Supplemental Methods

Genome mining

We mined publicly available reference genomes (NCBI) of 85 mammals, spanning 21 orders and feeding on a variety of diets (Fig. 1a) for *ADH7* gene sequences. We confirmed the automated NCBI gene annotations via TBlastN searches, using *ADH7* exon sequences from humans or a close relative as queries. For some exons the sequences of other *ADH* genes can be quite similar, resulting in multiple BLAST matches, however, we could exclude these hits by taking into account the genomic context and identities of the neighboring exons. Several genomes, especially for members of Carnivora, such as *Canis lupus familiaris* (domestic dog) and *Vulpes vulpes* (red fox), did not contain an annotation for *ADH7*, but we identified partial sequences through TBlastN searches. For some species all attempts to identify *ADH7* or partial sequences failed and the status of the gene as either functional or pseudogenised is, therefore, unknown. This was the case for *Choloepus hoffmanni* (Hoffmann's two-toed sloth), *Trichechus manatus* (West Indian manatee), *Oryctolagus cuniculus* (European rabbit), *Procavia capensis* (rock hyrax), platypus (*Ornithorhynchus anatinus*), and *Manis pentadactyla* (Chinese pangolin). Data for *Elephas maximus* and *Mammuthus primigenius* were obtained from published mapping data of reads mapped to the *Loxodonta africana* assembly [1].

All exon sequences were concatenated into coding sequences, translated and aligned with Geneious 9.1.8, and manually inspected for the presence of premature stop codons. We classified a sequence as a pseudogene if we observed at least one mutation that introduced a premature stop codon within the coding sequence of *AHD7*. Mutations that introduce stop codons can be in the form of point mutations that alter the codon to code for a stop codon (nonsense mutations) or in the form of frame-shift mutations, where an insertion or deletion alters the reading frame and introduces downstream stop codons. We acknowledge that the lack of an internal stop codon does not necessarily prove that a gene is not a pseudogene [2], so we classify genes lacking an internal stop codon as putatively functional for the purposes of this study.

Timing of gene loss

To estimate the timing of the loss of *ADH7* in branches with premature stop codons, we followed the method described in Meredith et al. [3]. This method uses ratios of non-synonymous to synonymous substitutions (dN/dS, omega) acting on pseudogenised, functional, and transitional (“mixed”) branches (Fig. S5), along with branch lengths to estimate when the shift from purifying to relaxed selection (signaling pseudogenisation) occurred. We calculated average dN/dS ratios with PAML using three-ratio models in codeml for two different codon substitution models (F1X4 and F3X4). We had to exclude some species from this analysis, because only partial sequences were available for *ADH7*: *Canis lupus*, *Orcinus orca*, *Tursiops truncatus*, *Orycteropus afer*, *Vulpes vulpes*. For species which had multiple *ADH7* sequences (marsupials), we included only one sequences, choosing the one with the highest average percentage of identical bases to the other mammals. We used a non-mammalian *ADH7* sequence as an outgroup (*Xenopus tropicalis*). The alignment used for PAML analyses was trimmed to exclude regions with a large number of gaps and manually adjusted to ensure that alignments remained in frame. For pseudogenised sequences, this required removing frameshift-causing indels and removing stop codons caused by point mutations. PAML removes sites in the alignment that contain gaps, so to avoid losing too much of our aligned sequences and retain enough sites to be informative, we filled gaps within exons with sequences from the sister taxon for some sequences. Control and input files for codeml analyses can be found at https://github.com/MareikeJaniak/Mammal_ADH_IV. Branch lengths were obtained from TimeTree [4]. The amount of time that a branch has been pseudogenic (T_p) is calculated by subtracting the amount of time that the branch is inferred to have been under purifying selection (T_f), which is determined by the following formula:

$$Tf = T - (\frac{T \times (\omega m - \omega p)}{(\omega f - \omega p)})$$

Where T is the length of the mixed branch, ω_m is the dN/dS ratio of the mixed branch, ω_p is the dN/dS ratio of pseudogenised branches, and ω_f is the dN/dS ratio of functional branches. Because the two different substitution models used to calculate dN/dS ratios resulted in slightly different estimates of T_p, we used the mean of the two numbers to determine gene inactivation dates. If pseudogenisation occurred on a terminal branch (*Equus*, *Ceratotherium*, *Cavia*, *Castor*, *Octodon*, *Dasypus*) T_p is equal to the inactivation date in millions of years ago. If gene loss occurred on an internal branch (Bovidae, Elephantidae, Cetacea, Carnivora), the length of branches leading to the tip was added to arrive at the inactivation date (Table S2).

Phylogenetic Logistic Regression

To test the hypotheses that *ADH7* was (a) more likely to be to be retained in frugivorous/nectarivorous lineages or (b) more likely to be lost in herbivorous lineages, we performed phylogenetic logistic regression analyses using two different methods in the R package phyolm [5], following [6]. Briefly, we implemented the logistic_IG10 and logistic_MPLE methods in the function “phyloglm” with (a) percent of fruit and/or nectar in the diet ([7]; Table S1) as the predictor variable or (b) percent of vegetative (non-fruit) plant material in the diet ([7]; Table S1) as the predictor variable and loss (1) or retention (0) of *ADH7* as the dependent trait. Input files and code for running these analyses in R can be found at https://github.com/MareikeJaniak/Mammal_ADH_IV.

RELAX

To test for changes in selective pressure acting on *ADH7* in lineages with frugivorous or nectarivorous diets versus those without, we used the method RELAX from the HyPhy package [8]. We tested the hypotheses that (1) selection is relaxed in species that do not consume fruit and/or nectar as part of their diet and (2) selection is intensified in species in which fruit and/or nectar make up the majority (>50%) of the diet. Given a codon-based alignment and phylogenetic tree, RELAX detects whether a specified subset of branches (“test branches”) is under relaxed or intensified selection compared to a specified subset of “reference branches.” For Model 1 (“Is selection on *ADH7* relaxed in species without fruit or nectar in their diet?”) we classified species with 0% fruit or nectar in their diet [7] as “test branches” and other species as “reference branches.” Internal branches were left unclassified, unless they led to two branches that were both categorized as “test” or “reference.” For Model 2 (“Is selection on *ADH7* intensified in species whose diets contain a majority of fruit and/or nectar?”) we classified species with >50% fruit or nectar in their diet as “test branches” and other species as “reference branches.” Internal branches were left unclassified unless they led to two branches that were both categorized as “test” or “reference.” The input file used for RELAX analyses can be found at https://github.com/MareikeJaniak/Mammal_ADH_IV.

Supplemental Tables & Figures

Table S1. Dietary information for species included in this study.

Species	Diet	%fruit[1]	%nectar[1]	%vegetative material[1]	Notes	Reference(s)
Canis lupus	carnivore	0	0	0		[9]
Equus caballus	herbivore	0	0	100		[10]
Delphinapterus leucas	carnivore	0	0	0		[11]
Bos taurus	herbivore	0	0	100		[12]
Capra hircus	herbivore	0	0	100		[13–15]
Ovis aries	herbivore	0	0	100		[15]
Pteropus vampyrus	frugivore	100	0	0	Fruit, some leaves and flowers	[16]
Rousettus aegyptiacus	frugivore	60	40	0	Fruit, some leaves and pollen	[17]
Desmodus rotundus	sanguivore	0	0	0		[18]
Eptesicus fuscus	insectivore	0	0	0		[19,20]
Myotis brandtii	insectivore	0	0	0		[21]
Myotis lucifugus	insectivore	0	0	0		[22]
Myotis davidii	insectivore	0	0	0		[23]
Miniopterus natalensis	insectivore	0	0	0		[24]
Rattus norvegicus	omnivore	20	0	10	Diet highly variable depending on habitat	[25,26]
Mus musculus	omnivore	0	0	60	Invertebrates, seeds	[27–29]
Cricetulus griseus	omnivore	0	0	50	Seeds, herbs, insects	[30]
Marmota marmota	omnivore	30	0	40		[31]
Galago moholi	gummivore-insectivore	0	60 (gum)	0		[32,33]
Daubentonia madagascariensis	insectivore-nectarivore	0	20	0		[34]
Microcebus murinus	frugivore-insectivore	30	10	20		[35,36]
Eulemur albifrons	omnivore	40	0	40		[37]
Saguinus oedipus	gummivore-insectivore	30	30 (gum)	0		[38]
Callithrix jacchus	gummivore-insectivore	30	40	0		[39]
Saimiri sciureus	insectivore-frugivore	20	0	20		[40,41]
Sapajus apella	omnivore	20	10	20		[42,43]
Papio anubis	omnivore	20	0	30		[44]
Chlorocebus aethiops	omnivore	40	0	20		[45]
Hylobates moloch	frugivore	70	0	20		[46]
Pongo abelii	frugivore	80	0	0		[47,48]
Gorilla gorilla	herbivore-frugivore	10	0	90		[49]
Pan troglodytes	frugivore	60	0	30		[50]

Homo sapiens	omnivore	60	0	30	Diet varies with population, location	
Tupaia chinensis	insectivore-frugivore	0	0 (*10)	10	Evidence of frugivory	[51–53]
Loxodonta africana	herbivore	0	0	100		[54,55]
Elephas maximus	herbivore	0	0	100		[56,57]
Mammuthus primigenius	herbivore	0	0	100		[58,59]
Elephantulus edwardii	insectivore-nectarivore	0	0 (*10)	0	Recently observed to feed on nectar	[60,61]
Balaenoptera acutorostrata	carnivore	0	0	0		[62]
Bos mutus	herbivore	0	0	100		[63]
Carlito syrichta	insectivore	0	0	0		[64]
Castor canadensis	herbivore	0	0	100		[65]
Cavia porcellus	herbivore	0	0	100		[66,67]
Ceratotherium simum	herbivore	0	0	100		[68]
Chinchilla lanigera	herbivore	0	0	100		[69]
Choloepus hoffmanni	folivore-frugivore	30	0	70		[70,71]
Dasypus novemcinctus	insectivore	0	0	0		[72,73]
Dipodomys ordii	omnivore	20	0	30	Seeds, insects, grasses	[74,75]
Eonycteris spelaea	nectarivore	0	100	0		[76,77]
Erinaceus europaeus	insectivore	0	0	0		[78]
Galeopterus variegatus	folivore	0	0	100	Occasional fruit and insects	[79]
Ictidomys tridecemlineatus	omnivore	0	0	40	Insects, seeds, grass	[80]
Manis pentadactyla	insectivore	0	0	0		[81]
Marmota flaviventris	herbivore-omnivore	30	0	40	Grasses, forbs, seeds	[82,83]
Mastomys coucha	omnivore	0	0	30		[84]
Meriones unguiculatus	granivore	10	0	50	Seeds	[85]
Microtus ochrogaster	herbivore	0	0	80		[86]
Monodon monoceros	carnivore	0	0	0		[87,88]
Mus caroli	omnivore	0	0	60	Invertebrates, seeds	[89]
Mus pahari	omnivore	0	0	60	Data from wild not available	
Neophocaena phocaenoides	carnivore	0	0	0		[90]
Octodon degus	herbivore	20	0	60		[91]
Odobenus rosmarus	carnivore	0	0	0		[92]
Orcinus orca	carnivore	0	0	0		[93,94]
Orycteropus afer	insectivore	0	0	0		[95]
Oryctolagus cuniculus	herbivore	0	0	100		[96]
Peromyscus leucopus	omnivore	20	0	0	Invertebrates, fruit, seeds	[97]
Peromyscus maniculatus	omnivore	20	0	0	Invertebrates, fruit, seeds	[97]
Phascolarctos	folivore	0	0	100	Specialized on eucalyptus	[98]

cinereus						
Phyllostomus discolor	omnivore	40	30	0		[99]
Physeter catodon	carnivore	0	0	0		[100]
Piliocolobus tephrosceles	folivore	20	0	80		[101,102]
Ornithorhynchus anatinus	carnivore	0	0	0		[103]
Procavia capensis	herbivore	0	0	100		[104]
Rhinolophus sinicus	insectivore	0	0	10		[105]
Rhinopithecus roxellana	folivore	10	0	80		[106,107]
Sarcophilus harrisii	carnivore	0	0	0		[108,109]
Sorex araneus	insectivore	0	0	0		[110,111]
Sus scrofa	omnivore	0 (*10)	0	60	Diet variable, includes fruit	[112]
Trichechus manatus	herbivore	0	0	100		[113]
Tursiops truncatus	carnivore	0	0	0		[114]
Urocitellus parryii	herbivore	0	0	40		[115]
Vulpes vulpes	carnivore	10	0	0	Occasional fruit	[116]
Zalophus californianus	carnivore	0	0	0		[117]

Notes for diet table:

1 From EltonTraits 1.0 [7]. *For some species, frugivory and/or nectarivory has been reported in the literature, but was listed as 0% in EltonTraits. For these species we used a conservative estimate of 10% fruit/nectar in the linear model.

Table S2. Calculations for dating pseudogenization events

Clade (branch on which gene was inactivated)	substitution model	ω mixed	ω functional	ω pseudogene	Mixed branch length (Ma)	Time pseudogenic (Ma)	Mean time pseudogenic (Ma)	Inactivation date (MYA)
Bovidae	F1X4	0.56399	0.12664	0.99106	31.3598531	15.86639799	15.86441248	40.46441248
	F3X4	0.59723	0.13005	1.05366		15.86242697		
Elephantidae	F1X4	0.56399	0.12664	0.99106	57.40394286	29.04330581	29.03967135	54.92467135
	F3X4	0.59723	0.13005	1.05366		29.03603688		
Cetacea	F1X4	0.56399	0.12664	0.99106	22.4598531	11.36347696	11.36205494	44.86205494
	F3X4	0.59723	0.13005	1.05366		11.36063292		
Equus	F1X4	0.56399	0.12664	0.99106	54.40072903	27.52384124	27.52039692	27.52039692
	F3X4	0.59723	0.13005	1.05366		27.5169526		
Castor	F1X4	0.56399	0.12664	0.99106	63.254405	32.0033248	31.99931992	31.99931992
	F3X4	0.59723	0.13005	1.05366		31.99531504		
Octodon	F1X4	0.56399	0.12664	0.99106	32.85536467	16.62304636	16.62096616	16.62096616
	F3X4	0.59723	0.13005	1.05366		16.61888597		
Cavia	F1X4	0.56399	0.12664	0.99106	35.7789995	18.10224825	18.09998294	18.09998294
	F3X4	0.59723	0.13005	1.05366		18.09771764		
Ceratotherium	F1X4	0.56399	0.12664	0.99106	54.40072903	27.52384124	27.52039692	27.52039692
	F3X4	0.59723	0.13005	1.05366		27.5169526		
Dasypus	F1X4	0.56399	0.12664	0.99106	100.5063633	50.85081095	50.84444751	50.84444751
	F3X4	0.59723	0.13005	1.05366		50.83808406		
Carnivora	F1X4	0.56399	0.12664	0.99106	57.75697092	29.22191901	29.2182622	48.68788528
	F3X4	0.59723	0.13005	1.05366		29.21460538		

Notes for table: Omega values (dN/dS ratios) were calculated with codeml in PAML and calculations were based on [3]. Branches on which pseudogenising mutations occurred were classified as "mixed," branches that post-date pseudogenisation as "pseudogene," and branches without premature stop codons as "functional." Some species were excluded from the alignment because only a small part of the sequence aligned cleanly to the other sequences. The amount of time that a branch has been pseudogenic (T_p) is calculated by subtracting the amount of time that the branch is inferred to have been under purifying selection (T_f), which is determined by the following formula:

$$Tf = T - \left(\frac{T \times (\omega m - \omega p)}{(\omega f - \omega p)}\right)$$

Where T is the length of the mixed branch, ω_m is the dN/dS ratio of the mixed branch, ω_p is the dN/dS ratio of pseudogenised branches, and ω_f is the dN/dS ratio of functional branches. Because the two different substitution models used to calculate dN/dS ratios resulted in slightly different estimates of T_p, we used the mean of the two numbers to determine gene inactivation dates. If pseudogenisation occurred on a terminal branch (*Equus*, *Delphinapterus*) T_p is equal to the inactivation date in millions of years ago. If gene loss occurred on an internal branch (Bovidae, Elephantidae), the length of branches leading to the tip was added to arrive at the inactivation date. All branch lengths were from TimeTree [4].

Table S3. Results of phylogenetic logistic regression analyses.

Hypothesis	**Method** (phyloglm function in R)	**α** (strength of phylogenetic signal)	**Intercept**	**Slope**	***p***
ADH7 loss predicted by herbivory	logistic_MPLE	0.006305245	-1.35102583	0.00030788	0.9603
	logistic_IG10	0.003992322	-1.3510919	0.0141951	0.04379
ADH7 retention predicted by frugivory/ nectarivory	logistic_MPLE	0.006327771	-0.07124251	-0.00058409	0.9329
	logistic_IG10	0.002991951	-0.8292794	0.000000	1.000000

Table S4. Results of the RELAX analyses of relaxed/intensified selection.

Hypothesis	**Model**	logL	# of parameters	AIC_c	Branch Set	ω1	ω2	ω3
							(percent of sites)	
No fruit/nectar -> relaxed selection	General descriptive	-13114.3	305	26849.6	Shared	0.00 (70.56%)	0.81 (29.44%)	1.00 (0.00%)
	Alternative	-13371.5	168	27082.2	Reference	0.00 (81.83%)	0.95 (18.14%)	9999843742.81 (0.02%)
					Test	0.12 (81.83%)	0.99 (18.14%)	381.31 (0.02%)
	Null	-13393.0	167	27123.3	Reference	0.01 (75.98%)	1.00 (24.00%)	1.00 (24.00%)
					Test	0.01 (75.98%)	1.00 (24.00%)	219.85 (0.02%)
	Partitioned descriptive	-13361.8	172	27071.0	Reference	0.00 (85.03%)	1.00 (13.77%)	2.86 (1.20%)
					Test	0.00 (67.78%)	1.00 (32.17%)	382.69 (0.05%)
High fruit/nectar -> intensified selection	General descriptive	-13116.0	305	26852.8	Shared	0.00 (64.67%)	0.19 (30.25%)	1.08 (5.07%)
	Alternative	-13374.4	168	27088.1	Reference	0.07 (53.89%)	0.17 (34.70%)	1.44 (11.41%)
					Test	0.00 (53.89%)	0.00 (34.70%)	3.20 (11.41%)
	Null	-13377.0	167	27091.2	Reference	0.00 (21.69%)	0.21 (73.74%)	2.77 (4.57%)
					Test	0.00 (21.69%)	0.21 (73.74%)	2.77 (4.57%)
	Partitioned descriptive	-13373.6	172	27094.6	Reference	0.00 (32.07%)	0.25 (63.41%)	2.46 (4.52%)
					Test	0.00 (58.59%)	0.00 (29.15%)	2.96 (12.27%)

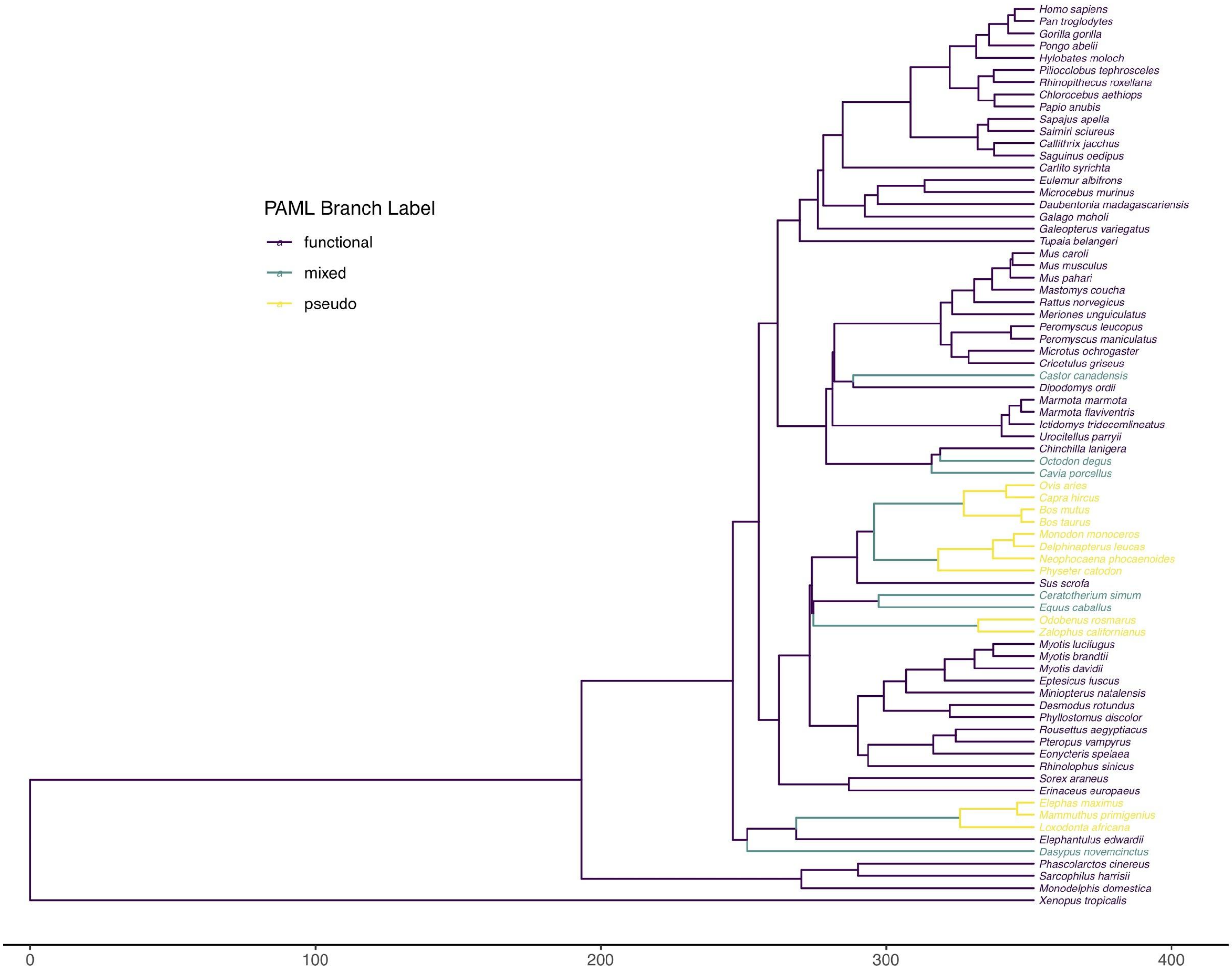

Figure S5. Phylogeny and branch classifications used in PAML analyses for timing of pseudogenisations.

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