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Comparative analysis of palmitoylation sites of serotonin (5-HT) receptors in vertebrates

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Abstract

Background: In the vertebrate central nervous system as well as in the periphery, serotonin, also known as 5-hydroxytriptamine (5-HT), function as a neurotransmitter, a hormone or a mitogen. 5-HT receptors are composed of 7 family 5-HT$_{1\text{-}7}$ receptors, comprising of 14 structurally and pharmacologically distinct 5-HT receptor subtypes. Previous experimental studies showed that mouse 5-HT$_{1A}$, 5-HT$_4$ and 5-HT$_7$ receptors are regulated by post-translational protein palmitoylation, the reversible attachment of the lipid palmitate to intracellular cysteine residues. Here, we further focused on conservation of these putative palmitoylation sites found in vertebrate 5-HT receptor orthologs.

Methods and Results: Analysis of sequence databases provides evidence to suggest that palmitoylation sites of these 5-HT receptors have been extremely conserved in the vertebrate lineages from jawless fishes to human, in spite of the divergence of 5-HT$_{1A}$, 5-HT$_4$ or 5-HT$_7$ receptors full-length amino acid sequences during molecular evolution.

Conclusion: Our findings mean that dynamic regulation of 5-HT receptors made possible by reversible post-translational protein palmitoylation may be critical for refined functions of the vertebrate serotonergic systems.

KEYWORDS
5-hydroxytryptamine, orthologs, post-translational protein palmitoylation, receptor, serotonin, vertebrate

1 | INTRODUCTION

Serotonin, also chemically known as 5-hydroxytryptamine (5-HT), is an important monoamine neurotransmitter and a local hormone, which diversely acts in the vertebrate central nervous system as well as in the various peripheral organs.[1] Serotonergic dysfunction induces multiple psychiatric disorders, including mood disorders, such as major depressive disorder and bipolar disorder.[2–5] Evolutionally, serotonin is widely used in invertebrates, plants, and even in unicellular organism.[1,6] In mammals, serotonin effects are mediated via 7 family 5-HT$_{1\text{-}7}$ receptors, comprising of 14 structurally and pharmacologically distinct subtypes: 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1E}$, 5-HT$_{1F}$, 5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_{2C}$, 5-HT$_3$, 5-HT$_4$, 5-HT$_{5A}$, 5-HT$_{5B}$, 5-HT$_6$, and 5-HT$_7$.[7] These 5-HT receptors are classified into G protein-coupled receptors (GPCRs) with the exception of the 5-HT$_3$ receptor, which is a ligand-gated sodium/potassium cation channel.[7,8] All GPCR-type 5-HT receptor isoforms have evolutionarily conserved 7 transmembrane regions with the extracellular N-terminal domain and the C-terminal cytoplasmic domain.

One key modification of mammalian 5-HT receptors is the reversible attachment of the lipid palmitate to intracellular cysteine residues. Previous biochemical studies showed that 4 5-HT receptor subtypes, mouse 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_4$, and 5-HT$_7$ receptors are palmitoylated within their C-terminal intracellular region like many other GPCRs.[9,10]

This process, post-translational protein palmitoylation, acts as a sticky "tag" that can direct channels and receptors to specific regions of the plasma membrane, or to specific intracellular membranes or vesicles.[11–14] Genetic evidence strongly links impaired palmitoylation to abnormal mammalian brain development and/or function, including human neuropsychiatric disorders.[15–20] Biochemical experiments have already determined the palmitoylation sites of mouse 5-HT$_{1A}$ receptor,[21] 5-HT$_4$ receptor,[22,23] and 5-HT$_7$ receptor.[24]

In this report, we further focused on conservation of 5-HT$_{1A}$, 5-HT$_4$, and 5-HT$_7$ receptor palmitoylation sites found in their vertebrate orthologs. Analysis of sequence databases provides evidence for the acquisition and conservation of palmitoylation mechanism in these 5-HT receptor regulations during vertebrate evolution.

2 | METHODS

For analysis of the 5-HT receptor orthologs, currently available protein sequences, cDNA sequences, expressed sequence tags (ESTs), and genomic sequences were obtained by searching the National Center for Biotechnology Information (NCBI) databases, GenBank, EST banks, elephant shark genome project (http://esharkgenome.imcb.a-star.edu.sg/), Joint Genome Institute (http://genome.jgi-psf.org), and the Ensembl database (http://www.ensembl.org/) by sequence homologies. Amino acid sequence alignments among 5-HT receptor orthologs were performed using a Basic Local Alignment Search Tool (BLASTP) search of the NCBI (https://blast.ncbi.nlm.nih.gov/ with BLOSUM62). For the evaluation of sequence homology between humans and animal species in each class, full-length data of 5-HT$_{1A}$, 5-HT$_{4(a)}$, and 5-HT$_{7(a)}$ receptor orthologs were used for calculation (Table 1). Both splice variants, 5-HT$_{4(a)}$ and 5-HT$_{7(a)}$ receptors, uniquely possess variant-specific palmitoylation sites in addition to the common palmitoylation sites of 5-HT$_4$ and 5-HT$_7$ receptor variants, respectively (for detail, see Figure 3A,B). To analyze the conservation and exchange of all palmitoylation sites, we select 5-HT$_{4(a)}$ and 5-HT$_{7(a)}$ receptors, rather than other 5-HT$_4$ and 5-HT$_7$ variants, for the homology comparison. Average of all identity percentages across 5-HT receptor orthologs is shown in the text.

A full phylogenetic tree of 5-HT$_{1A}$ orthologs can be found at: http://www.ensembl.org/Homo_sapiens/Gene/Compara_Tree?db=core;g=ENSG00000178394;r=5:63960356-63962507.

A full phylogenetic tree of 5-HT$_4$ orthologs can be found at: http://www.ensembl.org/Homo_sapiens/Gene/Compara_Tree?db=core;g=ENSG00000164270;r=5:148451032-148677235.

A full phylogenetic tree of 5-HT$_7$ orthologs can be found at: http://www.ensembl.org/Homo_sapiens/Gene/Compara_Tree?db=core;g=ENSG00000148680;r=10:90740823-90857698.

3 | RESULTS

Recent expansive progress in genome analyses revealed that many vertebrate species possess 5-HT$_{1A}$, 5-HT$_4$, and 5-HT$_7$ receptor orthologs (Figure 1). Generally speaking, structurally or functionally important amino acid residues are conserved during molecular evolution against mutation pressure. The homology comparison of full-length amino acid sequence of 5-HT$_{1A}$ receptor orthologs showed ~93% identity among mammalian species, ~80% identity between humans and birds, ~76% identity between humans and reptiles, ~74% identity between humans and amphibians, ~71% identity between humans and ray-finned fishes (the class Actinopterygii) (Figure 2A). The homology comparison of full-length amino acid sequence of 5-HT$_{4(a)}$ (or 5-HT$_{4\ \mathrm{isoform\ X2}}$) receptor orthologs showed ~96% identity among mammalian species, ~87% identity between humans and birds, ~88% identity between humans and reptiles, ~85% identity between humans and amphibians, ~76% identity between humans and ray-finned fishes (Figure 2B). The homology comparison of full-length amino acid sequence of 5-HT$_{7(a)}$ receptor orthologs showed ~95% identity among mammalian species, ~83% identity between humans and birds, ~86% identity between humans and reptiles, ~77% identity between humans and amphibians. 5-HT$_{7(a)}$-type splice variants have not been identified for fishes (Figure 2C). Random mutations are observed all over vertebrate 5-HT receptor sequences during vertebrate evolution. Sequence alignment among 5-HT$_{1A}$, 5-HT$_4$, or 5-HT$_7$ receptor orthologs revealed that cysteine residues at the putative palmitoylation sites are extremely conserved across vertebrates. (All currently available sequence data are shown in Table 1.) Details for each 5-HT receptor subtype are described below. While there is no strict consensus rule in amino acid sequence around known palmitoylated cysteines, hydrophobic residues (Ile, Leu, Val, and Phe) and positively charged basic residues (Arg and Lys) often locate around the palmitoylation sites, which might contribute to membrane binding.[11,25] Actually, hydrophobic residues exist around the putative palmitoylation sites in vertebrate 5-HT$_{1A}$, 5-HT$_4$, and 5-HT$_7$ receptor orthologs (Table 1). Arginine and lysine residues are notably detected around the putative palmitoylation sites in most vertebrate 5-HT$_{1A}$ and 5-HT$_7$ receptor orthologs. Concerning 5-HT$_4$ receptors, arginine, lysine, or histidine residues locate around the putative palmitoylation sites in almost all cartilaginous and ray-finned fishes, but not in coelacanth and tetrapods.

3.1 | 5-HT$_{1A}$ receptor palmitoylation sites in various vertebrate species

A previous work demonstrated that mouse 5-HT$_{1A}$ receptor is doubly palmitoylated at its C-terminal cysteine residues Cys417 and Cys420.[21] Stable palmitoylation is required for the receptor coupling to the G$_i$ protein that regulates the inhibition of downstream cAMP formation. Sequence comparison of total 216 vertebrate species from jawless fish (sea lamprey) to humans showed that the first palmitoylation site corresponding to mouse/human 5-HT$_{1A}$ receptor Cys417 is completely conserved in all vertebrate species without exception (Table 1). Concerning another palmitoylation site Cys420, 5-HT$_{1A}$ receptor orthologs of all tetrapods, non-tetrapod lobe-finned fish (the class Sarcopterygii), and cartilaginous fish, namely mammals, birds, reptiles, amphibians, coelacanth, and whale shark possess a

TABLE 1 The BLAST alignments of palmitoylation sites in vertebrate 5-HT_{1A}, 5-HT_4, and 5-HT_7 receptor orthologs

	Species (common name)	Species (Latin name)	identity (%)	5-HT1A C417C420	identity (%)	5-HT4 C328C329	5-HT4(a) C386	identity (%)	5-HT7 C401	5-HT7(a) A435C438	Superorder	Subclass
Phylum: Chordata	Subphylum: Vertebrata											
Superclass: Gnathostomata	Class: Mammalia											
Order: Primates	Human	*Homo sapiens*	100	IIKCKFCRQ	100	IILCCDDE	LESCF	100	LLQCQYR	QNADYCRKK	Magorder Boreoeutheria/Euarchontoglires	Eutheria
	Bonobo	*Pan paniscus*	99	IIKCKFCRQ	99	IILCCDDE	LESCF	100	LLQCQYR	QNADYCRKK		
	Chimpanzee	*Pan troglodytes*	99	IIKCKFCRQ	99	IILCCDDE	LESCF	100	LLQCQYR	QNADYCRKK		
	Western lowland gorilla	*Gorilla gorilla gorilla*	99	IIKCKFCRQ		IILCCDDE	N.D.	100	LLQCQYR	QNADYCRKK		
	Sumatran orangutan	*Pongo abelii*	99	IIKCKFCRQ		IILCCDDE	N.D.	99	LLQCQYR	QNADYCRKK		
	White-cheeked crested gibbon	*Nomascus leucogenys*	99	IIKCKFCRQ	99	IILCCDDE	LESCF		LLQCQYR	N.D.		
	Rhesus monkey	*Macaca mulatta*	98	IIKCKFCRQ	98	IILCCDDE	LESCF	99	LLQCQYR	QNADYCRKK		
	Crab-eating macaque	*Macaca fascicularis*	98	IIKCKFCRQ	98	IILCCDDE	LESCF	99	LLQCQYR	QNADYCRKK		
	Pig-tailed macaque	*Macaca nemestrina*	98	IIKCKFCRQ	98	IILCCDDE	LESCF	99	LLQCQYR	QNADYCRKK		
	Japanese macaque	*Macaca fuscata*	98	IIKCKFCRQ	98	IILCCDDE	LESCF		N.D.	N.D.		
	Celebes crested macaque	*Macaca nigra*	98	IIKCKFCRQ		N.D.	N.D.		N.D.	N.D.		
	Golden snub-nosed monkey	*Rhinopithecus roxellana*	98	IIKCKFCRQ	98	IILCCDDE	LESCF	99	LLQCQYR	QNADYCRKK		
	Black snub-nosed monkey	*Rhinopithecus bieti*	98	IIKCKFCRQ		IILCCDDE	N.D.	98	LLQCQYR	QNADYCRKK		
	Angola colobus	*Colobus angolensis palliatus*	98	IIKCKFCRQ		IILCCDDE	N.D.		LLQCQYR	N.D.		
	Green monkey	*Chlorocebus sabaeus*	98	IIKCKFCRQ	98	IILCCDDE	LESCF	99	LLQCQYR	QNADYCRKK		
	African green monkey	*Chlorocebus aethiops*	98	IIKCKFCRQ		N.D.	N.D.		N.D.	N.D.		
	Drill	*Mandrillus leucophaeus*	98	IIKCKFCRQ	98	IILCCDDE	LESCF		LLQCQYR	N.D.		
	Sooty mangabey	*Cercocebus atys*	98	IIKCKFCRQ		IILCCDDE	N.D.	99	LLQCQYR	QNADYCRKK		
	Olive baboon	*Papio anubis*	98	IIKCKFCRQ		IILCCDDE	N.D.	98	LLQCQYR	QNADYCRKK		
	Ma's night monkey	*Aotus nancymaae*	97	IIKCKFCRQ	98	IILCCDGE	LESCF	99	LLQCQYR	QTTDYCRKK		
	Common marmoset	*Callithrix jacchus*	97	IFKCKFCRQ	98	IILCCDGE	LESCF	99	LLQCQYR	QTTDYCRKK		
	White-headed capuchin	*Cebus capucinus imitator*	96	IIKCKFCRQ	98	IILCCDDE	LESCF	98	LLQCQYR	QTTDYCRKK		
	Bolivian squirrel monkey	*Saimiri boliviensis boliviensis*	96	IIKCKFCRQ	98	IILCCDGE	LESCF	98	LLQCQYR	QTTDYCRKK		
	Philippine tarsier	*Carlito syrichta*	96	IIKCKFCRQ	97	IILCCDDE	LESCF	97	LLQCQYR	QNSDYCRKK		
	Coquerel's sifaka	*Propithecus coquereli*	93	IIKCKFCRR	97	IILCCDDE	LESRF	97	LLQCQYR	QNSDYCRKK		
	Northern greater galago	*Otolemur garnettii*	94	IIKCKFCRQ	96	IILCCDDE	LESCF	97	LLQCQYR	QNSDYCRKK		
Order: Dermoptera	Sunda flying lemur	*Galeopterus variegatus*	94	IIKCKFCRQ		IILCCDDE	N.D.	95	LLQCQYR	QNSDYCRKK		
	Gray mouse lemur	*Microcebus murinus*	93	IIKCKFCRR	97	IILCCDDE	LESCF	97	LLQCQYR	QNSDYCRKK		
Order: Scandentia	Chinese tree shrew	*Tupaia chinensis*	93	IIKCKFCRR	98	IILCCDDE	LESCF	98	LLQCQYR	QNSDYCRKK		
Order: Rodentia	Degu	*Octodon degus*	91	IIKCKFCRR	94	IILCCDDE	LESCF	94	LLQCQYR	QNSDYCRKK		
	Long-tailed chinchilla	*Chinchilla lanigera*	92	IIKCKFCRR		IILCCDDE	N.D.	95	LLQCQYR	QNSDYCRKK		
	Guinea pig	*Cavia porcellus*	92	IIKCKFCRR		IILCCDDE	N.D.	92	LLQCQYR	QNSDYCRKK		
	Naked mole rat	*Heterocephalus glaber*	90	IIKCKFCRP	97	IILCCDDE	LESCF	95	LLQCQYR	QNSDYCRKK		
	Damara mole-rat	*Fukomys damarensis*	90	IIKCKFCR	96	IILCCDDE	LESCF	95	LLQCQYR	QNSDYCRKK		
	North American beaver	*Castor canadensis*	93	IIKCKFCRQ		IILCCDDE	N.D.	97	LLQCQYR	QNSDYCRKK		
	Ord's kangaroo rat	*Dipodomys ordii*	90	IIKCKFCRR		IILCCDDE	N.D.	95	LLQCQYR	QNSDYCRKK		
	Upper Galilee mountains blind Mole rat	*Nannospalax galili*	92	IIKCKFCRR		IILCCDDE	N.D.	96	LLQCQYR	QSSDYCRKK		
	House mouse	*Mus musculus*	88	IIKCKFCR	94	IILCCDDE	LESCF	95	LLQCQYR	QNCDHCGKK		
	Ryukyu mouse	*Mus caroli*	88	IIKCKFCRR	94	IILCCDDE	LESCF	95	LLQCQYR	QNSDHCGKK		
	Gairdner's shrewmouse	*Mus pahari*	87	IIKCKFCRR	95	IILCCDDE	LESCF	95	LLQCQYR	QNSDHCGKK		
	Mongolian jird	*Meriones unguiculatus*	91	IIKCKFCRR		IILCCDDE	N.D.	95	LLQCQYR	QNSDNCGKK		
	Norway rat	*Rattus norvegicus*	90	IIKCKFCRR	94	IILCCDDE	LESCF	95	LLQCQYR	QNSDHCGKK		
	Prairie deer mouse	*Peromyscus maniculatus bairdii*	91	IIKCKFCRR	91	VILCCDDE	LESCF	95	LLQCQYR	QNSDHCGKK		
	Prairie vole	*Microtus ochrogaster*	90	IIKCKFCRR		IILCCDDE	N.D.	94	LLQCQYR	QNSDHCKKK		
	Chinese hamster	*Cricetulus griseus*	90	IIKCKFCRR		IILCCDDE	N.D.	94	LLQCQYR	QNSDHCRDK		
	Golden hamster	*Mesocricetus auratus*	90	IIKCKFCRR		IILCCDDE	N.D.	94	LLQCQYR	QNSDHCSKK		
	Lesser egyptian jerboa	*Jaculus jaculus*	89	IIKCKFCRR	95	IILCCDDE	LESCF	95	LLQCQYR	QNSDYCRKK		
	Thirteen-lined ground squirrel	*Ictidomys tridecemlineatus*	92	IIKCKFCRR	96	IILCCDDE	LESCF		LLRCQYR	N.D.		
	Alpine marmot	*Marmota marmota marmota*	93	IIKCKFCSR	96	IILCCDDE	LESCF		LLRCQYR	N.D.		
Order: Lagomorpha	Rabbit	*Oryctolagus cuniculus*	94	IIKCKFCRR	97	IILCCDDE	LESCF	94	LLQCQYR	QNSDYCRKK		
	American pika	*Ochotona princeps*	91	IIKCKFCRR		IILCCDDE	N.D.	94	LLQCQYR	QNSDYCRKK		
Order: Carnivora	Cat	*Felis catus*	94	IIKCKFCRR		IILCCDDE	N.D.		LLQCQYR	N.D.	Magorder Boreoeutheria/Laurasiatheria	
	Amur tiger	*Panthera tigris altaica*	94	IIKCKFCRR		IILCCDDE	N.D.		LLQCQYR	N.D.		
	Leopard	*Panthera pardus*	94	IIKCKFCRR	97	IILCCDDE	LESCF	96	LLQCQYR	QNSDYCRKK		
	Cheetah	*Acinonyx jubatus*	93	IIKCKFCRR		IILCCDDE	N.D.		LLQCQYR	N.D.		
	Dog	*Canis lupus familiaris*	92	IIKCKFCRR	97	IILCCDDE	LESCF	98	LLQCQYR	QNSDYCRKK		
	Polar bear	*Ursus maritimus*	95	IIKCKFCRR		IILCCDDE	N.D.	97	LLQCQYR	QNSDYCRKK		
	Giant panda	*Ailuropoda melanoleuca*	95	IIKCKFCRR	93	IILCCDDE	LESCF	95	LLQCQYR	QNSDYCRKK		
	Ferret	*Mustela putorius furo*	94	IIKCKFCRR	97	IILCCDDE	LESCF	96	LLQCQYR	QNSDYCRKK		
	American mink	*Neovison vison*		N.D.		N.D.	N.D.		LLQCQYR	N.D.		
	Pacific walrus	*Odobenus rosmarus divergens*	95	IIKCKFCRG	97	IILCCDDE	LESCF		LLQCQYR	N.D.		
	Weddell seal	*Leptonychotes weddellii*	95	IIKCKFCRR		N.D.	N.D.		LLQCQYR	N.D.		
	Hawaiian monk seal	*Neomonachus schauinslandi*	94	IIKCKFCRR	97	IILCCDDE	LESCF	96	LLQCQYR	QNSDYCRKK		
Order: Pholidota	Malayan pangolin	*Manis javanica*	91	IIKCKFCRQ		SILCCDDE	N.D.	94	LLQCQYR	QNSDYCRKK		
Order: Perissodactyla	Southern white rhinoceros	*Ceratotherium simum simum*	93	IIKCKFCRR	96	IILCCDDE	LESCF	96	LLQCQYR	QNSDYCRKK		
	Przewalski's horse	*Equus przewalskii*	93	ILKCKFCRR		MILCCDDE	N.D.	96	LLQCQYR	QNSDYCRKK		
	Horse	*Equus caballus*	94	ILKCKFCRR		IILCCDDE	N.D.	93	LLQCQYR	QNSDYCRKK		
	Donkey	*Equus asinus*		N.D.		N.D.	N.D.	96	LLQCQYR	QNSDYCRKK		
Order: Chiroptera	Brandt's bat	*Myotis brandtii*	90	IIKCKFCRG		IILCCDDE	N.D.	97	LLQCQYR	QKSDCCRKK		
	David's myotis	*Myotis davidii*	89	IIKCKFCR	96	IILCCDDE	LESCF	95	LLQCQYR	QKSDCCRKK		
	Little brown bat	*Myotis lucifugus*	90	IIKCKFCRG	95	IILCCDDE	LESCF	83	LLQCQYR	QKSDCCRKK		
	Great roundleaf bat	*Hipposideros armiger*	92	ILKCKFGRQ		N.D.	N.D.	96	LLQCQYR	QNSDYCRKK		
	Chinese rufous horseshoe bat	*Rhinolophus sinicus*	92	IIKCKFGRQ	95	IILCCDDE	LESCF	96	LLQCQYR	QNSDYCRKK		
	Big brown bat	*Eptesicus fuscus*	90	IIKCKFCRG	96	IILCCDDE	LESCF	95	LLQCQYR	QKSDYCRKK		
	Natal long-fingered bat	*Miniopterus natalensis*	88	IVRCKFCRG	94	ITLCCDDE	LESCF	96	LLQCQYR	QKSDYCRKK		
	Large flying fox	*Pteropus vampyrus*	94	IIKCKFCRR	96	IILCCDDE	LESCF	95	LLQCQYR	QNSDYCRKK		
	Black flying fox	*Pteropus alecto*	94	IIKCKFCRR	96	IILCCDDE	LESCF	95	LLQCQYR	QNSDYCRKK		
	Egyptian fruit bat	*Rousettus aegyptiacus*	93	IIKCKFCRR		IILCCDDE	N.D.	96	LLQCQYR	QNSDYCRKK		
Order: Cetartiodactyla	Common minke whale	*Balaenoptera acutorostrata scammoni*	92	IIKCKFCRR		IILCCDDE	N.D.	95	LLQCQYR	QNSDYRRKK		
	Bottlenose dolphin	*Tursiops truncatus*	93	IikCKfCRr	93	IilCcDne	LesCF	96	LlqCQyr	QnsdyCRkk		
	Killer whale	*Orcinus orca*	93	IikCKfCRr	93	IilCcDne	LepCF	96	LlqCQyr	QnsdyCRkk		
	Yangtze River dolphin	*Lipotes vexillifer*	92	IIKCKFCRR	94	IILCCDNE	LESCF	95	LLQCQYR	QNSDYCRKK		
	Sperm whale	*Physeter catodon*	92	IikCKfCRr		IilCcDde	N.D.	96	LlqCQyr	QnsdyCRkk		
	Arabian camel	*Camelus dromedarius*	92	IIKCKFCRR	96	IILCCDDE	LESCF	94	LLQCQYR	QNSEYCRKK		
	wild Bactrian camel	*Camelus ferus*	93	IIKCKFCRR	92	IILCCDDE	LESCF	94	LLQCQYR	QNSEYCRKK		
	Bactrian camel	*Camelus bactrianus*	93	IIRCKFCRR		IILCCDDE	N.D.	94	LLQCQYR	QNSEYCRKK		
	Cattle	*Bos taurus*	92	IikCKfCRr	95	IilCcDde	LesCF	94	LlqCQyr	QnsdyCRkk		
	Wild yak	*Bos mutus*	92	IikCKfCRr	95	IilCcDde	LesCF	96	LlqCQyr	QnsdyCRkk		
	Zebu	*Bos indicus*	92	IikCKfCRr		IilCcDde	N.D.		LlqCQyr	N.D.		
	Water buffalo	*Bubalus bubalis*	91	IikCKfCRr	92	IilCcDde	LesCF	95	LlqCQyr	QnsdyCRkk		
	American bison	*Bison bison bison*	92	IIKCKFCRR	96	IILCCDDE	LESCF	94	LLQCQYR	QNSDYCRKK		
	White-tailed deer	*Odocoileus virginianus texanus*	92	IIKCKFCRR		IILCCDDE	N.D.	94	LLQCQYR	QNSDYCRKK		
	pig (wild boar)	*Sus scrofa*	91	IIKCKFCRR		IILCCDDE	N.D.	96	LLQCQYR	QKSDYCRKK		
	Goat	*Capra hircus*	91	IirCKfCRr	92	IilCcDde	LesCF		LlqCQyr	N.D.		
	Sheep	*Ovis aries*	92	IikCKfCRr	95	IilCcDde	LesCF		LlqCQyr	N.D.		
	Mouflon	*Ovis aries musimon*	92	IikCKfCRr	95	IilCcDde	LesCF		LlqCQyr	N.D.		
	Chiru	*Pantholops hodgsonii*	92	IikCKfCRr		IilCcDde	N.D.	96	LlqCQyr	QnsdyCRkk		
	Alpaca	*Vicugna pacos*	93	IikCKfCRr		IilCcDde	N.D.	95	LlqCQyr	QnsdyCRkk		
Order: Erinaceomorpha	Western European hedgehog	*Erinaceus europaeus*	89	IIKCKFCRR		IILCCDDD	N.D.	96	LLQCQYR	QNSDYCRKK		
Order: Soricomorpha	Star-nosed mole	*Condylura cristata*	92	IIKCKFCRQ	95	IILCCDDE	LESCF	96	LLQCQYR	QNSDYCRKK		
	European shrew	*Sorex araneus*	90	IIKCKFCRR		IILCCDDD	N.D.		LLQCQYR	N.D.		
Order: Afrosoricida	Cape golden mole	*Chrysochloris asiatica*	93	IIKCKFCRR	96	IILCCDDE	LESCF		LLQCQYR	N.D.	Afrotheria	
	Lesser hedgehog tenrec	*Echinops telfairi*	89	IIKCKFCRR		IILCCDDE	N.D.		LLKCQYR	N.D.		
Order: Macroscelidea	Cape elephant shrew	*Elephantulus edwardii*	90	IIKCKFCR	94	IILCCDDE	LESCF		LLRCRYR	N.D.		
Order: Tubulidentata	Aardvark	*Orycteropus afer afer*	92	IIKCKFCRR	97	IILCCDDE	LESCF		LLQCQYR	N.D.		
Order: Proboscidea	African savanna elephant	*Loxodonta africana*	90	IIKCKFCRR	96	IILCCDDE	LESCF		LLRCRYR	N.D.		
Order: Sirenia	Florida manatee	*Trichechus manatus latirostris*	92	IIKCKFCRR	95	IILCCDDE	LESCF		LLQCQYR	N.D.		
Order: Cingulata	Nine-banded armadillo	*Dasypus novemcinctus*	90	IIKCKFCRR	97	IILCCDDE	LESCF	96	LLQCQYR	QNSDYSRKK	Xenarthra	
Order: Didelphimorphia	Gray short-tailed opossum	*Monodelphis domestica*	84	IVRCKFCRQ		IILCCDEE	N.D.	84	LLQCRYR	QNSDYCRKK	Ameridelphia	Metatheria
Order: Dasyuromorphia	Tasmanian devil	*Sarcophilus harrisii*	80	IIRCKFCWR		IILCCDEE	N.D.	85	LLQCRYR	QNSDYSRKK	Australidelphia	
Order: Diprotodontia	Koala	*Phascolarctos cinereus*	83	IVRCKFCRQ		IILCCDEE	N.D.	84	LLQCRYR	QNSDYSRKK		
Order: Monotremata	Platypus	*Ornithorhynchus anatinus*	57	IVRCKFCRQ		IILCCGDE	N.D.	80	LLQCRYR	RNSDSSRKK	Monotremata	Prototheria

(Continues)

TABLE 1 (Continued)

Superclass: Gnathostomata	class: Aves									
Order: Psittaciformes	Kea	*Nestor notabilis*		N.D.		IILCCGDE	N.D.		LLQCRYR	N.D.
	Budgerigar	*Melopsittacus undulatus*	80	IIKCKFCRQ	88	IILCCGDE	QESCF		LLQCRYR	N.D.
	Turquoise-fronted amazon	*Amazona aestiva*	79	IVKCKFCRQ		IILCCGDE	N.D.	86	LLQCRYR	RSSDFSREK
Order: Passeriformes	American crow	*Corvus brachyrhynchos*	83	N.D.	80	IILCCGDE	QESCF	86	LLQCRYR	RSPDFSREK
	Hooded craw	*Corvus cornix cornix*	80	IIKCKFCRQ	84	IILCCGDE	QESCF	87	LLQCRYR	RSPDFSREK
	Canary	*Serinus canaria*	81	IIKCKFCRQ		IILCCGDE	N.D.	77	LLQCRYR	RSSDFSREK
	Collared flycatcher	*Ficedula albicollis*	79	IIKCKFCRQ	87	IILCCGDE	QESCF	80	LLQCRYR	RSPDFSREK
	Ground tit	*Pseudopodoces humilis*	81	IIKCKFCRQ	83	IILCCGDE	QESCF		LLQCRYR	N.D.
	Great tit	*Parus major*	81	IIKCKFCRQ	78	IILCCGDE	QESCF	81	LLQCRYR	RSSDFSREK
	Medium ground-finch	*Geospiza fortis*	73	IIKCKXXXX	81	IILCCGDE	QESCF		LLQCRYR	N.D.
	Zebra finch	*Taeniopygia guttata*	79	IIKCKFCQQ		IILCCGDE	N.D.		LLQCRYR	N.D.
	Society finch	*Lonchura striata domestica*	79	IIKCKFCQQ	82	IILCCGDE	QESCF	81	LLQCRYR	RSSDFSREK
	Rifleman	*Acanthisitta chloris*		N.D.		IILCCGDE	N.D.		LLQCRYR	N.D.
	White-throated sparrow	*Zonotrichia albicollis*	80	IIKCKFCRQ	85	IILCCGDE	QESCF	83	LLQCRYR	RSSDFSREK
	Golden-collared manakin	*Manacus vitellinus*	79	IIKCKFCRQ	89	IILCCGDE	QESCF		LLQCRYR	N.D.
	Blue-crowned manakin	*Lepidothrix coronata*	79	IINCKFCRQ	89	IILCCGDE	QESCF		LLQCRYR	N.D.
	Common starling	*Sturnus vulgaris*	80	IIKCKFCRQ		IILCCGDE	N.D.	81	LLQCRYR	RSPDFSREK
	Blackbird	*Turdus merula*	80	IIKCKFCRQ		N.D.	N.D.		N.D.	N.D.
Order: Falconiformes	Saker falcon	*Falco cherrug*	81	IIKCKFCKQ	89	IILCCGDE	QESCF		LLQCRYR	N.D.
	Peregrine falcon	*Falco peregrinus*	80	IIKCKFCKQ	89	IILCCGDE	QESCF		LLQCRYR	N.D.
Order: Bucerotiformes	Rhinoceros hornbill	*Buceros rhinoceros silvestris*	80	IIKCKFCRQ		IILCCGDE	N.D.		LLQCRYR	N.D.
Order: Piciformes	Downy woodpecker	*Picoides pubescens*	80	IIKCKFCRQ	90	IILCCGDE	QESCF		LLQCRYR	N.D.
Order: Coraciiformes	Carmine bee-eater	*Merops nubicus*		N.D.		IILCCGDE	N.D.	85	LLQCRYR	KSSDFSRES
Order: Cariamiformes	Red-legged seriema	*Cariama cristata*		N.D.		IILCCGDE	N.D.		LLQCRYR	N.D.
Order: Accipitriformes	White-tailed eagle	*Haliaeetus albicilla*		N.D.		IILCCGDE	N.D.		LLQCRYR	N.D.
	Bald eagle	*Haliaeetus leucocephalus*	81	IIKCKFCRQ		IILCCGDE	N.D.		LLQCRYR	N.D.
	Golden eagle	*Aquila chrysaetos canadensis*	81	IIKCKFCRQ	89	IILCCGDE	QESCF	81	LLQCRYR	RSSDFSREK
	Turkey vulture	*Cathartes aura*		N.D.		N.D.	N.D.		LLQCRYR	N.D.
Order: Strigiformes	Barn owl	*Tyto alba*		N.D.		N.D.	N.D.		LLQCRYR	N.D.
Order: Coliiformes	Speckled mousebird	*Colius striatus*		N.D.		IILCCGDE	N.D.		LLQCRYR	N.D.
Order: Leptosomatiformes	Cuckoo roller	*Leptosomus discolor*	80	IIKCKXXXX		IILCCGDE	N.D.		LLQCRYR	N.D.
Order: Trogoniformes	Bar-tailed trogon	*Apaloderma vittatum*	64	IIXXXXXXX		IILCCGDE	N.D.	88	LLQCRYR	RSSDFSREK
Order: Gaviiformes	Red-throated loon	*Gavia stellata*		N.D.		IILCCGDE	N.D.		LLQCRYR	N.D.
Order: Sphenisciformes	Emperor penguin	*Aptenodytes forsteri*	80	IVKCKFCRQ	90	IILCCGDE	HESCF		LLQCRYR	N.D.
	Adelie penguin	*Pygoscelis adeliae*	77	N.D.		IILCCGDE	N.D.		LLQCRYR	N.D.
Order: Procellariiformes	Northern fulmar	*Fulmarus glacialis*		N.D.		IILCCGDE	N.D.		LLQCRYR	N.D.
Order: Pelecaniformes	Dalmatian pelican	*Pelecanus crispus*		N.D.		IILCCGDE	N.D.		LLQCRYR	N.D.
	Crested ibis	*Nipponia nippon*	79	IIKCKFCRQ		IILCCGDE	N.D.		LLQCRYR	N.D.
	White-throated tinamou	*Tinamus guttatus*	85	N.D.	83	IILCCGDE	QESCF		LLQCRYR	N.D.
Order: Ciconiiformes	Little egret	*Egretta garzetta*		N.D.		IILCCGDE	N.D.		LLQCRYR	N.D.
Order: Suliformes	Great cormorant	*Phalacrocorax carbo*		N.D.		IILCCGDE	N.D.	87	LLQCKYR	RSSDFSREK
Order: Caprimulgiformes	Chuck-will's-widow	*Caprimulgus carolinensis*		N.D.		IILCCGNE	N.D.		LLQCRYR	N.D.
Order: Apodiformes	Chimney swift	*Chaetura pelagica*		N.D.	87	IILCCGDE	QESCF		LLQCRYR	N.D.
	Anna's hummingbird	*Calypte anna*	83	IIKCKFCRQ	89	IILCCGDE	QESCF		LLQCRYR	N.D.
Order: Podicipediformes	Great crested grebe	*Podiceps cristatus*		N.D.		IILCCGDE	N.D.		LLQCRYR	N.D.
Order: Phoenicopteriformes	American flamingo	*Phoenicopterus ruber ruber*		N.D.		IILCCGDE	N.D.		LLQCRYR	N.D.
Order: Pteroclidiformes	Yellow-throated sandgrouse	*Pterocles gutturalis*		N.D.		IILCCGDE	N.D.		LLQCRYR	N.D.
Order: Columbiformes	Rock pigeon	*Columba livia*	79	IIKCKFCRE	88	IILCCGVE	QESCF		LLQCRYR	N.D.
	Band-tailed pigeon	*Patagioenas fasciata monilis*	80	IIKCKFCRQ	88	IILCCGVE	QESCF		LLQCRYR	N.D.
Order: Gruiformes	Grey crowned crane	*Balearica regulorum gibbericeps*	70	IIKXXXXXX		IILCCGDE	N.D.		LLQCRYR	N.D.
	Sunbittern	*Eurypyga helias*		N.D.		IILCCGDE	N.D.		LLQCRYR	N.D.
	Brown mesite	*Mesitornis unicolor*		N.D.		IILCCGDE	N.D.		LLQCRYR	N.D.
Order: Otidiformes	Macqueen's bustard	*Chlamydotis macqueenii*		N.D.		IILCCGDE	N.D.		LLQCRYR	N.D.
Order: Opisthocomiformes	Hoatzin	*Opisthocomus hoazin*	85	IIKCKFCRQ		IILCCGDE	N.D.		LLQCRYR	N.D.
Order: Charadriiformes	Ruff	*Calidris pugnax*	81	IIKCKFCRQ	89	IILCCGDE	QESCF	81	LLQCRYR	RSSDFSREK
	Killdeer	*Charadrius vociferus*	68	IILCKFCRQ	89	IILCCGDE	QESCF		LLQCRYR	N.D.
Order: Cuculiformes	Common cuckoo	*Cuculus canorus*	80	IIKCKFCRQ	88	IILCCGDE	QESCF		LLQCRYR	N.D.
Order: Musophagiformes	Red-crested turaco	*Tauraco erythrolophus*		N.D.		IILCCGDE	N.D.		LLQCRYR	N.D.
Order: Galliformes	Scaled quail	*Callipepla squamata*	81	IIKCKFCRQ		N.D.	N.D.		LLQCRYR	N.D.
	Japanese quail	*Coturnix japonica*	80	IIKCKFCRQ	88	IILCCGDE	QESCF	80	LLQCRYR	RSSDFSREK
	Northern bobwhite	*Colinus virginianus*	81	IIKCKFCRQ		IILCCGDE	N.D.		LLQCRYR	N.D.
	Chicken	*Gallus gallus*	80	IIKCKFCRQ	89	IILCCGDE	QESCF	80	LLQCRYR	RSSDFSREK
	Turkey	*Meleagris gallopavo*	81	N.D.	89	IILCCGDE	QESCF	87	LLQCRYR	RSSDFSREK
	Helmeted guineafowl	*Numida meleagris*	80	IIKCKFCRQ	89	IILCCGDE	QESCF		LLQCRYR	N.D.
Order: Anseriformes	Mallard	*Anas platyrhynchos*		N.D.	89	IILCCGDE	QESCF	88	LLQCRYR	RSSDFSREK
	Swan goose	*Anser cygnoides*	81	IIKCKFCRQ						
	Chinese geese	*Anser cygnoides domesticus*			89	IILCCGDE	QESCF	87	LLQCRYR	RSSDFSREK
Order: Struthioniformes	Southern ostrich	*Struthio camelus australis*		N.D.	89	IILCCGDE	QESCF		LLQCRYR	N.D.
Order: Tinamiformes	White-throated tinamou	*Tinamus guttatus*	85	N.D.					LLQCRYR	N.D.
Order: Apterygiformes	Kiwi	*Apteryx australis mantelli*	82	N.D.	89	IILCCGDE	QESCF		LLQCKYR	N.D.
Superclass: Gnathostomata	class: Reptilia									
Order: Crocodilia	Saltwater crocodile	*Crocodylus porosus*	76	IVKCKFCRQ	87	IILCCGDE	QESCF	84	LLQCRYR	RSCDYSRKK
	American alligator	*Alligator mississippiensis*	76	IVKCKFCRQ	87	IILCCGDE	QESCF	84	LLQCRYR	RSSDYSRKK
	Chinese alligator	*Alligator sinensis*	81	IVKCKFCRQ	87	IILCCGDE	QESCF		LLQCRYR	N.D.
	Gharial	*Gavialis gangeticus*	76	IVKCKFCRQ	87	IILCCGDE	QESCF	84	LLQCRYR	RSSDYSRKK
Order: Testudines	Green sea turtle	*Chelonia mydas*	81	IIKCKFCRQ	88	IILCCGDE	QESCF		LLRCRYR	N.D.
	Chinese soft-shelled turtle	*Pelodiscus sinensis*	84	IIKCKFCRQ	88	IILCCGDE	QESCF	89	LLQCRYR	RNSDYARKK
	Painted turtle	*Chrysemys picta bellii*	81	IIKCKFCRQ	89	IILCCGDE	QESCF		LLQCRYR	N.D.
Order: Squamata	Green anole	*Anolis carolinensis*	71	IVRCKFCRQ	88	IILCCGDE	QESCF		LLQCRYR	N.D.
	Schlegel's Japanese gecko	*Gekko japonicus*	73	IVKCKFCRQ		IILCCGDE	N.D.	81	LLQCRYR	RSSDYSRKK
	Central bearded dragon	*Pogona vitticeps*	73	IVRCKFCTQ	88	IILCCGDE	QESCF	89	LLQCRYR	RSSDYSRKK
	Burmese python	*Python bivittatus*	74	IVRCKFCAP		IILCCGDE	N.D.	89	LLQCRYR	RNSDYSRRK
	King cobra	*Ophiophagus hannah*		N.D.		IILCCGDE	N.D.		LLQCRYR	N.D.
	Pit vipers	*Protobothrops mucrosquamatus*	72	IVRCKFCMP	87	IILCCGDE	QESCF		LLQCRYR	N.D.
	Common garter snake	*Thamnophis sirtalis*	73	IVRCKFCLP		IILCCGDE	N.D.		LLQCRYR	N.D.
Superclass: Gnathostomata	class: Amphibia									
Order: Anura	Western clawed frog	*Xenopus (Silurana) tropicalis*	76	IIKCKFCRQ	84	IILCCGDE	QESCF		LLQCRYR	-TRRHSREH
	African clawed frog	*Xenopus laevis*	72	IIKCKFCRQ	85	IILCCGDE	QESCF	74	LLQCRYR	RTRRQSREN
	High Himalaya frog	*Nanorana parkeri*	75	IIKCKFCRQ	86	IILCCGDE	QESCF	79	LLQCKYR	K--NYSRED
Superclass: Gnathostomata	class: Sarcopterygii									
Order: Coelacanthiformes	coelacanth	*Latimeria chalumnae*	79	IIKCKFCRQ	84	IILCCGDE	QESCF		LLQCRYR	N.D.
Superclass: Gnathostomata	class: Actinopterygii									
Order: Cypriniformes	Common carp	*Cyprinus carpio*	74	ILKCKCIRQ		N.D.	N.D.		LLSCRYR	N.D.
	Common carp	*Cyprinus carpio*	69	IIKCHFCRP					LIRCRYR	N.D.
		Sinocyclocheilus anshuiensis	75	ILKCKCIRQ	77	IILCCGHK	QESCF		LLSCHYR	N.D.
		Sinocyclocheilus anshuiensis	70	IIKCHFCRP						
	Golden-line barbel	*Sinocyclocheilus grahami*	75	ILKCKCIRQ	78	IILCCGHK	QESCF		LLRCRYR	N.D.
	Golden-line barbel	*Sinocyclocheilus grahami*	74	ILKCKCIRQ					LLRCRYQ	N.D.
	Golden-line barbel	*Sinocyclocheilus grahami*	70	IIKCHFCRP					LIRCRYR	N.D.
		Sinocyclocheilus rhinocerous	75	ILKCKCIRQ	76	IILCCGHK	QESCF		LLRCRYR	N.D.
		Sinocyclocheilus rhinocerous	69	IIKCHFCRP					LIRCRYR	N.D.
	Zebrafish	*Danio rerio*	75	ILKCKCIRQ	76	IILCCGHK	QESCF		LLRCRYR	N.D.
	Zebrafish	*Danio rerio*	69	IIKCHFCRP					LIRCRYR	N.D.
Order: Perciformes	Burton's mouthbrooder	*Haplochromis burtoni*	75	IIKCKFHRP	77	IILCCGRQ	HESCL		LLRCRYR	N.D.
	Burton's mouthbrooder	*Haplochromis burtoni*	67	IIKCHXXXX					LLRCRYR	N.D.
	Nile tilapia	*Oreochromis niloticus*	76	IIKCKFHRP	76	IILCCGRQ	HESCL		LLRCRYR	N.D.
	Nile tilapia	*Oreochromis niloticus*	67	IIKCHFCRP					LLRCRYR	N.D.
	Mozambique tilapia	*Oreochromis mossambicus*	67	IIKCHFCRP		N.D.	N.D.		N.D.	N.D.
	Lyretail cichlid	*Neolamprologus brichardi*	76	IIKCKFHRP	76	IILCCGRQ	HESCL		LLRCRYR	N.D.
	Nyerere's Victoria cichlid	*Pundamilia nyererei*	75	IIKCKFHRP		N.D.	N.D.		LLRCRYR	N.D.
	Nyerere's Victoria cichlid	*Pundamilia nyererei*	67	IIKCHFCRP					LLRCRYR	N.D.
	Asian sea bass	*Lates calcarifer*	71	ILKCKFHRP	76	IILCCGRK	HESCL		LLRCRYR	N.D.
	Asian sea bass	*Lates calcarifer*	68	IIKCHFCRP					LLRCRYR	N.D.
	Spiny chromis	*Acanthochromis polyacanthus*	75	IIKCKFHRP		IILCCGRK	N.D.		LLRCRYR	N.D.
	Spiny chromis	*Acanthochromis polyacanthus*	68	ILKCHFCRP					LLRCRYR	N.D.

(Continues)

TABLE 1 (Continued)

	Zebra mbuna	*Maylandia zebra*	75	IIKCKFHRP	76	IILCCGRQ	HESCL		LLRCRYR	N.D.
	Zebra mbuna	*Maylandia zebra*	67	IIKCHFCRP					LLRCRYR	N.D.
	Bicolor damselfish	*Stegastes partitus*	75	IIKCKFHRP	76	IILCCGRK	HESCL		LLRCRYR	N.D.
	Bicolor damselfish	*Stegastes partitus*	67	ILKCHFCRP					LLRCRYR	N.D.
	Mudskipper	*Boleophthalmus pectinirostris*	73	ILRCKFHRP		IILCCGRK	N.D.		LLRCRYR	N.D.
	Croceine croaker	*Larimichthys crocea*	74	ILRCKFHRP	79	IILCCGRK	HESCL		LLRCRYR	N.D.
	Croceine croaker	*Larimichthys crocea*	67	IIKCHFCRP					LLRCRYR	N.D.
	Ballan wrasse	*Labrus bergylta*	72	IIRCKFHRA		N.D.	N.D.		LLRCRYR	N.D.
	Ballan wrasse	*Labrus bergylta*	68	ILKCHFCRP					LLRCRYR	N.D.
Order: Cyprinodontiformes		*Austrofundulus limnaeus*	71	IIKCKFHRA	76	IILCCGRK	RESCL		LLRCRYR	N.D.
	Mummichog	*Fundulus heteroclitus*	72	IIKCKFHRP	73	IILCCGRK	HESCL		LLRCRYR	N.D.
	Mummichog	*Fundulus heteroclitus*	66	IIKCHFCKN					LLRCRYR	N.D.
	Sheepshead minnow	*Cyprinodon variegatus*	72	IIKCKFHRP		N.D.	N.D.		LLRCRYR	N.D.
	Sheepshead minnow	*Cyprinodon variegatus*	66	IIKCHFCKP					LLRCRYR	N.D.
	Turquoise killifish	*Nothobranchius furzeri*	73	IIKCKFHRA	74	IILCCGRK	HESCL		LLRCRYR	N.D.
	Turquoise killifish	*Nothobranchius furzeri*	66	IIKCYFCKP					LLRCRYR	N.D.
	Mangrove rivulus	*Kryptolebias marmoratus*	72	IIKCKFHRP	75	IILCCGRK	RESCL		LLRCRYR	N.D.
	Mangrove rivulus	*Kryptolebias marmoratus*	66	IIKCHFCRP					LLRCRYR	N.D.
	Guppy	*Poecilia reticulata*	72	IIKCKFHRP	74	IILCCGRK	RESCL		LLRCRYR	N.D.
	Guppy	*Poecilia reticulata*	66	IIKCHFCKP					LLRCRYR	N.D.
	Amazon molly	*Poecilia formosa*	72	IIKCKFHRP	74	IILCCGRK	RESCL		LLRCRYR	N.D.
	Amazon molly	*Poecilia formosa*	66	IIKCHFCKP					LLRCRYR	N.D.
	Sailfin molly	*Poecilia latipinna*	72	IIKCKFHRP	74	IILCCGRK	RESCL		LLRCRYR	N.D.
	Atlantic molly	*Poecilia mexicana*		N.D.	74	IILCCGRK	RESCL		LLRCRYR	N.D.
	Platyfish	*Xiphophorus maculatus*	71	IIKCKFHRP		IILCCGRK	N.D.		LLRCRYR	N.D.
Order: Beloniformes	Japanese medaka	*Oryzias latipes*	72	IIKCKFHRP	76	IILCCGRK	HESCL		LLRCRYR	N.D.
	Japanese medaka	*Oryzias latipes*	67	IIKCHFCKP					LLRCRYR	N.D.
Order: Batrachoidiformes	Gulf toadfish	*Opsanus beta*	71	ILKCKFHRA		N.D.	N.D.		N.D.	N.D.
Order: Scorpaeniformes	Black rockcod	*Notothenia coriiceps*	70	IIRCKFHRA		VILCCGDE	N.D.		LLRCRYR	N.D.
	Black rockcod	*Notothenia coriiceps*	67	IIKCHFCRP					LLRCRYR	N.D.
	False kelpfish	*Sebastiscus marmoratus*		N.D.		N.D.	N.D.		N.D.	N.D.
Order: Characiformes	Red pirahna	*Pygocentrus nattereri*	69	IIKCHFCRP	77	IILCCGHR	QESCF		LLSCRYR	N.D.
	Red pirahna	*Pygocentrus nattereri*							IICCRYR	N.D.
	Mexican tetra	*Astyanax mexicanus*							LLRCRYR	N.D.
	Mexican tetra	*Astyanax mexicanus*	68	IIKCHFCRP	78	IILCCGHK	QESCF		IICCRYR	N.D.
Order: Esociformes	Northern pike	*Esox lucius*	70	IIRCKFHRP	77	IILCCGRK	PESCF		LLRCRYR	N.D.
	Northern pike	*Esox lucius*							ILLCRYR	N.D.
Order: Clupeiformes	Atlantic herring	*Clupea harengus*	70	IVKCKNFRPIIC		IILCCGRR	N.D.		LLRCRYR	N.D.
	Atlantic herring	*Clupea harengus*							LIRCRYR	N.D.
Order: Salmoniformes	Rainbow trout	*Oncorhynchus mykiss*	71	IIKCKFHRQ	75	IILCCGRK	QESCF		LLRCRYR	N.D.
	Rainbow trout	*Oncorhynchus mykiss*							ILLCRYR	N.D.
	Coho salmon	*Oncorhynchus kisutch*	72	IIRCKFHRP		N.D.	N.D.		LLRCRYR	N.D.
	Coho salmon	*Oncorhynchus kisutch*	71	IIKCKFHRQ					ILLCRYR	N.D.
	Atlantic Salmon	*Salmo salar*	71	IIRCKFHRP	76	IILCCGRK	QESCF		LLRCRYR	N.D.
	Atlantic Salmon	*Salmo salar*	71	IIKCKFHRQ					ILLCRYR	N.D.
Order: Tetraodontiformes	Japanese pufferfish	*Takifugu rubripes*	73	ILRCKFHRH		N.D.	N.D.		LLRCRYR	N.D.
	Japanese pufferfish	*Takifugu rubripes*	66	IIKCHFCRA					LLRCRYR	N.D.
	Spotted green pufferfish	*Tetraodon nigroviridis*	70	ILRCKFHRX		N.D.	N.D.		N.D.	N.D.
Order: Pleuronectiformes	Tongue sole	*Cynoglossus semilaevis*	73	IIKCKFHRP		IILCCGRK	N.D.		LLRCRYR	N.D.
	Olive flounder	*Paralichthys olivaceus*	72	IIKCKFHRP	79	IILCCGRK	SESCL		LLRCRYR	N.D.
	Olive flounder	*Paralichthys olivaceus*	66	IIKCHFCRP					LLRCRYR	N.D.
	European flounder	*Platichthys flesus*	77	IIKCKFHRP		N.D.	N.D.		N.D.	N.D.
Order: Gasterosteiformes	Tiger tail seahorse	*Hippocampus comes*	76	IVKCKFHRP	75	SILCCGRK	AESCL		LLRCRYR	N.D.
	Tiger tail seahorse	*Hippocampus comes*	68	IIKCHFCRP					LLRCRFR	N.D.
Order: Siluriformes	Channel catfish	*Ictalurus punctatus*	66	IIKCHFCRK	78	IILCCGHQ	QESCF		LLACRYR	N.D.
	Channel catfish	*Ictalurus punctatus*							LI---YR	N.D.
Order: Anguilliformes	Swamp eel	*Monopterus albus*	75	IIKCKFHRP	77	IILCCGRK	HESCL		LLRCRYR	N.D.
	Swamp eel	*Monopterus albus*	68	IIKCHFCRP					LLRCRYR	N.D.
Order: Osteoglossiformes	Asian arowana	*Scleropages formosus*	75	IVKCKFYR	78	IILCCGHK	QESCL		LLRCRYR	N.D.
Order: Lepisosteiformes	Spotted gar	*Lepisosteus oculatus*	79	IIKCKFCR	78	IILCCGQE	QESCF		LLRCRYR	N.D.
	Spotted gar	*Lepisosteus oculatus*							LLQCRYR	N.D.
superclass: Gnathostomata	class: Chondrichthyes									
Order: Orectolobiformes	Whale shark	*Rhincodon typus*	75	IIKCKFCKQ		RILCCGDR	N.D.		LLRCRYR	N.D.
	Whale shark	*Rhincodon typus*							LLRCKYR	N.D.
Order: Chimaeriformes	Australian ghostshark	*Callorhinchus milii*	76	IIKCKF		TILCCGNR	N.D.		LLRCRYR	N.D.
	Australian ghostshark	*Callorhinchus milii*							LLRCQYR	N.D.
superclass: Agnatha	class: Petromyzontida									
Order: Petromyzontiformes	Sea lamprey	*Petromyzon marinus*	62	IIKCKFCRQ		N.D.	N.D.		N.D.	N.D.
phylum: Chordata	subphylum: Cephalochordata									
Order: Amphioxiformes	Belcher's lancelet	*Branchiostoma belcheri*	49	ILFGRR	47	RILSCWSCCDVDN	N.D.		N.D.	N.D.
phylum: Chordata	subphylum: Urochordata									
Order: Enterogona	Sea squirt	*Ciona intestinalis*	32	LFRWNRRR		N.D.	N.D.		N.D.	N.D.

Amino acid sequences around putative palmitoylation sites corresponding to human 5-HT_{1A} receptor Cys417 and Cys420; those corresponding to human 5-HT_4 receptor Cys328, Cys329, and human 5-$HT_{4(a)}$ receptor Cys386; those corresponding to human 5-HT_7 receptor Cys401 and human 5-$HT_{7(a)}$ receptor Ala435 and Cys438 in vertebrate 5-HT receptor orthologs are shown. Percent identities between orthologs across 2 species were obtained by performing BLAST search (with BLOSUM62) with full-length amino acid sequence of human 5-HT receptor orthologs. N. D., sequence not determined.

cysteine residue there (Table 1). In these animals, the corresponding site is exceptionally substituted or lost only in 2 bat species (great roundleaf bat and Chinese rufous horseshoe bat) and ghost shark. Similar to many other teleost fish genes, most teleost fishes have more than 2 different types of 5-HT_{1A} receptor. Redundant 5-HT_{1A} receptor orthologs in teleost fishes are consistent with their additional whole-genome duplication that occurred in the ancestor of teleosts.[26,27] Basically, there exist 2 cysteine residues at least in one ortholog of most ray-finned fishes. Characteristic sequences "-IIK**C**HF**C**RP-stop" or "-IIK**C**KFHRP-stop" and their several variations are detected in the C-termini of fish 5-HT_{1A} receptor orthologs. Even sea lamprey 5-HT_{1A} receptor holds both putative palmitoylation sites "-IIK**C**KF**C**RQ-stop."

3.2 | 5-HT_4 receptor palmitoylation sites in various vertebrate species

5-HT_4 receptors are coupled to G_s protein that stimulate adenylyl cyclases to produce cAMP. Some splice variants of the 5-HT_4 receptor have been identified for many vertebrate species. For instance, there exist at least 11 5-HT_4 receptor splice variants in humans.[28] These variants show different tissue distribution and exhibit different functional activities.[28,29] Mouse 5-$HT_{4(a)}$ (also called 5-$HT_{4\ \text{isoform}\ X2}$) receptor contains 387 amino acids that is palmitoylated at distinct sites, Cys328/Cys329 and Cys386.[22,23] While Cys328/Cys329 are commonly conserved among all mouse splice variants, Cys386 specifically locates close to the C-terminus

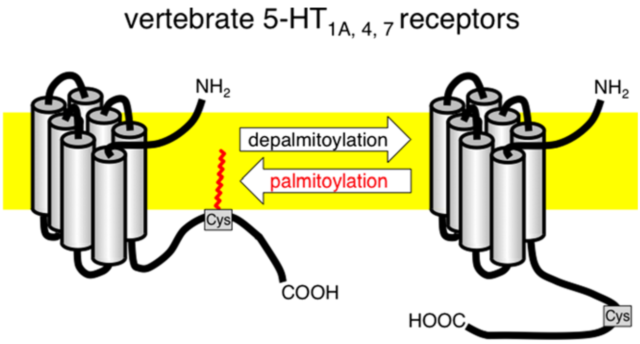
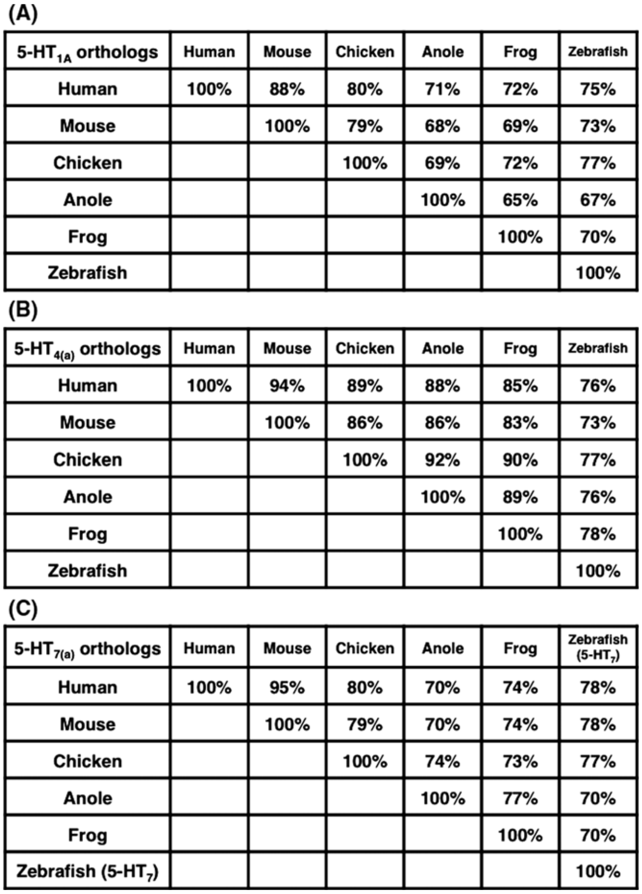


FIGURE 1 Palmitoylation of vertebrate 5-HT receptors. Schematic structure of 5-HT_{1A}, 5-HT_4, and 5-HT_7 receptors and their C-terminal palmitoylation sites. 5-HT receptors contain 7 transmembrane domains and intracellular C-terminal domain. Squared “Cys” represents multiple palmitoylation sites of 5-HT_{1A}, 5-HT_4, and 5-HT_7 receptors

of 5-$HT_{4(a)}$-type variant (Figure 3A). The 5-$HT_{4(a)}$ receptor C-terminus around the palmitoylation site (-Ser-**Cys**386-Phe-stop) may also form a class I PDZ ligand (-Ser/Thr-Xaa-ϕ-stop, where ϕ represents a hydrophobic residue), which controls protein-protein interactions between receptors and PDZ domain-containing binding proteins.[30] Previous reports showed that agonist stimulation-dependent palmitoylation on both sites is involved in regulation of receptor constitutive activity.[22,23] Palmitoylation sites corresponding to mouse 5-HT_4 receptor Cys328/Cys329 are completely conserved in all vertebrate 5-HT_4 receptor orthologs including other splice variants than 5-$HT_{4(a)}$ receptor (227 vertebrate species examined, Table 1). In addition to these common palmitoylation sites among 5-HT_4 receptor splice variants, 5-$HT_{4(a)}$ receptor-specific C-terminal palmitoylation site Cys386 and PDZ ligands are almost completely conserved in known 5-$HT_{4(a)}$-type splice variants (Table 1). This palmitoylation site is exceptionally absent only in sifaka 5-$HT_{4(a)}$ receptor in total 142 vertebrate species.

3.3 | 5-HT_7 receptor palmitoylation sites in various vertebrate species

Several isoforms exist in mammalian 5-HT_7 receptors.[28] Both humans and mouse possess 3 5-HT_7 receptor C-terminal splice variants. Dynamic palmitoylation of mouse 5-$HT_{7(a)}$ receptor regulates cAMP formation by activating adenylyl cyclase via a G_s-mediated signaling pathway.[24] Mouse 5-$HT_{7(a)}$ (or 5-$HT_{7\ \text{isoform}\ 1}$) receptor holds 3 palmitoylation sites at Cys404, Cys438, and Cys441 (Figure 3B). Mouse 5-HT_7 receptor Cys404 corresponds to human 5-HT_7 receptor Cys401, which is commonly conserved in all splice variants of vertebrate 5-HT_7 receptor orthologs (240 vertebrate species examined, Table 1). In contrast to this common palmitoylation site, cysteine residue corresponding to mouse 5-$HT_{7(a)}$ receptor Cys438 is specifically found only in 2 species, mouse and saltwater crocodile (Table 1). In other 114 vertebrate species, Ser dominantly exists at the corresponding sites, which is substituted to Ala, Thr, or Pro in some species.



(A)

5-HT_{1A} orthologs	Human	Mouse	Chicken	Anole	Frog	Zebrafish
Human	100%	88%	80%	71%	72%	75%
Mouse		100%	79%	68%	69%	73%
Chicken			100%	69%	72%	77%
Anole				100%	65%	67%
Frog					100%	70%
Zebrafish						100%

(B)

5-$HT_{4(a)}$ orthologs	Human	Mouse	Chicken	Anole	Frog	Zebrafish
Human	100%	94%	89%	88%	85%	76%
Mouse		100%	86%	86%	83%	73%
Chicken			100%	92%	90%	77%
Anole				100%	89%	76%
Frog					100%	78%
Zebrafish						100%

(C)

5-$HT_{7(a)}$ orthologs	Human	Mouse	Chicken	Anole	Frog	Zebrafish (5-HT_7)
Human	100%	95%	80%	70%	74%	78%
Mouse		100%	79%	70%	74%	78%
Chicken			100%	74%	73%	77%
Anole				100%	77%	70%
Frog					100%	70%
Zebrafish (5-HT_7)						100%

FIGURE 2 The BLAST alignments of vertebrate 5-HT receptors. A, 5-HT_{1A} receptor orthologs; B, 5-$HT_{4(a)}$ receptor orthologs; C, 5-$HT_{7(a)}$ receptor orthologs. Percent identity among orthologs across any 2 species was obtained by performing BLAST search (with BLOSUM62) with full-length amino acid sequences of vertebrate 5-HT receptor orthologs. *Homo sapiens* (human being), *Mus musculus* (mouse), *Gallus gallus* (chicken), *Anolis carolinensis* (green anole), *Xenopus laevis* (African clawed frog), *Danio rerio* (zebrafish) are compared as representative of each vertebrate class

The class Mammalia recognizes 3 subclasses: the Prototheria (platypus and several species of echidna), the Metatheria (extant Marsupialia, eg, koala), and the Eutheria (extant Placentalia, eg, mouse) (Figure 4A). Eutherian (placental) mammals further comprise 4 superorders; Afrotheria, Xenarthra, Laurasiatheria, and Euarchontoglires. All of the eutherian species diverged from the same root around 100 million years ago in the early Cretaceous period.[31,32] Mouse 5-$HT_{7(a)}$ receptor Cys441 corresponds to human 5-$HT_{7(a)}$ receptor Cys438, which is extremely conserved in almost all eutherian 5-$HT_{7(a)}$-type splice variants (83 eutherian species examined, Table 1). The site is exceptionally substituted only in 2 species, minke whale and armadillo. Moreover, many molecular studies based on DNA analysis have supported an integrated classification as the magorder Boreoeutheria.[31–33] The Boreoeutheria is a clade that is composed of 2 superordinal sister taxa Laurasiatheria (cat, etc.) and Euarchontoglires (humans, mouse, etc.). Our analysis showed that 81

(A)

mouse 5-HT$_4$ receptor splice variants

isoform X1/(b): 388 aa (C328, C329)
LIILCCDDERYKRPPILGQTVPCSTTTINGSTHVLRDTVECGGQWESRCHLTATSPLVAAQPSDT

isoform X2/(a): 387 aa (C328, C329, C386)
LIILCCDDERYKRPPILGQTVPCSTTTINGSTHVLRYTVLHSGHHQELEKLPIHNDPESLESCF

isoform X3: 377 aa (C328, C329)
LIILCCDDERYKRPPILGQTVPCSTTTINGSTHVLRTSDYDTYLQSLGSDKVSI

isoform X4/(e): 371 aa (C328, C329)
LIILCCDDERYKRPPILGQTVPCSTTTINGSTHVLSFPLLFRNRPVPV

isoform X5: 368 aa (C328, C329)
LIILCCDDERYKRPPILGQTVPCSTTTINGSTHVLRAGGDQLVTP

(B)

mouse 5-HT$_7$ receptor splice variants

isoform 1/(a): 448 aa (C404, C438, C441)
LLQCQYRNINRKLSAAGMHEALKLAERPERSEFVLQNCDHCGKKGHDT

isoform 2: 470 aa (C404)
LLQCQYRNINRKLSAAGMHEALKLAERPERSEFVLMTGASGVQKALENLPWGNGVNTGIKAVNSVALTKL

isoform 3: 435 aa (C404)
LLQCQYRNINRKLSAAGMHEALKLAERPERSEFVL

human 5-HT$_7$ receptor splice variants

isoform (a): 445 aa (C401, A435, C438)
LLQCQYRNINRKLSAAGMHEALKLAERPERPEFVLQNADYCRKKGHDS

isoform (b): 432 aa (C401)
LLQCQYRNINRKLSAAGMHEALKLAERPERPEFVL

isoform (d): 479 aa (C401, C435(?))
LLQCQYRNINRKLSAAGMHEALKLAERPERPEFVLRACTRRVLLRPEKRPPVSVWVLQSPDHHNWLADKM
LTTVEKKVMIHD

FIGURE 3 Palmitoylation sites in the 5-HT receptor splice variants. A, C-terminal sequence alignment of the mouse 5-HT$_4$ receptor splice variants; B, C-terminal sequence alignments of the mouse and the human 5-HT$_7$ receptor splice variants. Consensus amino acid sequence among splice variants is underlined. Palmitoylation sites are marked in red. The box shows the canonical class I PDZ ligand specifically located on the C-terminus of 5-HT$_{4(a)}$ receptor

5-HT$_{7(a)}$ receptor orthologs contain the corresponding cysteine residue in total 82 boreoeutherian species (Figure 4B). In metatherians, opossum holds cysteine residue at the corresponding site, whereas Tasmanian devil and koala lack the palmitoylation site. The serine residue is shared at the corresponding site in Tasmanian devil, koala, platypus, sauropsids (all birds and all reptiles), and all amphibians. 5-HT$_{7(a)}$-type splice variants have not been detected in fishes.

4 | DISCUSSION

Sequence comparison of 5-HT receptor orthologs made it possible to clarify the process of acquisition, conservation, substitution, or loss of these protein modification sites in vertebrate evolution. As described above, sea lamprey 5-HT$_{1A}$ receptor ortholog has completely same sequence with humans in its C-terminus "-IIK**C**KF**C**RQ-stop." There exists no palmitoylation site in nonvertebrate chordates such as lancelet and sea squirt. Thus, acquisition event of these palmitoylation sites in the 5-HT$_{1A}$ receptor may occur in the common vertebrate ancestor around 500 million years ago in the late Cambrian to the early Ordovician periods. These sites are evolutionarily conserved against mutation pressure throughout vertebrate species. They have been partly lost later in limited species. Conserved expression pattern of 5-HT$_{1A}$ receptor orthologs was observed in most of the brain regions between sea lamprey and other vertebrates.[34] In conclusion, C-terminal palmitoylation sites of 5-HT$_{1A}$ receptor, which is likely to play crucial roles in the vertebrate central nervous system, are completely conserved in the vertebrate lineage from the superclass Agnatha (jawless fishes) to the superclass Gnathostomata (jawed vertebrate).

Similarly, all 5-HT$_4$ receptor splice variants have the common palmitoylation sites. Even the lancelet 5-HT$_4$ receptor ortholog

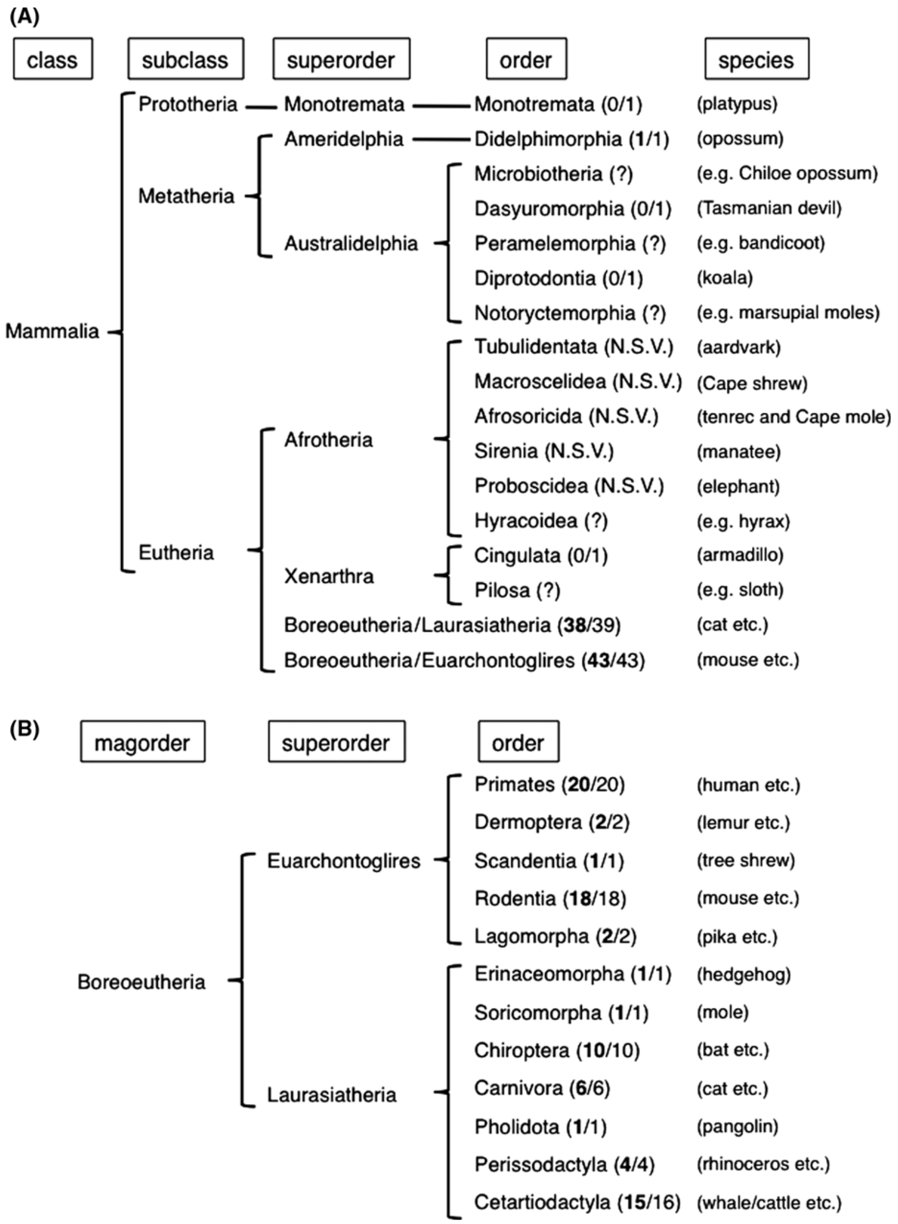


FIGURE 4 Conserved palmitoylation motifs in the mammalian 5-HT$_{7(a)}$ receptors. A, Classification of mammalian species and existence of C-terminal putative palmitoylation site corresponding to mouse 5-HT$_{7(a)}$ receptor Cys441/human 5-HT$_{7(a)}$ receptor Cys438. Numbers of putative palmitoylation site-containing species (shown in bold) per those of examined species in 17 reported mammalian orders are indicated. "?" means that sequence information is currently unavailable for indicated orders. N. S. V.: *n*o information has been reported on 5-HT$_{7(a)}$-type *s*plice *v*ariant sequence yet; B, Classification of boreoeutherian species and existence of C-terminal putative palmitoylation site corresponding to mouse 5-HT$_{7(a)}$ receptor Cys441/human 5-HT$_{7(a)}$ receptor Cys438. Numbers of putative palmitoylation site-containing species (shown in bold) per those of examined species in 12 mammalian orders are indicated

possesses a primitive palmitoylation sequence "-RILS**C**WS**CC**DVDN-." Repetitive deletions and substitutions have presumably occurred to generate the common palmitoylation motif for vertebrate 5-HT$_4$ receptors "typically, -IIL**CC**GDE-," followed by Gly to Asp mutation in mammals "typically, -IIL**CC**DDE-" (Figure 5A). 5-HT$_{4(a)}$ receptor-specific C-terminal palmitoylation site corresponding to mouse/human Cys386 had appeared in the ancestor of bony fishes. 5-HT$_{4(a)}$-type splice variants have not been reported for cartilaginous fishes, and no information about any 5-HT$_4$ receptor is currently available for cyclostomes (hagfishes and lampreys). Future analysis about 5-HT$_4$ receptor orthologs of hagfishes, lampreys, more sharks, and rays will reveal the detailed history of acquisition and establishment of palmitoylation sites among 5-HT$_4$ receptor variants. Reversible palmitoylation and depalmitoylation cycle of this 5-HT$_{4(a)}$ receptor-specific site may be critical for dynamic regulation of 5-HT$_{4(a)}$ receptor localization and membrane trafficking through its binding to PDZ domain-containing scaffold proteins.

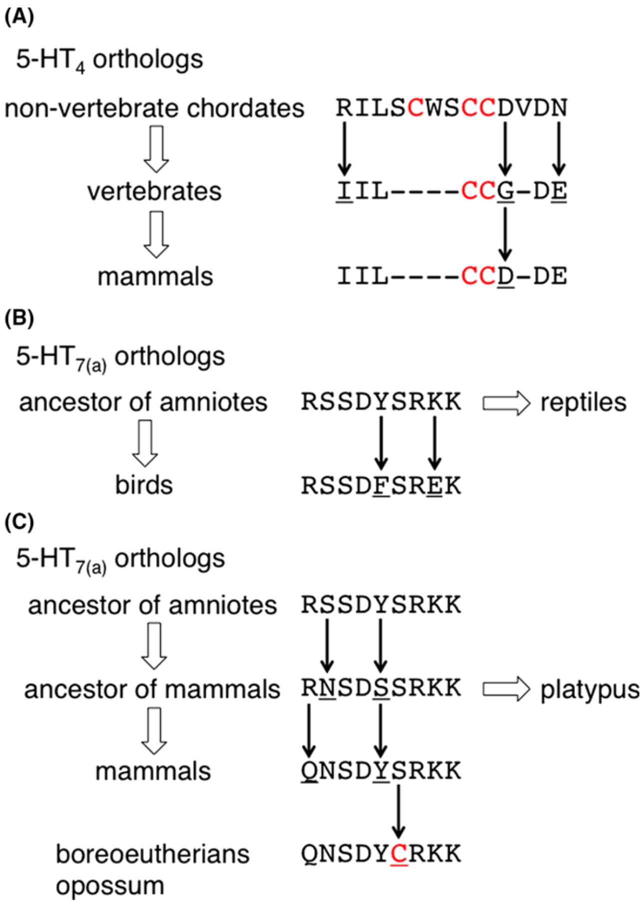


FIGURE 5 Evolutionary models of stepwise mutations around palmitoylation sites in vertebrate 5-HT receptor orthologs. A, A model of mutations in 5-HT_4 receptor orthologs developed from a putative ancestor of chordates to mammals. Sequence of the ancestral chordate is predicted from nonvertebrate chordate, lancelet; B, A model of mutations in 5-$HT_{7(a)}$ receptor orthologs developed from a putative ancestor of amniotes to birds. Sequence of the ancestral amniote is predicted from reptiles; C, A model of mutations in 5-$HT_{7(a)}$ receptor orthologs developed from a putative ancestor of amniotes to mammals. Sequence of the ancestral mammal is predicted from platypus. (-): amino acid deletions; arrows: amino acid changes. Putative palmitoylation sites are marked in red

Likewise, all tetrapod 5-HT_7 receptor orthologs have the common palmitoylation site corresponding to mouse Cys404/human Cys401. The origin and development of the palmitoylation motifs in 5-HT_7 receptors still remains unclear because there is no available information on lancelet, sea squirt, and cyclostomes. By contrast, 5-$HT_{7(a)}$-type variant appeared in the ancestor of tetrapods, in which serine residue locates at the corresponding site to mouse Cys441/human Cys438. Both reptiles and birds hold serine residue at the corresponding site (Figure 5B). Cysteine residue at the palmitoylation site on 5-$HT_{7(a)}$ receptor C-terminus is almost completely conserved in all orders belonging to boreoeutherians (Figure 5C). Highly conserved 5-$HT_{7(a)}$ receptor C-terminal motif around the palmitoylation site in boreoeutherian lineages strongly suggests that this C-terminal motif was shared at least in the common ancestor of boreoeutherians (Figure 4B). This feature was acquired at a certain point during the mammalian evolution. It is still difficult to speculate when acquisition events of this 5-$HT_{7(a)}$ receptor-specific modification site initially took place, because 5-$HT_{7(a)}$-type variants have not been identified for afrotherian mammals (Figure 4A). On the other hand, Gray short-tailed opossum possesses cysteine residue at the corresponding site in its 5-$HT_{7(a)}$ receptor. This sequence enables us to predict that a replacement of Ser to Cys specifically happened in this marsupial species and in the common ancestor of boreoeutherians. In contrast, Tasmanian devil and koala hold serine residue there. The difference may reflect the marsupial divergence history in Australasia and the Americas. Another possibility is that 5-$HT_{7(a)}$ receptor-specific palmitoylation site was initially established in the common ancestor between the metatherian and the eutherian lineages and has been lost later in afrotherians, xenarthrans, and most metatherian species.

Further accumulation of sequence data will fill in the blanks of the sequence list concerning vertebrate 5-$HT_{4(a)}$-type variants and tetrapod 5-$HT_{7(a)}$-type variants and will reveal the timeline of establishment and divergence of these palmitoylation sites-containing motifs in more detail. Especially, sequence information on urochordates (ascidians or sea squirts), cephalochordates (lancelets), and cyclostomes will clarify the initial acquisition of the mechanism of 5-HT receptor palmitoylation.

Dynamic regulation of 5-HT receptors made possible by reversible post-translational protein palmitoylation may be critical for more effective membrane localization and trafficking in refined functions of the vertebrate tissues. Previous researches have revealed that many other GPCRs are functionally regulated by their direct palmitoylation.[35,36] So far, we have shown that palmitoylation sites of ionotropic glutamate receptors (iGluRs), the major excitatory neurotransmitter receptors in vertebrate central nervous system, and those of iGluRs-binding proteins are extremely conserved in various species of whole vertebrate.[37–39] Furthermore, palmitoylation sites of hyperpolarization-activated cyclic nucleotide-gated (HCN)-2 channel [40] and water channel aquaporin (AQP)-4[41] are conserved across vertebrates. By contrast, palmitoylation sites of dopamine D_1-like, D_1 and D_5, receptors, are broadly found in vertebrates and invertebrates.[42] Future genome analysis would permit us to understand detailed history of acquisition and refinement of the post-translational protein palmitoylation in vertebrates.

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CONFLICT OF INTEREST

The authors declare no conflict of interest for this article.

DATA REPOSITORY

The authors do not deposit any data because this manuscript does not use data.

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INFORMED CONSENT

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REGISTRY AND THE REGISTRATION NO. OF THE STUDY/TRIAL

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AUTHOR CONTRIBUTION

TK executed data collection and analyzed the results. TH conceived this project, analyzed the results, and wrote the manuscript.

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