NM 001080919 XM 001251492
B. taurus SULT1C4 XM 010810036
M. fascicularis SULT1C4 XM 005575210
H. sapiens SULT1C4 AF055584.1 re
T. vulpecula SULT1C4 XM 036757222
M. domestica SULT1C4-like XM 001372508 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
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
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
H. sapiens SULT1E1 Y11195.1 re
rat/mouse SULT1E1
O. anatinus SULT1E1 XM 029073465 LOC100081825
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
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
H. sapiens NM 014351 SULT4A1
H. sapiens SULT2A1 NM 003167
0.20
SULT1Cs
SULT1B1
SULT1E1
SULT1D1
SULT1As

Figure 4-5. **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.

	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 4-1. 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.

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

Table 4-2. Details about liver samples used in this analysis.

Chapter 5

Conclusion and Future investigation

Through these analyses, I have comprehensively characterized the XMEs in Carnivora from evolutionary, genetic and enzymatic perspectives.

In *Chapter 1*, I investigated the CYP-specific duplication and loss event in Carnivorans, and I identified specific expansion of CYP 2Cs and 3As in omnivorous animals such as the brown bear, black bear, badger, and dog. Further, phylogenetic analysis of CYP2Cs revealed the possible orthologs of CYP2C21s, 2C41s, and 2C23s in Carnivora. In contrast, CYP3As diverged differently between Caniformia and Feliformia. These features indicated that, even among Carnivora, genetic features of CYP may be different and this highlighted the importance of appropriately extrapolating pharmacokinetic or toxicokinetic data from experimental animals to wild Carnivorans.

In *Chapter 2*, research was conducted to identify the evolutional history of UGTs in Carnivoran species. I found specific gene expansion of UGT1As in Canidae, the brown bear, and the black bear. Further, I found similar genetic duplication in UGT2Bs in Canidae, and some Mustelidae and Ursidae. Additionally, I discovered contraction or complete loss of UGT1A7-12 in phocids, felids, and some otariids and Mustelids. These results strongly suggest a completely different evolution of UGTs in Carnivora, similarly to CYPs, and further demonstrate the importance of analyzing the various XMEs in Carnivora.

In *Chapter 3*, I clarified the genetic properties of SULTs in a wide range of mammals, but focusing on carnivorans and using in silico genetic analyses. I

found genetic deficiencies in the SULT1E1 and SULT1D1 isoforms in all pinnipeds analyzed and nonsense mutations in SULT1Cs in several carnivorans, including pinnipeds. I further investigated the enzyme activity of SULT1E1 in vitro using liver cytosols from pinnipeds. Using a SULT1E1 probe substrate, I found restricted estradiol sulfonation in pinnipeds, whereas other mammals had relatively high sulfonation. These results suggest that SULT1E1 activity is severely reduced or completely absent in pinnipeds. SULT1E1 activity catalyzes the metabolism of estrogens, drugs, and environmental toxins, which further suggests that these carnivorans may be highly susceptible to a wide range of xenobiotics.

Through these analyses, I clarified the evolutionary, genetic, and enzymatic properties of CYPs, UGTs, and SULTs in various carnivorans. This information is crucial for appropriate extrapolation of pharmacokinetic or toxicokinetic data to wild carnivorans. Further detailed studies regarding isoform-specific in vivo analysis, expression patterns on each organ, and relationship with in vivo pharmacokinetics, are essential for evaluating the toxicological effect of xenobiotics on wild Carnivorans.

Future research

Although I had a comprehensive selection of Carnivora for genetic analysis, species gaps and blanks exist for some families, such as Procyonidae, Mephitidae, Ailuridae in Caniformia, Vivveridae, Herpestidae, Prionodontidae, Nandinnidae, Eupleridae, and Hyaenidae in Feliformia. These families include various endangered species [47,57]. Recent progress in improving NGS sheds light on analysis of XMEs of wild animals, and significantly more species genomic data will be available in the future. These genomic analyses enable us to investigate population level genomics in wild animals and to investigate individual variation [67,259–261]. Besides humans, cats and dogs have shown some genetic variation of the XMEs, and these investigations should also be considered.

I utilized in vitro analysis for SULT enzymatic features in this dissertation. In vitro analysis is effective for identifying XMEs enzymatic features, however sample collection from wild fauna is a serious limitation of the method. Freshly frozen liver tissue samples are required for these analyses, and such samples are rarely available for wild animals. Although genomic analyses could provide a substitute for the basic information acquired from XMEs, there are huge differences between genetic information and functional enzymatic features in the

body. Expression patterns and distribution of XMEs in various organs, recombinant analysis of each XMEs isoforms in wild animals, and in silico analysis or simulations such as 3D-docking simulations and quantitative structure-activity relationship (QSAR) are potential alternative approaches that could be used to address these shortcomings.

XMEs expression is regulated by various factors including xenobiotics, hormones, infection, inflammation, cholestasis, and gut microbiome [20,32,59,262,263]. These regulatory mechanisms are evolutionarily important for individuals to adapt to their own foraging habit or environment. Thus, these regulating features should also be investigated.

In conclusion, we require further investigation, mostly through in vivo related analyses, to provide a deeper understanding of XMEs.

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Japanese abstract

第 1 章：序論

農薬、残留性有機汚染物質、医薬品などを含む化学物質は時に環境汚染物質として環境中に放出され、野生動物の個体、および生態系に対して継続的な影響を与えている。化学物質代謝酵素はそれら環境化学物質の化学的性質を変化させ、「解毒」する酵素として知られており、第 I 相反応から第 III 相反応に大別される。第 I 相反応は酸化、還元、加水分解などの反応で、第 II 相反応は抱合反応、第 III 相反応は代謝後の細胞外排出反応である。これら反応を通し化学物質は多くの場合毒性が弱い形に変換され、体外へ排泄されやすくなる。これら代謝酵素群が関与する化学物質代謝能は動物種差が大きいため化学物質の感受性を動物種間で大きく左右する大きな要因である。そのため、環境汚染物質に継続的に暴露されている野生哺乳類において化学物質代謝酵素の評価は急務となる。
哺乳類の中における「食肉目」動物はネコ科、イヌ科、クマ科、イタチ科、アザラシ科などを含むグループである。これらは生態系の高次に位置し、残留性の高い環境汚染物質の生物学的濃縮の影響を受けやすい。さらに、アンブレラ種として、生態系の保全の鍵となる種群であり、環境汚染物質に対する影響評価が重要な種である。そこで本研究では、これら食肉目動物における、化学物質代謝酵素の性状を、in vitro 解析と in silico 解析の双方を用いて、進化的、遺伝的、酵素的な側面から包括的に解明を試みた。

第 2 章：Cytochrome P450 1-3 ファミリーの動物種特異的な重複と欠損

本章では Cytochrome P450（CYP）に関しての解析を詳細に述べている。CYP は第 I 相反応に関わる化学物質代謝酵素であり、最も重要な化学物質代謝酵素として医薬品を含む外因性物質や、ステロイド、脂肪酸、コレステロールなどの内因性物質など多岐にわたる化学物質代謝に関与している。特にその中でも 1-3 ファミリーが主な外因性物質代謝に関与している分子種群といわれている。これら CYP をコードしている遺伝子は特徴的な重複と欠損を繰り返してその遺伝子の多様性を増やしており、進化の観点から動物種間の化学物質代謝を比較するうえで、この遺伝子重複・欠損は必須な情報である。そこで本章ではゲノムデータベースにおける食肉目動物の CYP1-3 の遺伝子情報を網羅的に比較、解析することで進化的な背景を明らかにした。

その結果、雑食性の動物であるクマ（ヒグマ、アメリカクロクマ)、アナグマ、イヌにおいて異物代謝に重要なサブファミリーである CYP2C と 3A において特徴的な遺伝子重複が確認され、これは恒常的に暴露される植物二次代謝産物に対応するために遺伝的に進化してきた可能性が示唆された。また、系統樹解析を行った結果 CYP2C は食肉目種では CYP2C21, 2C41, 2C23 のグループに分けられることが明らかとなった。さらに 3A ではネ

コ亜目とイヌ亜目では全く異なった遺伝子進化を遂げていることが明らかとなった。これらの結果から、同じ食肉目内でも CYP 分子種の進化に大きな差があり、これらの進化的背景の情報は、より適切な野生動物での化学物質代謝の推定に必須であると考えられる。

第 3 章：グルクロン酸転移酵素の遺伝的重複および欠損

本章では第 1 章では評価していない第 II 相反応にかかわる酵素のうち、主要な解毒酵素であるグルクロン酸転移酵素（UGT）に着目した。第 1 章でも明らかになったように食肉目でも解毒酵素には大きな種差があり、特に食性との関連が先行研究から示唆されている。UGT は第 II 相反応として CYP などの酸化、水酸化反応の後にグルクロン酸を化学物質に付加する反応であるが、CYP の代謝の後に、多くの化学物質が代謝的活性化を示すため「解毒」の意味では特に重要となる。UGT は 1A,2A,2B サブファミリーに大別され、特に 1A と 2B が肝臓で高発現し、異物代謝に関与しているといわれている。特に食肉目では先行研究よりネコ科動物、鰭脚類において一分子種である UGT1A6 の遺伝的な欠損、および in vitro における活性低下が明らかとなっている。そのため、これらに近い食肉目動物での遺伝的な種網羅的な比較評価はより正確な野生食肉目での UGT の性状解析につながる。本章では第 1 章同様に、データベースを用いた UGT 遺伝子の網羅的解析を行った。その結果イヌ科、ヒグマ、アメリカクロクマにおいて UGT1A の遺伝的拡大（遺伝子数の増加）が確認された。同様に UGT2B ファミリーにおいてもイヌ科とヒグマ、アメリカクロクマでは遺伝的拡大が確認され、一部のイタチ科でも同様の可能性が示唆された。さらに UGT1A7-12 分子種群において鰭脚類、ネコ科、一部のイタチ科では完全な欠損、または遺伝的縮小が確認された。この結果より一部 CYP と同様の動物で UGT も遺伝的に拡張していることが明らかとなり、これらクマ科、イヌ科の動物では化学物質に対しての強い代謝能が示唆された。一方、ネコ科や鰭脚類などでは先行研究で報告されていた UGT1A6 の欠損以外にもより広範にわたる遺伝子の縮小が明らかとなり、化学物質に対する弱い代謝能が示唆される結果となる。

第 4 章：硫酸転移酵素の遺伝的、および酵素学的な性状の特徴

本章では第 3 章で評価した UGT と同様に第二相反応に重要な硫酸転移酵素（SULT）に着目した。SULT は UGT と基質特異性を共有することが多く、より包括的な化学物質代謝の評価には UGT 同様に重要である。UGT と同様に種々の外因性物質代謝に関与すると同時に、ステロイドホルモン、ドパミンなどの神経伝達物質、甲状腺ホルモンなどの内因性物質代謝に重要な酵素である。そこで本章では前章同様の遺伝的解析を行うと同時に、野生動物の肝臓を用いた In vitro 解析により酵素学的な評価を行った。

その結果、遺伝的解析により SULT1E1 といわれるエストロン代謝に重要な分子種、および SULT1D1 分子種が鰭脚類では遺伝的に欠損していることが明らかとなった。さらに in vitro 解析にて SULT1E1 が主に代謝するエストラジオールを用いた代謝活性試験を行い、

その結果 SULT1E1 の活性が鰭脚類では著しく低いことが示唆された。この結果から鰭脚類ではエストロゲンや種々の医薬品、環境汚染物質に対して硫酸抱合能が弱い可能性が示唆された。また、SULT1E1 は内因性物質代謝にも大きく関与しているため、食肉目動物間でもエストロゲン代謝に対する種差が大きい可能性が示唆された。

第 5 章　総括

本研究より食肉目動物で「解毒」に重要な化学物質代謝酵素における包括的な性状を明らかにした。その結果、進化的に近い食肉目内でも非常に多岐にわたる化学物質代謝酵素の進化を経ていることが明らかとなった。特にヒグマやアメリカクロクマ、アナグマでの CYP の特徴的重複、ヒグマ、クロクマ、イヌ科での UGT の遺伝的拡張、UGT の鰭脚類、ネコ科における遺伝的収縮、更には SULT1E1 の鰭脚類での欠損などが明らかとなった。本研究での知見は、化学物質代謝酵素の性状をより正確に評価することを可能とし、実験動物で得られた化学物質代謝酵素の知見を的確に外挿することへの一助となる。さらに、化学物質に対する生体防御機構として重要なこれら酵素を評価することで環境化学物質に対して感受性の高い動物を推定する上でも重要な研究であったと考えている。