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Cytochrome P450 2B diversity in a dietary specialist—the red tree vole (*Arborimus longicaudus*)

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Although herbivores rely on liver enzymes to biotransform plant secondary metabolites ingested in plant-based diets, only a few enzymes from a handful of species have been characterized at the genomic level. In this study, we examined cytochrome P450 2B (*CYP2B*) sequence diversity and gene copy number in a conifer specialist, the red tree vole (*Arborimus longicaudus*). We fed captive individuals exclusively Douglas-fir (*Pseudotsuga menziesii*) foliage, cloned and sequenced their liver *CYP2B* cDNA, and estimated *CYP2B* gene copy number. We identified 21 unique *CYP2B* nucleotide sequences, and 20 unique *CYP2B* amino acid sequences. Gene copy number of *CYP2B* was estimated at 7.7 copies per haploid genome. We compared red tree vole *CYP2B* with *CYP2B* sequences of a generalist, the prairie vole (*Microtus ochrogaster*), found in GenBank. Our study revealed that the *CYP2B* enzymes of red tree voles possess unique sequences compared to *CYP2B* enzymes of other herbivorous species. The unique combination of amino acid residues at key substrate recognition sites of *CYP2B* enzymes may underlie the ability of the red tree vole to specialize on a highly toxic diet of Douglas-fir.

Key words: cytochrome P450 enzymes, detoxification, diet, red tree vole, terpenes

The majority of mammalian species are either herbivorous or ingest significant quantities of plant material in their diet (Ley et al. 2008). To deter herbivory, most plants produce plant secondary metabolites (PSMs). PSMs cause various effects on herbivores, including diuresis, cellular damage, and decreased digestion (Freeland and Janzen 1974; Dearing et al. 2005). The toxic load of PSMs can be especially high for dietary specialists, which satisfy their dietary needs by consuming almost exclusively a single plant species, often abundant in PSMs. For example, the diet of Stephens' woodrat (*Neotoma stephensi*) consists of > 90% one-seeded juniper (*Juniperus monosperma*—Vaughan 1982), which contains high quantities of terpenes. The daily dose of terpenes that *N. stephensi* ingests and tolerates in its diet is equivalent to or higher than the lethal dose for terpenes in humans when scaled to body size (Gscheidmeier and Fleig 1996; Troulakis et al. 1997; Dearing et al. 2000; Saeidnia 2014). To enable consumption of a plant-based diet and reduce exposure to toxic PSMs, herbivores rely on hundreds of hepatic enzymes (Dearing et al. 2005).

Recent work suggests that enzymes of the cytochrome P450 2B subfamily (*CYP2B*) play a critical role in the detoxification of PSMs by herbivores (Malenke et al. 2012; Kitanovic et al. 2018). Pharmacological blocking of *CYP2B* in herbivorous woodrats (genus *Neotoma*) decreases their ability to feed on their natural diet of juniper foliage, which is high in terpenes (Skopec et al. 2013). Furthermore, the ingestion of a juniper diet induces *CYP2B* expression in woodrats and results in greater enzyme activity (Haley et al. 2007; Skopec et al. 2007; Magnanou et al. 2009). *In vitro* studies indicate that purified woodrat *CYP2B* enzymes display high affinities for α-pinene, a key monoterpene present in juniper (Wilderman et al. 2014).

In this study, we focused on identifying and quantifying *CYP2B* genes in an herbivore specialist, the red tree vole (*Arborimus longicaudus*; Fig. 1). The diet of red tree voles consists primarily of Douglas-fir (*Pseudotsuga menziesii*) needles, although in some areas they consume western hemlock (*Tsuga heterophylla*), Sitka spruce (*Picea sitchensis*), or grand fir (*Abies grandis*—Walker 1928, 1930; Benson and Borell 1931).

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Fig. 1.—Red tree vole (*Arborimus longicaudus*) perched on a Douglas-fir branch inside its enclosure at the Oregon State University captive colony (photo credit: Chad A. Marks-Fife).

They also consume a small amount of bark and cambium from leftover twigs after the needles have been removed (Maser 1966; Forsman et al. 2009). Needles belonging to these tree species contain resin ducts high in terpene content, which function as chemical defenses against pathogens and herbivores (Phillips and Croteau 1999; Langenheim 2003; Kelsey et al. 2009). Although red tree voles generally reduce their exposure to terpenes by removing resin ducts from needles before consumption (Howell 1926), they often consume entire needles or portions of needles with the resin ducts intact (Kelsey et al. 2009).

The hepatic detoxification mechanisms of this unique dietary specialist have not been described to date. Therefore, to advance our knowledge of the detoxification enzymes of mammalian specialists, we sequenced *CYP2B* cDNA in the red tree vole and compared features of its active site (i.e., the chemical character and volumes of specific side chains, and active site volume), to those of other herbivorous mammals. The red tree vole may have adapted to its unique diet through the evolution of *CYP2B* enzymes that process Douglas-fir terpenes with high catalytic efficiency. Thus, we predicted that red tree vole *CYP2B* enzymes would differ in several physical or chemical features from the *CYP2B* enzymes of other herbivorous mammals, which may enhance the processing of terpenes in the diet. In addition, we estimated *CYP2B* gene copy number in red tree voles, as multiple gene copies of cytochrome P450 enzymes enable animals to produce higher quantities of enzymes that increase the detoxification of xenobiotics and PSMs (Ingelman-Sundberg 2005; Zanger et al. 2007).

Materials and Methods

Capture and diet of red tree voles.—The Oregon State University (OSU) captive colony of red tree voles was created from 10 adult and several pre-dispersal age young founder animals that were captured between February and July 2013 in coastal conifer forests near Harlan, Benton County, Oregon (Marks-Fife 2016). Red tree voles used in our study were all born in captivity, with "W" animals conceived in the wild, and "C" animals conceived in captivity (Table 1). The animals were maintained in captivity exclusively on a diet of Douglas-fir needles. The conventional approach of demonstrating the induction of detoxification enzymes after introduction of a PSM was not possible in this case, as red tree voles require a diet of conifer needles to survive and are not known to feed on artificial diets. Every day, faux trees in each enclosure were replenished with fresh Douglas-fir branches so that red tree voles foraged for their food, simulating natural behavior. The branches were misted with water and the cages were equipped with water bottles, providing the animals with sufficient water supply. Within 120–420 days after birth, the animals were sacrificed, and their livers were removed and stored at −80°C in RNAlater (Thermo Fisher Scientific, Waltham, Massachusetts). This study conforms to guidelines of the American Society of Mammalogists for the use of wild animals in research (Sikes et al. 2016), and all procedures were performed according to an IACUC-issued ACUP protocol. Red tree vole specimens from individuals used in this study have been deposited in the Burke Museum of Natural History and Culture, Seattle, Washington and Oregon State University, Department of Fisheries and Wildlife, Corvallis, Oregon (Supplementary Data SD1).

CYP2B cloning and sequencing.—Total RNA was isolated from 14–20 mg of red tree vole (n = 5) liver tissue (RNAqueous-4PCR Kit; Ambion, Naugatuck, Connecticut), which had been stored in RNAlater at −80°C. The isolated RNA served as template for *CYP2B* cDNA synthesis using a *CYP2B*-specific reverse primer, H7 (Table 2; High-Capacity cDNA Reverse Transcription Kit; Thermo Fisher Scientific). *CYP2B* cDNA was then amplified in PCR with Herculase II Fusion DNA Polymerase (Agilent Technologies, Santa Clara, California) and primer pair L6d and H7b (Table 2). Primer H7 was previously designed to study *CYP2B* in the desert woodrat, *Neotoma lepida*, using *CYP2B* cDNA sequence alignments from Norway rat (*Rattus norvegicus*) and house mouse (*Mus musculus*) in GenBank (Malenke et al. 2012). Primers L6d and H7b were previously used to amplify *CYP2B* in *N. albigula* and *N. stephensi* (Kitanovic et al. 2018). Red tree vole *CYP2B* cDNA was amplified using a range of PCR conditions: 95°C: 1–2′; 95°C: 30″, 62°C: 40″, 68°C: 1′30″-2′ for 35–40 cycles; and final extension at 68°C: 0–4′. We added 3′A overhangs with Taq Polymerase (Thermo Fisher Scientific) to blunt-ended

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Table 1.—*CYP2B* clones found in individual red tree voles (*Arborimus longicaudus*). To determine *CYP2B* sequence variation and gene copy number, we used 5 and 8 adult individuals, respectively. Though all individuals were born in captivity, "W" individuals were conceived in the wild and "C" individuals were conceived in captivity. Individuals C10 and C12 were siblings, whereas individuals C03 and C04, as well as C35 and W08, were half-siblings. "Unique *CYP2B* clones" refers to unique *CYP2B* nucleotide sequences. NA = not applicable.

Individual ID	Sex	Age (days)	Number of analyzed *CYP2B* clones	Number of unique *CYP2B* clones	Number of *CYP2B* variants	*CYP2B* gene copy number (haploid)
C03	F	360	9	3	3	7.8
C04	M	300	NA	NA	NA	9.8
C10	F	240	NA	NA	NA	6.5
C12	M	300	8	3	1	7.9
C35	M	120	8	7	5	6.8
W08	F	420	8	4	2	7.2
W09	M	420	7	7	4	8.5
W28	F	240	NA	NA	NA	7.3

Table 2.—Red tree vole (*Arborimus longicaudus*) primer names, sequences, and uses.

Primer name	Primer sequence (5′→3′)	Primer use
NL_CYP2B_H7 (reverse)	GACACCTGGCCACCTCAG	Synthesis of *CYP2B* cDNA
NA_NS_CYP2B_L6d (forward)	TGRYYASACCAGGACCATG	Amplification of *CYP2B* cDNA
NA_NS_CYP2B_H7b (reverse)	ACCTGGCCACCTCAGCCCAG	Amplification of *CYP2B* cDNA
AL_CYP2B_exon9 (forward)	AAAGCGCATTTGTCTTGG	qPCR primers for *A. longicaudus CYP2B*
AL_CYP2B_exon9_2 (reverse)	TCAATGTCCTTAGGAGCCA	qPCR primers for *A. longicaudus CYP2B*
NS_SOD1_exon4_2b (forward)	GCATGTGGGAGACCTGG	qPCR primers for *A. longicaudus SOD1*
AL_SOD1_exon4 _1 (reverse)	GGAATGTTCTCCTGAGAGTGAG	qPCR primers for *A. longicaudus SOD1*
NA_NS_SOD1_deg1 (forward)	GAYATCAKTGCTTATCCACC	Amplification of internal portion of *SOD1*
NA_NS_SOD1_deg1 (reverse)	GTCATCTTSTTTCTCRTGGAC	Amplification of internal portion of *SOD1*

PCR products purified with the GeneJET PCR Purification Kit (Thermo Fisher Scientific). PCR products were then ligated into the Topoisomerase I-activated pCR2.1-TOPO vector (Thermo Fisher Scientific). TOP10 cells (One Shot Chemically Competent *Escherichia coli* cells; Thermo Fisher Scientific) were transformed with pCR2.1-TOPO vector containing *CYP2B* cDNA, and blue-white colony screening was used to identify TOP10 colonies with *CYP2B* cDNA. Positive (white) TOP10 colonies containing plasmid DNA (*CYP2B*-pCR2.1-TOPO) were selected and propagated overnight at 37°C in Luria-Bertani broth (Thermo Fisher Scientific) with 50 µg/ml ampicillin. Plasmid DNA was isolated from the liquid culture using the QIAprep Spin Miniprep Kit (Qiagen, Germantown, Maryland) or ZR Plasmid Miniprep–Classic Kit (Zymo Research, Irvine, California), and sequenced at the University of Utah DNA Sequencing Core Facility. *CYP2B* nucleotide sequences were assembled with the ApE application (http://biologylabs.utah.edu/jorgensen/wayned/ape/) and translated into amino acid sequences using the ExPASy Bioinformatics Resource Portal (http://www.expasy.org/).

Identification of CYP2B substrate recognition site variants.—Substrate recognition sites (SRSs) are linear stretches of amino acids that comprise part of the cytohrome P450 enzyme active site, which were previously identified in *CYP2B* enzymes (Gotoh 1992). We categorized red tree vole *CYP2B* clones into *CYP2B* variants according to their unique combinations of 10 amino acid residues located in SRSs previously identified through mutagenesis studies as critical to enzyme function (residue positions 101, 104, 108, 114, 206, 209, 290, 297, 363, and 367—Domanski and Halpert 2001). These *CYP2B* variants were then organized and numbered based on the volume of the amino acid residue at each of these 10 selected positions (Zamyatnin 1972). *CYP2B* variants were listed according to amino acid identity at position 101−from variants containing larger isoleucine (Ile) to variants with smaller valine (Val) (Table 3). For example, variants 1–2 possess an Ile at position 101, whereas variants 3–10 contain the smaller Val. These 2 sets of *CYP2B* variants were further subdivided at position 104, with variants 1–2, 3, and 4–10 possessing Val, phenylalanine (Phe), or leucine (Leu), respectively, at this position. The same organizing process for these variants was repeated at each subsequent selected position. Some variants contain several *CYP2B* clones that differ in nucleotide sequence outside of these 10 positions.

To calculate cumulative residue volumes for each red tree vole *CYP2B* variant, we summed the volumes of residues 101, 104, 108, and 114 (Table 3) and compared the mean cumulative residue volume of red tree vole *CYP2B* variants with that of the prairie vole *CYP2B* variants. In addition, we calculated the active site volume of homology models for each *CYP2B* variant. Using the X-ray crystal structures of *CYP2B35* (PDB ID: 5E58, www.rcsb.org) and *CYP2B37* (PDB ID: 5E0E), homology models were made for each *CYP2B* variant using MODELLER 9.18 (Webb and Sali 2016), and the active site volume was calculated using Voidoo (Kleywegt and Jones 1994; Uppsala Software Factory, Uppsala, Sweden).

Estimation of CYP2B gene copy number.—We used quantitative PCR (qPCR) to estimate *CYP2B* gene copy number in red tree voles. Genomic DNA was isolated from 12–22 mg of red tree vole liver tissue (Quick-gDNA MiniPrep Kit; Zymo

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Table 3.—Red tree vole (*Arborimus longicaudus*) *CYP2B* variants were organized according to cumulative residue volume (CRV) of residues 101, 104, 108, and 114. ASV is "active site volume" of each *CYP2B* variant. Shaded in gray are key amino acid residues that differ among red tree vole *CYP2B* variants. NA = not applicable.

Variant	Key residues in *CYP2B* substrate recognition sites													CRV (Å^3)	ASV (Å^3)	# individuals
	101	104	108	114	206	209	290	294	297	363	367	477	478			
1	I	V	F	I	F	I	I	S	F	I	V	L	T	663.3	240.1	1
2	I	V	T	F	F	T	I	S	F	V	V	L	T	612.7	220.2	1
3	V	F	M	I	F	I	I	S	L	V	V	L	T	659.5	211.6	2
4	V	L	V	I	F	I	I	S	F	A	V	L	T	613.4	225.0	1
5	V	L	V	I	F	I	I	S	L	V	V	L	T	613.4	247.0	1
6	V	L	V	I	F	T	Q	S	F	A	A	L	T	613.4	269.9	1
7	V	L	V	I	I	I	Q	S	F	V	V	L	T	613.4	228.8	1
8	V	L	V	I	I	I	Q	S	F	A	A	L	T	613.4	289.8	5
9	V	L	V	V	I	I	Q	S	F	A	V	L	T	586.7	287.1	1
10	V	L	V	V	I	I	Q	S	F	A	A	L	T	586.7	349.2	1
M. ochrogaster CYP2B1	I	V	F	I	F	I	I	S	F	I	V	F	G	663.3	224.3	NA
M. ochrogaster CYP2B1-like	I	F	V	V	F	I	I	S	F	L	V	F	T	636.6	265.5	NA
M. ochrogaste CYP2B1-like	I	I	V	V	I	L	I	T	F	L	V	L	T	613.4	189.0	NA
N. albigula CYP2B (v. 43)	V	V	F	I	F	T	I	S	F	I	V	F	G	636.6	209.2	NA
N. stephensi CYP2B (v. 13)	I	I	I	I	F	M	N	S	F	A	A	I	G	666.8	284.0	NA

Table 4.—*CYP2B* sequence diversity in red tree voles (*Arborimus longicaudus*).

Number of analyzed *CYP2B* clones	Number of unique *CYP2B* nucleotide sequences	Number of unique *CYP2B* amino acid sequences	Number of *CYP2B* variants	Number of *CYP2B* variants in > 1 individual
40	21	20	10	2

Research), which had been stored at −80°C. Using the alignment of 21 unique red tree vole *CYP2B* cDNA sequences (MUSCLE, http://www.ebi.ac.uk/Tools/msa/muscle/; Table 4), we designed primers to amplify a 111 base pair (bp) fragment of exon 9 in *CYP2B* (Table 2). Red tree vole *CYP2B* gene copy number was then estimated relative to *SOD1*, which served as a single copy reference gene. To design primers to amplify a fragment of *SOD1*, exon 4 of red tree vole *SOD1* was first amplified using degenerate primers, and then sequenced (Table 2). The forward and reverse degenerate primers, with sequences based on alignments of *N. lepida*, *R. norvegicus*, and *M. musculus SOD1*, annealed to intron 3 and exon 5 of *SOD1*, respectively (Kitanovic et al. 2018). Primers were then designed for amplification of red tree vole *SOD1* exon 4 to generate a 97 *SOD1* bp fragment in qPCR (Table 2). Reactions for qPCR, done in triplicate for each individual, were prepared in 96-well plates using 2x Apex qPCR Master Mix (Apex BioResearch Products, Houston, Texas), followed by cycling in CFX96 Real-Time System C1000 Touch Thermal Cycler (Bio-Rad, Hercules, California) at 95°C: 10′; then 95°C: 30″ and 60°C: 30″ for 40 cycles. We generated a standard curve to determine qPCR efficiencies of *CYP2B* and *SOD1* primer pairs, which were both 98% (Livak and Schmittgen 2001). Using the $2^{-\Delta C'}{}_T$ method, we then estimated *CYP2B* gene copy number in 8 red tree vole individuals (Livak and Schmittgen 2001); 5 of those individuals had been used previously to obtain *CYP2B* sequences. Consistent with amplification of a single PCR product, a single melt peak for each qPCR was observed, as well as single bands on a 1.2% agarose gel for selected qPCR samples. Sequences of amplified *CYP2B* and *SOD1* exon fragments were confirmed for a representative red tree vole individual.

Results

The primers shown in Table 2 yielded *CYP2B* cDNA from red tree voles with 1,476 bp from start to stop codon, which translated to a protein sequence of 491 amino acid residues. This is consistent with findings for the prairie vole *CYP2B1* and *CYP2B1*-like sequences (*Microtus ochrogaster*, NCBI reference sequences XM_005372156.1 and XM_013354871.1; Broad Institute, Cambridge, Massachusetts), as well as *CYP2B* sequences from most other mammalian species determined to date (Nelson 2009). The best matches in the nucleotide database at NCBI for red tree vole *CYP2B* cDNA sequences were prairie vole *CYP2B1* and *CYP2B1*-like sequences, with coverage ranging between 91% and 95%, and e-values of 0.0.

We obtained high-quality nucleotide sequences for 40 red tree vole *CYP2B* cDNA clones (Tables 1 and 4). Unique *CYP2B* nucleotide and amino acid sequences were found across approximately one-half of the clones (Table 4). Sequence data for unique *CYP2B* cDNA clones were deposited in GenBank and can be found under accession numbers MG851823–MG851846. According to the identity of 10 amino acid residues located in SRSs, we classified red tree vole *CYP2B* enzymes

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into 10 *CYP2B* variants (Table 3; Supplementary Data SD2). Only 2 of these *CYP2B* variants (3 and 8) were found in more than 1 individual, of which variant 8 was present in all individuals ($n = 5$).

The chemical and physical properties of amino acid side chains found in previously identified SRSs determine substrate binding specificity and catalytic efficiency of *CYP2B* enzymes (Zhao and Halpert 2007). We compared the mean cumulative residue volume of red tree vole *CYP2B* variants to that of prairie vole *CYP2B* variants. The mean cumulative residue volume of red tree vole *CYP2B* variants (mean ± *SE*: 617.6 ± 8.1) was not significantly different from that of the prairie vole (mean ± *SE*: 637.8 ± 14.4, $P = 0.15$, *t*-test). However, *CYP2B* variants of red tree voles contained noteworthy residues at positions known to influence *CYP2B* enzyme activity. Variants 6–10 had a glutamine (Gln) residue at position 290, and all 10 red tree vole *CYP2B* variants possessed a Leu at position 477 and a threonine (Thr) at position 478 (Table 3). The *CYP2B* enzymes from other rodent species contain different residues at these positions (Malenke et al. 2012; Kitanovic et al. 2018). The unique combination of *CYP2B* residues in the red tree vole may support the binding and catalysis of a novel portfolio of PSMs in their diet compared to other rodent species.

We also found that the mean active site volume is likely significantly different among red tree vole *CYP2B* variants depending on the presence or absence of Ala at residue 363 (Ala [mean ± *SE*: 284.2 ± 17.9 Å^3] versus non-Ala [mean ± *SE*: 229.5 ± 5.7 Å^3], $P = 0.05$, *t*-test; Table 3). The mean active site volume did not differ significantly between the 2 vole species (generalist [mean ± *SE*: 256.9 ± 12.8 Å^3] versus specialist [mean ± *SE*: 226.3 ± 18.0 Å^3], $P = 0.30$, *t*-test).

We obtained *CYP2B* gene copy number values in 8 red tree vole individuals, ranging from 6.5 to 9.8 copies per haploid genome (Table 1). Mean *CYP2B* gene copy number per haploid genome in the red tree vole species was 7.7 ± 0.4 (mean ± *SE*; Table 1).

Discussion

The sequence identity of hepatic biotransformation ("detoxification") genes used by mammalian herbivores to process dietary toxins is largely unexplored, and yet these enzymes represent an essential mechanism for the processing of toxic diets. In this study, we characterized *CYP2B* sequence diversity and gene copy number in the red tree vole (*A. longicaudus*) sustained on its natural diet of Douglas-fir. We found that red tree voles expressed a variety of *CYP2B* enzymes in their liver. One of these *CYP2B* variants (variant 8) was present across all surveyed individuals, suggesting that it may be essential for the metabolism of terpenes. Other studies have also demonstrated that PSMs in Douglas-fir, such as α-pinene, induce expression of *CYP2B* enzymes and are substrates for this enzyme (Malenke et al. 2012; Wilderman et al. 2013). The large number of *CYP2B* gene copies coupled with the expression of multiple *CYP2B* variants per individual suggests that *CYP2B* enzymes are vital to the metabolism of PSMs in the diet of the red tree vole.

The differentiation of the *CYP2B* sequences in the dietary specialist red tree vole and generalist prairie vole suggests that these enzymes may have evolved in concert with the host diet. Red tree voles share no *CYP2B* variants with prairie voles. Moreover, the identities of amino acids located at the selected positions in *CYP2B* SRSs differ substantially (9 of 13) between the 2 vole species. Extensive prior mutagenesis studies have established the functional importance of these residues (Domanski and Halpert 2001). Two of the most unusual substitutions relative to most other *CYP2B* enzymes are Gln-290 in red tree voles and Thr-478 in both vole species (He et al. 1992; Nelson 2009; Malenke et al. 2012; Okamatsu et al. 2017). Most *CYP2B* enzymes characterized to date possess hydrophobic amino acids at position 290 and Gly at position 478. Prior site-directed mutagenesis studies of residue Asp-290 in *Canis familiaris CYP2B11* showed that the Asp290Ile mutant enzyme activity was 51% of wild-type enzyme activity for dihydroxylation of 2,2′,3,3′,6,6′-hexachlorobiphenyl (Waller et al. 1999). Likewise, mutation of residue Gly-478 in *R. norvegicus CYP2B1* significantly altered the product profile of androstenedione, testosterone, and *N*-benzyl-1-aminobenzotriazole metabolism (He et al. 1992; Kent et al. 1997). The *CYP2B1* Gly478Thr and Gly478Ser mutants displayed greatly decreased androstenedione and testosterone metabolism relative to wild-type enzyme (He et al. 1992). Thus, red tree vole *CYP2B* variants containing Gln-290 and Thr-478 likely have altered substrate specificities relative to previously reported *CYP2B* enzymes.

Additionally, most red tree vole *CYP2B* variants (variants 3–10) contain Val and Leu at positions 101 and 104, respectively. In prairie voles, residue 101 is a conserved Ile, and residue 104 is hydrophobic, but not conserved. Prairie vole *CYP2B* variants also possess features not found in any of the red tree vole *CYP2B* variants identified to date. At positions 363 and 477, some prairie vole *CYP2B* variants contain Leu and Phe, respectively, which are absent at those positions in red tree vole *CYP2B* variants. Such diversity in amino acid residues found in the SRSs of these *CYP2B* variants in red tree voles and prairie voles may impact their substrate specificities, which would likely support their abilities to consume different diets. Further analysis is required to determine which residues in red tree vole *CYP2B* SRSs interact with specific PSMs from Douglas-fir by performing structure-function mutagenesis studies and modeling of mutant *CYP2B* variants (Zhao and Halpert 2007).

CYP2B enzymes possess active sites characterized by plasticity, which allows them to accommodate substrates of varying sizes (Mr 80–800—Wilderman and Halpert 2012). However, physical properties of amino acids found in SRSs, such as their side chain volumes, may restrict the types of PSMs that dock and bind to the active site. Furthermore, the side chain volumes of amino acids in SRSs may also influence the volume of the active site, which plays an important role in determining *CYP2B* substrate specificity. According to this study, the mean cumulative residue volume and mean active site volume of red tree vole *CYP2B* enzymes were comparable to those of

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the prairie vole, suggesting that chemical properties of these amino acid side chains (i.e., functional groups), rather than their physical properties, may play a greater role in distinguishing specific substrates between the 2 species. Previously, we identified differences in *CYP2B* mean cumulative residue volumes between woodrat specialist and generalist species, and found that the specialist woodrat had a greater mean cumulative residue volume of *CYP2B* variants than the generalist woodrat (Kitanovic et al. 2018). Thus, no general patterns emerge with respect to residue volumes in SRSs across closely related pairs of specialist and generalist species (Stephens' woodrat versus white-throated woodrat; red tree vole versus prairie vole).

However, the active site volume of red tree vole *CYP2B* variants depended on the presence of Ala at residue 363, as previously seen in *CYP2B* variants in woodrats (Kitanovic et al. 2018). This suggests that *CYP2B* enzymes with different active site volumes may have evolved to accommodate the range of PSMs in the diet of the red tree vole. In the prairie vole, no *CYP2B* variants containing Ala-363 have been found. However, because the prairie vole *CYP2B* sequence data set is very small, with only 3 prairie vole *CYP2B* or *CYP2B*-like sequences available on GenBank, it is possible that prairie vole *CYP2B* variants containing Ala-363 may yet be discovered.

Many species possess multiple gene copies of detoxification enzymes, which are associated with elevated transformation rates of drugs in humans (Ingelman-Sundberg 2005) or pesticides in insects (Bass et al. 2014). Multiple gene copies of enzymes that target high concentrations of PSMs in a specialized diet may also provide an adaptive advantage to specialists by increasing enzyme quantity. We found that the red tree vole has multiple copies of *CYP2B* genes. Although the technique we used cannot distinguish between active genes and pseudogenes, the data on the number of unique *CYP2B* sequences per individual, assuming heterozygosity across loci, indicate that red tree voles have at least 4 copies of functional *CYP2B* genes. This number is a conservative underestimate, as it is unlikely that all animals are heterozygous across all *CYP2B* loci. Thus, specialists, such as red tree voles, may generally benefit from possessing high *CYP2B* gene copy numbers, because they likely contribute to increased *CYP2B* expression. Recent studies in woodrats suggest a positive correlation between *CYP2B* gene copy number and *CYP2B* enzyme expression in liver (Kitanovic et al. 2018; T. J. Orr, University of Utah, pers. comm.), and the same may be true for red tree voles.

The species-specific *CYP2B* sequences found in the red tree vole likely represent adaptations to its highly specialized diet of Douglas-fir, which contains a unique profile of toxic PSMs, dominated by terpenes such as α- and β-pinene (Kelsey et al. 2009). The *CYP2B* sequences of the generalist prairie vole differ from those of the red tree vole at key SRS positions that play a role in substrate binding and catalysis, likely because the prairie vole encounters a different set of PSMs in its diet. The plant species consumed by prairie voles, such as dandelion (*Taraxacum officinale*), clover (*Trifolium* spp.), and alfalfa (*Medicago sativa*—Batzli and Cole 1979), are abundant in flavonoids, isoflavonoids, and saponins (Saviranta et al. 2010; Rafińska et al. 2017). Though terpenes have been identified in *T. officinale*, the major ones—lupeol, α-amyrin, β-sitosterol, and betulin (Díaz et al. 2018)—differ from those found in the red tree vole's diet of Douglas-fir. In addition, the high copy number of CYP P450 genes may be a common phenomenon among specialist herbivores enabling the production of high levels of detoxification enzymes.

This study lays the groundwork for future functional studies on *CYP2B* enzymes in red tree voles. The *CYP2B* sequences provided herein can be used to generate recombinant red tree vole *CYP2B* enzymes for identification of specific substrates and evaluation of *CYP2B* catalytic efficiencies. Such future work will help expand our understanding of the enzymatic mechanisms that herbivores use to process PSMs in their diets.

Supplementary Data

Supplementary data are available at *Journal of Mammalogy* online.

Supplementary Data SD1.—Museum voucher IDs for red tree vole (*Arborimus longicaudus*) specimens.

Supplementary Data SD2.—List of red tree vole (*Arborimus longicaudus*) *CYP2B* variants with associated *CYP2B* clones.

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