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Opossum alcohol dehydrogenases: Sequences, structures, phylogeny and evolution
Evidence for the tandem location of *ADH* genes on opossum chromosome 5

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ABSTRACT

BLAT (BLAST-Like Alignment Tool) analyses and interrogations of the recently published opossum genome were undertaken using previously reported rat ADH amino acid sequences. Evidence is presented for six opossum ADH genes localized on chromosome 5 and organized in a comparable ADH gene cluster to that reported for human and rat ADH genes. The predicted amino acid sequences and secondary structures for the opossum ADH subunits and the intron–exon boundaries for opossum ADH genes showed a high degree of similarity with other mammalian ADHs, and four opossum ADH classes were identified, namely ADH1, ADH3, ADH6 and ADH4 (for which three genes were observed: ADH4A, ADH4B and ADH4C). Previous biochemical analyses of opossum ADHs have reported the tissue distribution and properties for these enzymes: ADH1, the major liver enzyme; ADH3, widely distributed in opossum tissues with similar kinetic properties to mammalian class 3 ADHs; and ADH4, for which several forms were localized in extrahepatic tissues, especially in the digestive system and in the eye. These ADHs are likely to perform similar functions to those reported for other mammalian ADHs in the metabolism of ingested and endogenous alcohols and aldehydes. Phylogenetic analyses examined opossum, human, rat, chicken and cod ADHs, and supported the proposed designation of opossum ADHs as class I (ADH1), class III (ADH3), class IV (ADH4A, ADH4B and ADH4C) and class VI (ADH6). Percentage substitution rates were examined for ADHs during vertebrate evolution which indicated that ADH3 is evolving at a much slower rate to that of the other ADH classes.

1. Introduction

Mammalian alcohol dehydrogenases (ADH; EC 1.1.1.1) exist as a family of enzymes which metabolize a broad range of alcohol and carbonyl compounds and are encoded by at least six classes of *ADH* genes [1–3]. Further mammalian *ADH* gene multiplicity may occur as a result of recent gene duplication events during mammalian evolution generating multiple non-allelic *ADH* genes [4,5] or following *ADH* pseudogene formation [6]. ADH subunit multiplicity may also be generated by allelic variation of *ADH* genes [7–10] or by differential splicing events during *ADH* mRNA transcription see [11]. Although complex in nature, the nomenclature for mammalian ADH genes and encoded proteins has taken account of the extensive gene and protein multiplicity reported for this enzyme [1].

Mammalian liver class I ADHs in particular have been intensively investigated because of their roles in ethanol metabolism and in neurotransmitter, retinoid and bile acid metabolism see [12]. Human liver class I ADHs comprise 3 genetically distinct subunits (α, β and γ) which form six homodimeric and heterodimeric isozymes [7]. Other ADH classes, with the exception of ADH4, are also localized in mammalian liver and include the class II enzyme ADH2, which is divided into two groups, human and rabbit forms and the rodent forms [13–15]. Class III ADH, ADH3, is widely distributed in mammalian tissues and functions in formaldehyde metabolism, involving S-hydroxymethyl glutathione, a conjugation product formed from glutathione and formaldehyde [16,17]; while ADH5 and ADH6 have been investigated only at the DNA and RNA level and analyzed for tissue distribution [12]. Mammalian class IV ADH, or ADH4, occurs in extra-hepatic tissues, particularly in stomach, intestine and eye [18–20], and may play a role in the first pass clearance of ingested alcohols and carbonyl compounds [10,21] and in retinoid metabolism [17,22]. Mammalian ADHs are zinc dependent dimeric enzymes which require NAD/NADH as coenzyme for

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catalytic activity, with each subunit consisting of about 375 amino acid residues [23,24].

Evolutionary studies on vertebrate ADHs have shown that class III ADH3 represents the primordial form and that both class I and class III ADHs are present in vertebrate fish and have co-existed for more than 500 million years [3,25]. These *ADH* genes have continued to diverge throughout vertebrate evolution and have undergone further gene duplication, generating additional *ADH* gene classes, as well as multiple *ADH* genes within an *ADH* class following more recent gene duplication.

This report outlines the predicted sequences, structures, phylogeny and evolution of *ADH* genes and enzymes in a South American marsupial, the gray short-tailed opossum (*Monodelphis domestica*), an established laboratory animal used to study the genetics of eye cancer and heart disease [26–28]. *In silico* methods were used to predict the amino acid sequences and secondary structures for opossum ADHs and gene locations for opossum *ADH* genes, using data from the recently released opossum genome sequence [29]. This paper extends previous biochemical genetic studies on opossum ADHs which reported the tissue distribution and biochemical properties of class I, class III and multiple class IV ADHs [30]. Phylogenetic analyses also describe the relationships and potential evolutionary origins of the opossum *ADH1*, *ADH3*, multiple *ADH4* and *ADH6* predicted gene and enzyme sequences with other previously reported mammalian and vertebrate ADHs.

2. Materials and methods

2.1. In silico opossum ADH gene and protein identification

BLAT (BLAST-Like Alignment Tool) *in silico* studies were undertaken using the UC Santa Cruz web browser (http://genome.ucsc.edu/cgi-bin/hgBlat) [31,32] with the default settings. UniProtKB/Swiss-Prot Database (http://au.expasy.org) and GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) sequences for rat ADH1, ADH2, ADH3, ADH4, ADH5 and ADH6A (Table 1) were used to interrogate the opossum genome sequence. Gene locations, predicted gene structures and ADH protein subunit sequences were observed for each ADH examined for those regions showing identity with the respective opossum *ADH* gene products.

2.2. Predicted secondary structures for opossum ADH gene products

Predicted secondary structures for rat ADH1, ADH2, ADH3, ADH4, ADH5 and ADH6A and for opossum ADH1, ADH3,

Table 1
Alcohol dehydrogenase (ADH) genes and enzymes examined.

Animal	*ADH* gene	*ADH* gene lineage	GenBank mRNA (or *N-scan ID)	UNIPROT ID	No. of amino acids	Chromosome location	Strand	Exons	Subunit	Alternate Gene Name
Human	*ADH1A*	*ADH1*	BX647987	P07327	375	4: 100,419,608–100,427,845	Negative	9	α	*ADH1*
	ADH1B	*ADH1*	BC033009	P00325	375	4: 100,450,947–100,459,066	Negative	9	β	*ADH2*
	ADH1C	*ADH1*	BC066227	P00326	375	4: 100,479,759–100,488,026	Negative	9	γ	*ADH3*
	ADH2	*ADH2*	BC002319	P08319	380	4: 100,266,770–100,282,954	Negative	9	Π	*ADH4*
	ADH3	*ADH3*	AK226177	P11766	374	4: 100,212,749–100,225,390	Negative	9	X	*ADH5*
	ADH4	*ADH4*	X76342	P40394	374	4: 100,555,660–100,569,813	Negative	9	σ/μ	*ADH7*
	ADH5	*ADH5*	BX647987	P28332	368	4: 100,345,104–100,356,445	Negative	9		*ADH6*
Rat	*ADH1*	*ADH1*	BC062403	P06757	376	2: 235,801,479–235,810,238	Positive	9	A	
	ADH2	*ADH2*	BC127504	Q64563	377	2: 235,953,574–235,969,179	Positive	9		*ADH4*
	ADH3	*ADH3*	AY310136		374	2: 235,981,154–235,990,634	Positive	9	X	
	ADH4	*ADH4*	*chr2.1432	P41682	374	2: 235,753,870–235,762,009	Positive	9		*ADH7*
	ADH5	*ADH5*	BC083782	Q5XI95	376	2: 235,916,404–235,937,398	Positive	9		*ADH6*
	ADH6A	*ADH6*	XM215715		375	2: 235,830,413–235,848,639	Positive	9		
	ADH6B	*ADH6*	XM227745		349	2: 235,868,290–235,885,810	Positive	9		
Mouse	*ADH1*	*ADH1*	BC13477	P00329	375	3: 137,942,695–137,952,902	Positive	9	A	
	ADH2	*ADH2*	BC100729	Q9QYY9	369	3: 138,081,089–138,092,137	Positive	9		*ADH4*
	ADH3	*ADH3*	AK146949	P28474	374	3: 138,108,251–138,117,768	Positive	9	B	
	ADH4	*ADH4*	AK9588	Q64437	374	3: 137,884,677–137,891,879	Positive	9	C	*ADH7*
Horse	*ADH1S*	*ADH1*	NM1081945	P00328	374	3: 59,314,912–59,324,682	Negative	9	S	
	ADH1E	*ADH1*	NM1082528	P00327	375	3: 59,311,838–59,327,348	Negative	9	E	
Opossum	*ADH1*	*ADH1*	*chr5.6.012		375	5: 51,904,684–51,919,994	Positive	9		
	ADH3	*ADH3*	*chr5.6.013		374	5: 52,074,904–52,084,744	Positive	9		
	ADH4A	*ADH4*	XP1369839		374	5: 51,768,316–51,783,274	Positive	9		
	ADH4B	*ADH4*	X̂P1369773		374	5: 51,644,038–51,659,611	Positive	9		
	ADH4C	*ADH4*	X̂P1369808		376	5: 51,702,617–51,735,349	Positive	9		
	ADH6	*ADH6*	N̂W1581967		375	5: 51,971,321–51,977,316	Positive	8		
	ADHΨ1	*ADH4*			61	5: 51,990,956–51,991,138	Positive	1		
	ADHΨ2	*ADH3*			147	5: 52,003,384–52,005,501	Positive	6		
Chicken	*ADH1*	*ADH1*	U73654		375	4: 61,555,669–61,565,489	Negative	9		
	ADH3	*ADH3*	AJ720203		374	4: 61,561,878–61,565,489	Negative	9		
Cod	*ADH1*	*ADH1*		P26325	375					
	ADH3L	*ADH3*		P81601	376					
	ADH3H	*ADH3*		P81600	376					

GenBank mRNA (or cDNA) IDs identify previously reported sequences (see http://www.ncbi.nlm.nih.gov/Genbank/); *N-scan IDs identify gene predictions using the N-SCAN gene structure prediction software provided by the Computational Genomics Lab at Washington University in St. Louis, MO, USA (see http://genome.ucsc.edu); UNIPROT refer to UniprotKB/Swiss-Prot IDs for individual ADHs (see http://kr.expasy.org). Sources for ADH sequences are provided by the above. Opossum ADH4A, ADH4B and ADH4C protein sequences (XP136839; XP1369773; and XP1369808) were obtained from a blast using predicted opossum ADH4 sequences (derived from a blat of the opossum genome using the rat ADH4 sequence http://genome.ucsc.edu) and web tools of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

ADH4A, ADH4B, ADH4C and ADH6 were obtained using the PSIPRED v2.5 web site tools provided by Brunel University (http://bioinf.cs.ucl.ac.uk/psipred/psiform.html) [33].

2.3. Alignment of mammalian ADH active site residues

Alignments of a key ADH active site binding region (residues 112–134 for rat ADH1) were undertaken using a ClustalW-technique [34] (http://www.ebi.ac.uk/clustalw/) and previously reported sequences for human ADH1A, ADH1B, ADH1C, ADH2, ADH3, ADH4 and ADH5; horse ADH1E and ADH1S; mouse ADH1, ADH2, ADH3 and ADH4; rat ADH1, ADH2, ADH3, ADH4, ADH5 and ADH6A; and predicted opossum ADH1, ADH3, ADH4A, ADH4B, ADH4C and ADH6 sequences (Table 1).

2.4. Phylogenetic studies and sequence divergence

Phylogenetic trees were constructed using a ClustalW-derived amino acid alignment of ADH protein sequences, obtained with default settings and corrected for multiple substitutions [34] (http://www.ebi.ac.uk/clustalw/). An alignment score was calculated for each aligned sequence by first calculating a pairwise score for every pair of sequences aligned. Alignment ambiguous regions, including the amino and carboxyl termini, were excluded prior

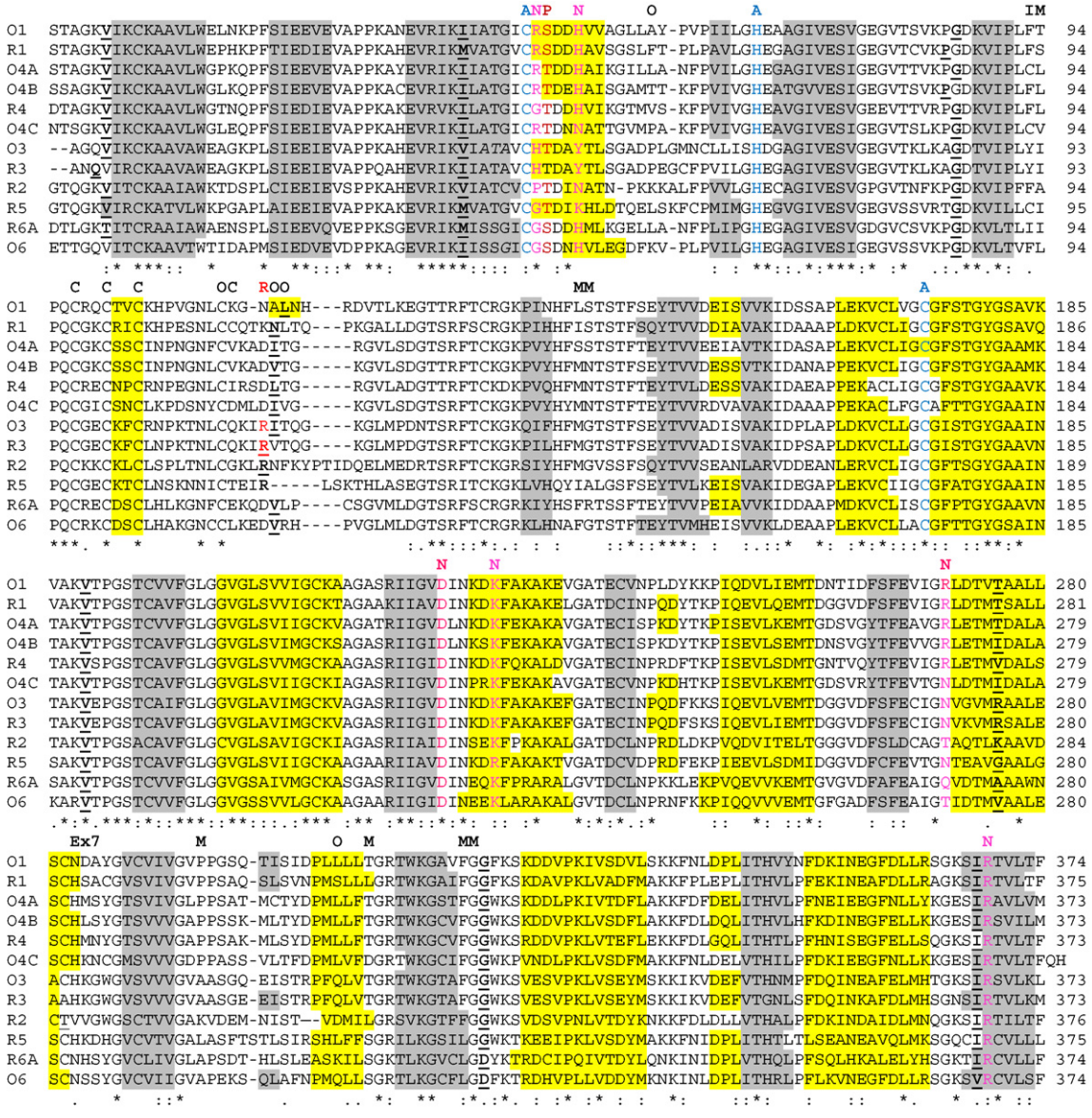

Fig. 1. Amino acid sequence alignments for rat and opossum alcohol dehydrogenases (ADHs)
See Table 1 for sources of ADH sequences; O1-opossum ADH1; R1-rat ADH1; O4A-opossum ADH4A; O4B-opossum ADH4B; O4C-opossum ADH4C; R4-rat ADH4; O3-opossum ADH3; R3-rat ADH3; R2-rat ADH2; R5-rat ADH5; R6A-rat ADH6A; O6-opossum ADH6; * shows identical residues; 2 alternate residues; 3 alternate residues; bold font shows known or predicted exon junctions; predicted exon boundaries and exon numbers are shown as **Ex1**| **Ex2**| etc; predicted β-sheet (gray shade) and α-helix (yellow shade) secondary structures are shown. Key residue identification is based on previous 3D studies of human and horse homologues and likely predicted roles for amino acid residues [24,37–44]: A-residues binding active site Zinc (blue); C-Cysteine residues binding structural Zinc; I-inner active site substrate binding residues; M-mid region active site substrate binding residues; O-outer region active site substrate binding residues; P-Ser/Thr residue involved in reaction mechanism (brown); R-Arg binding of S-hydroxymethyl glutathione by ADH3 (red); D-Asp/Glu charge clamp residue for dimer formation; N-coenzyme binding (pink). Initiation methionine is not shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 2
Percentage identities for rat, opossum, chicken and cod ADH amino acid sequences.

ADH	rat1	opo1	chi1	cod1	rat2	rat3	opo3	chi3	cod3L	cod3H	rat4	opo4A	opo4B	opo4C	rat5	rat6A	opo6
rat1	100	**74**	**71**	56	55	63	63	66	59	60	64	68	66	64	57	54	59
opo1	**74**	100	**72**	54	52	61	60	62	56	57	68	69	69	63	57	55	60
chi1	**71**	**72**	100	56	54	63	63	67	62	63	69	68	68	65	58	55	60
cod1	56	54	56	100	52	62	61	61	61	61	51	54	53	52	51	46	47
rat2	55	52	54	52	100	56	55	56	53	56	51	51	52	53	50	49	48
rat3	63	61	63	62	56	100	**90**	**87**	**77**	**81**	60	60	61	60	57	51	52
opo3	63	60	63	61	55	**90**	100	**85**	**75**	**81**	58	59	60	59	57	51	50
chi3	66	62	67	61	56	**87**	**85**	100	**76**	**81**	61	61	63	62	56	52	55
cod3L	59	56	62	61	53	**77**	**75**	**76**	100	**81**	54	55	56	56	52	48	50
cod3H	60	57	63	61	56	**81**	**81**	**81**	**81**	100	56	59	58	58	53	49	51
rat4	64	68	69	51	51	60	58	61	54	56	100	**74**	**78**	**70**	57	52	53
opo4A	68	69	68	54	51	60	59	61	55	59	**74**	100	**82**	**74**	56	56	54
opo4B	66	69	68	53	52	61	60	63	56	58	**78**	**82**	100	**74**	56	51	53
opo4C	64	63	65	52	53	60	59	62	56	58	**70**	**74**	**74**	100	57	51	53
rat5	57	57	58	51	50	57	57	56	52	53	57	56	56	57	100	52	51
rat6A	54	55	55	46	49	51	51	52	48	49	52	56	51	51	52	100	**64**
opo6	59	60	60	47	48	52	50	55	50	51	53	54	53	53	51	**64**	100

Numbers show the percentage of amino acid sequence identities: rat1-rat ADH1; opo1-opossum ADH1; chi1-chicken ADH1; cod1-cod ADH1; rat2-rat ADH2; rat3-rat ADH3; opo3-opossum ADH3; chi3-chicken ADH3; cod3L-cod ADH3L; cod3H-cod ADH3L; rat4-rat ADH4; opo4A-opossum ADH4A; opo4B-opossum ADH4B; opo4C-opossum ADH4C; rat5-rat ADH5; rat6A-rat ADH6A; opo6-opossum ADH6. Numbers in bold show higher sequence identities for ADHs from the same class.

to phylogenetic analysis yielding alignments of 298 residues of human, rat, opossum, chicken and cod ADH sequences (Table 1). Pairwise scores were calculated using the number of identities in the best alignment divided by the number of residues compared. Scores were initially calculated as percent identity scores and were converted to distances by dividing by 100 and subtracting from 1.0 to give the number of differences per site. The extent of divergence for the rat ADH1, ADH2, ADH3, ADH4, ADH5 and ADH6A subunits with the opossum ADH1, ADH3, ADH4 and ADH6 subunits were determined using the SIM-Alignment tool for Protein Sequences (http://au.expasy.org/tools/sim-prot.html) [35,36].

3. Results

3.1. Alignments of predicted opossum ADH amino acid sequences with rat ADH sequences

The deduced amino acid sequences for six predicted opossum ADH subunits (designated as ADH1, ADH3, ADH4A, ADH4B, ADH4C and ADH6) are shown in Fig. 1 together with previously reported sequences for rat ADH1, ADH2, ADH3, ADH4, ADH5 and ADH6A (see Table 1). The ADH alignments showed high levels of sequence identities for the corresponding ADH family sequences for rat and opossum ADH1-, ADH3-, ADH4- and ADH6-like sequences, respectively (Table 2). This supports a proposal that they are products of the same *ADH* gene family in each case. In contrast, the sequences for rat and opossum ADHs from different gene families showed lower levels of identity (51–69%) (Table 2). Comparisons of predicted opossum ADH sequences with rat ADH sequences also enabled identification of key residues which may contribute to catalysis and function (Figs. 1 and 2). These included active site residues (rat ADH1 numbers used) which bind to the catalytic zinc and/or substrate (Cys46; Ser48; His67; Cys175); structural zinc binding cysteines (residues 97, 100, 103 and 111); and coenzyme binding residues, Arg47, His51; Asp224; Lys228; Arg272 and Arg370 (Table 3) (see [37–43]). ADH residues aligning with active site substrate binding residues were also identified: inner pocket Ser48 was retained for opossum ADH1 and ADH6 but substituted for opossum ADH3 and ADH4 sequences with Thr48; and inner pocket Phe93 was also retained for the opossum ADH1, ADH4B and ADH6 sequences but substituted for the opossum ADH3 (Tyr92), ADH4A and ADH4C (Cys93) sequences. Other previously identified ADH1 substrate binding and coenzyme binding residues underwent several substitutions, although variation within families was lower than sequence comparisons of different ADH families (Table 3). Opossum ADH3 retained key ADH class III residues, Asp57 and Arg115, which support S-hydroxymethyl glutathione binding, whereas opossum ADH1 and ADH4 sequences lacked these residues (Fig. 1) [41]. Rat ADH2 retained Arg115 but substituted Asp57 with Lys56; rat ADH5 retained an acidic residue (Glu57) for Asp57 of rat ADH3 as well as Arg115; whereas rat ADH6A and opossum ADH6 have substituted both of these residues. Consecutive glycine residues (Gly262 and Gly263) were observed for rat ADH1, ADH2, ADH3 and ADH5 and opossum ADH3 sequences, but were absent from rat ADH4 and ADH6A and from opossum ADH1, ADH4 and ADH6 sequences.

Fig. 3 illustrates the alignment of human, horse, mouse rat and opossum ADH sequences for a region previously shown to bind substrates at the active site [37,42]. With the exception of rat ADH1, which has an extra amino acid in this region (119Gln) [45], the mammalian ADH1, ADH3 and ADH6 sequences examined showed a consistent gap of 4 residues, in comparison with the human, rat and mouse ADH2 sequences. In contrast, human and rat ADH5 and mammalian ADH4 sequences exhibited a gap of five amino acid residues in this region.

3.2. Predicted secondary structures for opossum ADHs

Predicted secondary structures for opossum ADH1, ADH3, ADH4 and ADH6 subunits and for rat ADH1, ADH2, ADH3, ADH4, ADH5 and ADH6A subunits, were compared in Fig. 1. Similar α-helix β-

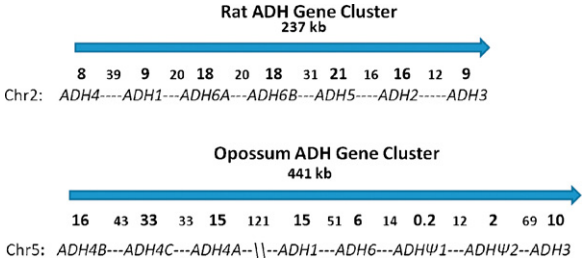

Fig. 2. Schematic representation of rat and opossum alcohol dehydrogenase (ADH) genes on chromosomes 2 and 5, respectively. Kb refers to kilobases of DNA. Size of ADH genes is presented in larger font as compared with the distance between ADH genes in smaller font.

Table 3
Predicted key alcohol dehydrogenase (ADH) amino acid residues for rat (r) ADH1, ADH3, ADH4, ADH5 and ADH6A and for opossum ADH1, ADH3, ADH4A, ADH4B, ADH4C and ADH6.

Predicted function	rADH1	oADH1	rADH3	oADH3	rADH4	oADH4A	oADH4B	oADH4C	rADH5	rADH2	rADH6A	oADH6
S binding inner region	Ser48	Ser48	Thr46	Thr46	Thr48	Thr48	Thr48	Thr48	Thr48	Thr48	Ser48	Ser14
	Phe93	Phe93	Tyr92	Tyr92	Phe93	Cys93	Phe93	Cys93	Cys93	Cys93	Ile93	Phe59
S binding middle region	Phe141	Phe140	Phe139	Phe139	Phe139	Phe139	Phe139	Tyr139	Tyr140	Phe144	Ser150	Ala106
	Ile142	Leu141	Met140	Met140	Met140	Ser140	Met140	Met140	Ile141	Met145	Phe151	Phe107
	Val295	Val294	Val293	Val293	Ala293	Leu293	Ala293	Asp293	Ala294	Ala298	Leu294	Val260
	Leu310	Leu309	Val308	Val308	Phe308	Phe308	Phe308	Phe308	Phe310	Ile311	Leu309	Leu275
	Ile319	Val318	Ala317	Ala317	Val317	Thr317	Val317	Ile317	Ile309	Phe320	Cys318	Phe284
	Phe320	Phe319	Phe318	Phe318	Phe318	Phe318	Phe318	Phe318	Leu310	Phe321	Leu319	Leu285
S binding outer region	Leu57	Leu57	Pro56	Pro56	Met57	Leu57	Met57	Met57	Leu58	Lys56	Leu57	Phe23
	Phe58	Ala58	Glu57	Leu57	Val58	Leu58	Thr58	Pro58	Ser59	Lys57	Leu58	Lys24
	Leu110	Leu110	Leu109	Leu109	Leu110	Phe110	Leu110	Tyr110	Ile111	Leu110	Phe110	Cys76
	Asn116	Leu116	Arg115	Arg115	Leu116	Ile116	Val116	Ile116	Leu117	Asn116	Val116	Val82
	Leu117	Asn117	Val116	Ile116	Thr117	Thr117	Thr117	Val117	Ser118	Phe117	Leu117	Arg83
	Ser298	Gly297	Ser296	Ser296	Ser296	Ser296	Ser296	Ala296	Ser297	Asp301	Ser297	Glu263
	Met307	Leu306	Phe305	Phe305	Phe305	Phe305	Phe305	Phe305	His307	Val308	Ser306	Met273
Coenzyme binding	Arg47	Arg47	His45	His45	Gly47	Arg47	Arg47	Arg47	Gly47	Pro47	Gly47	Gly13
	Ala231	Ala230	Ala229	Ala229	Gln229	Glu229	Glu229	Glu229	Ala230	Pro234	Pro230	Ala196
	Arg271	Arg270	Asn269	Asn269	Arg269	Arg269	Arg269	Asn269	Asn271	Thr275	Gln271	Thr237
	Arg364	Arg363	His362	His362	Tyr362	Tyr362	Ser362	Lys362	Lys364	Asn365	His363	Arg329
Charge clamp +ve*	Lys105	Lys103	Lys100	Lys100	Arg104	Lys99	Lys99	Lys105	Lys102	Lys101	Arg98	Lys65
Charge clamp −ve	Glu257	Glu256	Glu255	Glu255	Asp255	Glu255	Glu255	Glu255	Asp256	Glu260	Glu256	Glu222
Active site Zn binding	Cys46	Cys46	Cys44	Cys44	Cys46	Cys46	Cys46	Cys46	Cys46	Cys46	Cys46	Cys12
	His67	His67	His66	His66	His67	His67	His67	His67	His68	His67	His67	His33
	Cys175	Cys174	Cys174	Cys174	Cys173	Cys173	Cys173	Cys173	Cys174	Cys173	Cys174	Cys140
Structural Zn binding	Cys97	Cys97	Cys96	Cys96	Cys97	Cys97	Cys97	Cys97	Cys98	Cys97	Cys97	Cys63
	Cys100	Cys100	Cys99	Cys99	Cys100	Cys100	Cys100	Cys100	Cys101	Cys100	Cys100	Cys66
	Cys103	Cys103	Cys102	Cys102	Cys103	Cys103	Cys103	Cys103	Cys104	Cys103	Cys103	Cys69
	Cys111	Cys111	Cys110	Cys110	Cys111	Cys111	Cys111	Cys111	Cys112	Cys111	Cys111	Cys77
S-hydroxymethyl glutathione binding	Leu57	Leu57	Asp55	Asp55	Met57	Leu57	Met57	Met57	Glu57	Lys56	Leu57	Phe23
	Lys115	Asn114	Arg115	Arg115	Asp115	Asp115	Asp115	Asp115	Arg116	Arg115	Asp115	Asp81

Identification of predicted key catalytic and structural amino acid residues is based on 3D structural studies from several sources [24,37–44]; S refers to substrate; inner, middle and outer region refers to substrate binding regions of the ADH active site. See Fig. 1 for the complete amino acid sequences for rat and opossum ADHs.

sheet structures were observed for all of the rat and opossum ADH subunits examined and comparable structures were predicted in each case, which resemble the secondary structures previously reported [43]. These included key regions for the enzyme such as the α-helix located near the four cysteine residues binding the structural zinc atom and the subunit–subunit binding site at His105 (rat ADH1); the α-helix and β-sheet structures on either side of Cys46 (binding the catalytic zinc atom) and Arg47 (charge relay transfer role during catalysis); and the two α-helices on either side of active site Cys175 (also binding the catalytic zinc atom). Opossum and rat ADH3 however lacked a predicted β-sheet structure prior to the Zinc binding His67 in comparison with the other ADHs examined.

3.3. Predicted gene locations and exonic structures for opossum ADH1, ADH3 and ADH4 genes

Table 1 and Fig. 3 summarize the predicted locations for opossum ADH1, ADH3, ADH6 and three ADH4-like genes based upon BLAT interrogation of the opossum genome [29], using the reported sequences for rat ADH1 [47], ADH2 [14], ADH3 [48], ADH4 [20], ADH5 and ADH6 [12] and the UC Santa Cruz Web Browser [32]. All of the predicted opossum *ADH* genes were located on chromosome 5 in a large (441 kbs) cluster of six genes. In addition, two ADH-like pseudogenes, designated as ADHΨ1 and ADHΨ2, were also observed in this region with the predicted gene order of *ADH4B–ADH4C–ADH4A–ADH1–ADH6–ADHΨ1–ADHΨ2–ADH3*. This was compared with the predicted gene locations for rat ADH genes, which were also located on one chromosome (chromosome 2) and located within a 237 kb cluster, with the following gene order: *ADH4–ADH1–ADH6A–ADH5–ADH2–ADH3*. The opossum ADH genes were apparently transcribed on the positive DNA strand, as for the rat *ADH* genes. This is in contrast with human, horse and chicken *ADH* genes which are transcribed on the negative strand. Predicted exonic start sites for the opossum and rat *ADH* genes were examined with each gene having nine exons in each case, whereas the opossum ADHΨ1 and ADHΨ2 pseudogenes contained fewer exons (one and six exons, respectively). BLAT analyses using the rat ADH2 and ADH5 sequences for interrogation of the opossum genome did not reveal any unique opossum ADH2- and ADH5-like sequences.

3.4. Phylogeny and divergence of vertebrate ADH sequences

A phylogenetic tree (Fig. 4) was calculated by the progressive alignment of human, rat, opossum, chicken and cod ADH amino acid sequences which showed clustering into six main groups (ADH1–ADH6). The three human ADH1 sequences were grouped together on the ADH1 branch of the phylogram, as were the three opossum ADH4 sequences on the mammalian ADH4 branch, indicating that these *ADH* genes are products of recent gene duplication events of the respective ancestral *ADH1* and *ADH4* genes, respectively. Table 2 summarizes the percentages of sequence identities for the ADHs examined. Opossum ADH4 subunits shared a higher level of identity with each other (74% and 81%) and with rat ADH4 (70–78%) than with those from other ADH classes. In addi-

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ADH1A HUMAN          CLKNDVSN----PQGTLQDGTSRFTC 133
ADH1B HUMAN          CLKNDLGN----PRGTLQDGTRRFTC 133
ADH1C HUMAN          CLKNDLGN----PRGTLQDGTRRFTC 133
ADH1E HORSE          CLKNDLSM----PRGTMQDGTSRFTC 133
ADH1S HORSE          CLKN-LSM----PRGTMQDGTSRFTC 132
ADH1  MOUSE          CSRSDLLM----PRGTLREGTSRFSC 133
ADH1  RAT            CCQTKNLTQ---PKGALLDGTSRFSC 134
ADH1  OPOSSUM        CKGNALNH----RDVTLKEGTTRFTC 133

ADH4  HUMAN          CIRSDIT-----GRGVLADGTTRFTC 132
ADH4  MOUSE          CIRSDLT-----GCGVLADGTTRFTC 132
ADH4  RAT            CIRSDLT-----GRGVLADGTTRFTC 132
ADH4A OPOSSUM        CVKADIT-----GRGVLSDGTSRFTC 132
ADH4B OPOSSUM        CVKADVT-----GKGVLSDGTTRFTC 132
ADH4C OPOSSUM        CDMLDIV-----GKGVLSDGTSRFTC 132

ADH3  HUMAN          CQKIRVTQ----GKGLMPDGTSRFTC 132
ADH3  MOUSE          CQKIRVTQ----GKGLMPDGTSRFTC 132
ADH3  RAT            CQKIRVTQ----GKGLMPDGTSRFTC 132
ADH3  OPOSSUM        CQKIRITQ----GKGLMPDNTSRFTC 132

ADH2  HUMAN          CGKISNLKSPASDQQLMEDKTSRFTC 138
ADH2  MOUSE          CGKLRNFKYPTIDQELMEDRTSRFTC 127
ADH2  RAT            CGKLRNFKYPTIDQELMEDRTSRFTS 137

ADH5  HUMAN          CIQFKQSK-----TQLMSDGTSRFTC 133
ADH5  RAT            CTEIRLSK-----THLASEGTSRITC 133

ADH6A RAT            CEKQDVLP----CSGVMLDGTSRFSC 132
ADH6  OPOSSUM        CLKEDVRH----PVGLMLDGTSRFTC  98
                     *                : * *::*
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Fig. 3. Amino acid sequence alignments for a substrate binding region of human, horse, mouse, rat and opossum ADH1; human, mouse and rat ADH2; human, mouse, rat and opossum ADH3; human, mouse, rat and opossum ADH4; human and rat ADH5; and rat ADH6A and opossum ADH6.
See Table 1 for sources of ADH sequences; * shows identical residues for rat ADH1, ADH2, ADH3, ADH4, ADH5 and ADH6A, and for opossum ADH1, ADH3, ADH4A, ADH4B, ADH4C and ADH6; 2 alternate residues observed.

tion, opossum ADH1 shared a higher level of sequence identity with rat (74%) and chicken ADH1 (72%); opossum ADH3 shared a very high sequence identity with rat (90%) and chicken ADH3 (85%); and opossum ADH6 shared more sequence identity with rat ADH6A (64%) than with other ADH classes. The average amino acid sequence divergence rates for the mammalian ADH classes were also calculated using the average genetic distances observed for these ADHs and the dates for the common ancestors of eutherian and metatherian mammals and birds (Table 4). The results were indicative of a lower amino acid substitution rate for ADH3 (~0.03% per million years of evolution) as compared with ADH4 (~0.06%), ADH1 (0.06–0.11%), ADH6 (0.08–0.09%), ADH2 (0.14–0.16%) and ADH5, which showed the fastest amino acid substitution rate for mammalian ADHs of ~0.18% per million years of mammalian evolution.

4. Discussion

Several ADHs have been previously reported in the gray short-tailed opossum, *Monodelphis domestica*, with similar properties with other mammalian ADHs [30]. Three classes of opossum ADHs were described: class I or opossum liver ADH1, with preferential activity towards ethanol as substrate and showing sensitivity to pyrazole inhibition; class III ADH, being inactive towards ethanol as substrate and insensitive to pyrazole inhibition; and class IV ADH, requiring high concentrations of ethanol for activity and with a preference towards medium chain alcohol substrates [30]. The PAGE-IEF results also showed that opossum ADH4 exhibited several forms of activity which are located in various opossum tissues, including the cornea, stomach and esophagus.

The release of the opossum genome sequence [29] enabled this current study of opossum ADHs using BLAT techniques to interrogate the genome and to predict sequences for *ADH* genes and encoded ADH subunits. The results provide evidence for six *ADH* genes and encoded ADH proteins in the opossum, for which the amino acid sequences are consistent with classification into four ADH classes: class I (*ADH1*); class III (*ADH3*); class IV (3 *ADH4* genes: *ADH4A*, *ADH4B* and *ADH4C*); and class VI (*ADH6*) (Fig. 1). The respective *ADH* genes are predicted to be localized within a gene cluster

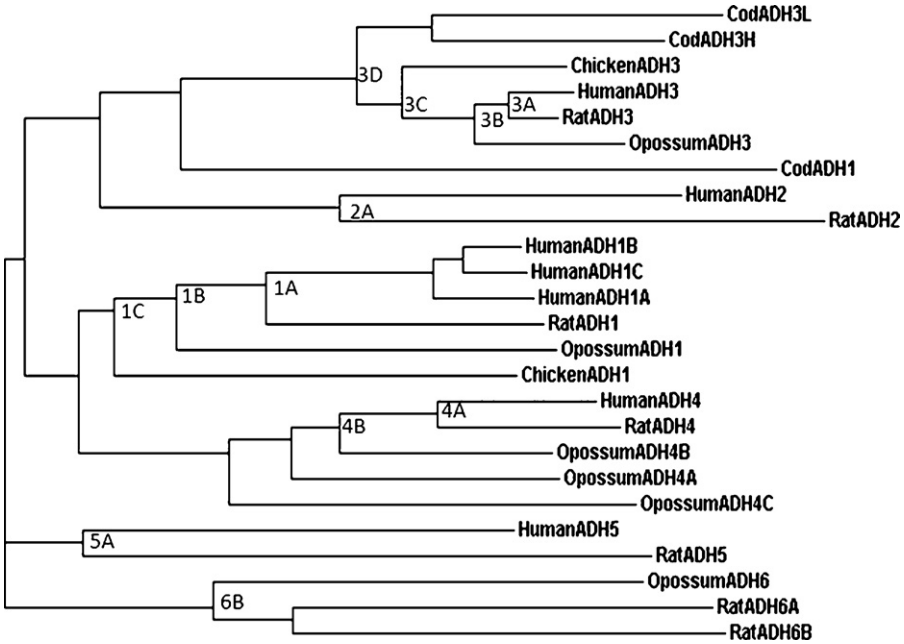

Fig. 4. Phylogenetic tree of selected vertebrate alcohol dehydrogenase (ADH) sequences. Each branch of the tree is labeled with the gene name followed by the species name. Predicted common ancestral genes are identified: 1A (human and rat ADH1); 1B (opossum, rat and human ADH1) and 1C (mammalian ADH1and chicken ADH1); 2A (human and rat ADH2); 3A (human and rat ADH3); 3B (opossum, human and rat ADH3); 3C (chicken and mammalian ADH3); 3D (cod, chicken and mammalian ADH3); 4A (human and rat ADH4); 4B (human and rat ADH4 and opossum ADH4B); 5A (human and rat ADH5); and 6B (opossum and rat ADH6). Note the clustering into six ADH groups (ADH1–6) and the likely sequence of gene duplication events: *ADH3* → *ADH1* → *ADH2* → *ADH5*/*ADH6* → *ADH4* during vertebrate ADH gene evolution.

Table 4
Genetic distance and amino acid substitution rate predictions for human, rat, opossum, chicken and cod alcohol dehydrogenases (ADHs).

ADH gene common ancestor	Common ancestor MY ago*	Genetic distance	Percentage substitution rate/MY
1A	84–99	0.09 ± 0.01	0.1
1B	173–193	0.12 ± 0.01	0.06–0.07
1C	300–320	0.18 ± 0.01	0.06
2A	84–99	0.14 ± 0.04	0.14–0.16
3A	84–99	0.03 ± 0.001	0.03
3B	173–193	0.05 ± 0.005	0.03
3C	300–320	0.07 ± 0.01	0.02
3D	500	0.1 ± 0.02	0.02
4A	84–99	0.06 ± 0.01	0.06
4B	173–193	0.09 ± 0.01	0.05
5A	84–99	0.17 ± 0.01	0.18
6B	173–193	0.15 ± 0.01	0.08–0.09

Common ancestors are identified in Fig. 4 and include 1A (human and rat ADH1); 1B (opossum, rat and human ADH1) and 1C (mammalian ADH1and chicken ADH1); 2A (human and rat ADH2); 3A (human and rat ADH3); 3B (opossum, human and rat ADH3); 3C (chicken and mammalian ADH3); 3D (cod, chicken and mammalian ADH3); 4A (human and rat ADH4); 4B (human, rat and opossum ADH4); 5A (human and rat ADH5); 6B (opossum and rat ADH6). Substitution rate is presented as a percentage of amino acid substitutions per million years. MY – million years ago. Dates for common ancestors were obtained from [48–50].

on chromosome 5 (Fig. 3), with each gene containing nine exons, in identical or similar positions to those previously reported (or predicted) for other mammalian *ADH* genes (Fig. 1). Three opossum *ADH4* genes were also described and designated as *ADH4A*, *ADH4B* and *ADH4C*, which were located in tandem with the opossum *ADH1*, *ADH3* and *ADH6* genes (Table 1; Fig. 3). In addition, two ADH-like pseudogenes were observed (ADHΨ1 and ADHΨ2), within the ADH gene cluster on chromosome 5 (Table 1; Fig. 2). BLAT analyses of the opossum genome using rat ADH2 and ADH5 sequences were unsuccessful in locating opossum *ADH2*- and *ADH5*-like genes which may be explained by gaps in the published opossum genome sequence in the corresponding regions for these genes or an absence of these ADH genes on the opossum genome.

Predicted amino acid sequences for the opossum ADHs showed high levels of identity in each case with the corresponding class of rat ADHs (Fig. 1; Table 2): ADH1 (74%), ADH3 (90%), ADH4 (74% or 82%) and ADH6 (64%), which is consistent with these *ADH* genes being classified within the same mammalian *ADH* gene family. This is supported by the phylogenetic analyses of mammalian ADH genes (Fig. 4) which showed that the opossum ADH sequences clustered with the corresponding ADH class of genes for human and rat. The phylogenetic studies also examined human, rat, opossum and chicken ADH sequences with those for cod ADH3 (ADH3L and ADH3H) and ADH1 sequences. The results supported previous studies which concluded that ADH3 represents the primordial vertebrate ADH gene [25,3], from which subsequent gene duplication events have generated several ADH gene classes during vertebrate evolution. A likely order for *ADH* gene evolution is suggested by this study as follows: *ADH3* → *ADH1* → *ADH2* → *ADH5*/*ADH6* → *ADH4*, with further gene duplication events occurring during primate evolution for *ADH1* (generating *ADH1A*, *ADH1B* and *ADH1C* genes) [4,5], and during marsupial evolution (generating the *ADH4C*, *ADH4A* and *ADH4B* genes on the opossum genome).

Given the sequence homologies with those for rat ADHs of the same ADH class, and the retention of family specific kinetic properties and the key amino acid residues described earlier, it is proposed that opossum ADHs contribute to metabolic functions previously reported for the eutherian mammalian ADH classes. For opossum ADH3, this may include metabolic roles in formaldehyde and long chain fatty alcohol metabolism [16,41]; for opossum ADH1, a major role in the clearance of aliphatic and aromatic alcohols from the body [10]; and for the opossum ADH4 enzymes, major roles in the first pass clearance of ingested aliphatic and aromatic alcohols within the digestive system [10], in the metabolism of retinoid compounds in extrahepatic tissues [17,22], and in the metabolism of lipid peroxidation products in the eye and other tissues of the body [18,46,47].

In summary, BLAT analyses of the recently published opossum genome [29] have been undertaken using the amino acid sequences reported for rat ADHs for interrogation of the genome. Evidence is reported for at least six opossum *ADH* genes which were localized on chromosome 5 in a comparable *ADH* gene cluster to that observed for human and rat *ADH* genes. In addition, the predicted amino acid sequences and secondary structures for the opossum ADH subunits showed a high degree of similarity with the corresponding classes of mammalian ADHs, and four opossum ADH classes were identified, namely ADH1, ADH3, ADH6 and three forms of ADH4 (designated as ADH4A, ADH4B and ADH4C). This is supported by a previous biochemical analysis of opossum ADHs which examined the tissue distribution and properties for these enzymes, showing that ADH1 is the major liver enzyme; ADH3 is widely distributed in opossum tissues and has similar kinetic properties to mammalian class 3 ADHs; and with several forms of ADH4 localized in extrahepatic tissues, especially in the digestive system and in the eye. Phylogenetic analyses undertaken with opossum, human, rat, chicken and cod ADHs, supported the proposed designation of opossum ADHs as class I (ADH1), class III (ADH3), class IV (ADH4A, ADH4B and ADH4C) and class VI (ADH6) and their differential functions in the metabolism of ingested and endogenous alcohols and aldehydes in tissues of the opossum. In addition, percentage substitution rates were examined for ADHs during vertebrate evolution which indicated that ADH3 is evolving at a much slower rate to that of the other ADH classes.

Conflict of interest statement

None.

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