LETTER

Largest Vertebrate Vomeronasal Type 1 Receptor Gene Repertoire in the Semiaquatic Platypus

Wendy E. Grus, Peng Shi,[1] *and Jianzhi Zhang*

Department of Ecology and Evolutionary Biology, University of Michigan

Vertebrate vomeronasal chemoreception plays important roles in many aspects of an organism's daily life, such as mating, territoriality, and foraging. Vomeronasal type 1 receptors (V1Rs) and vomeronasal type 2 receptors (V2Rs), 2 large families of G protein–coupled receptors, serve as vomeronasal receptors to bind to various pheromones and odorants. Contrary to the previous observations of reduced olfaction in aquatic and semiaquatic mammals, we here report the surprising finding that the platypus, a semiaquatic monotreme, has the largest V1R repertoire and nearly largest combined repertoire of V1Rs and V2Rs of all vertebrates surveyed, with 270 intact genes and 579 pseudogenes in the V1R family and 15 intact genes, 55 potentially intact genes, and 57 pseudogenes in the V2R family. Phylogenetic analysis shows a remarkable expansion of the V1R repertoire and a moderate expansion of the V2R repertoire in platypus since the separation of monotremes from placentals and marsupials. Our results challenge the view that olfaction is unimportant to aquatic mammals and call for further study into the role of vomeronasal reception in platypus physiology and behavior.

Vertebrates use olfaction to locate food, avoid predators, and identify mates, among other activities. Most vertebrates have 2 olfactory systems: the main olfactory system (MOS) and the vomeronasal system (VNS). The MOS is traditionally thought to detect environmental odorants, whereas the VNS recognizes intraspecific pheromonal cues, although this distinction has been blurred by recent reports that both systems can perceive both types of signals (Restrepo et al. 2004; Baxi et al. 2006). Nonetheless, a distinct set of chemoreceptors is expressed in each system: odorant receptors (ORs) and trace amine–associated receptors (TAARs) in the MOS and vomeronasal type 1 receptors (V1Rs) and vomeronasal type 2 receptors (V2Rs) in the VNS (Mombaerts 2004; Liberles and Buck 2006). These receptors form 4 evolutionarily unrelated large families of 7 transmembrane G protein–coupled receptors. Our previous study of 5 orders of placental and marsupial mammals showed that the among-species size variation of the V1R repertoire exceeds that of all other mammalian protein families (Grus et al. 2005). Recently, the genome sequence of the platypus *Ornithorhynchus anatinus*, a semiaquatic monotreme mammal, became available. It is of significant interest to examine platypus V1Rs for 2 reasons. First, the platypus represents the final of the 3 major mammalian groups (placentals, marsupials, and monotremes) whose V1Rs have yet to be examined. Second, olfaction is widely believed to be unimportant to aquatic mammals. For example, morphological components of MOS and VNS are absent or highly degenerated in cetaceans (whales, porpoises, and dolphins) and sirenians (manatees and Dugongs) (Oelschlager 1992; Meisami and Bhatnagar 1998). The platypus data open the door for a genomic test of the (un)importance of olfaction to aquatic mammals, as all previously studied mammalian V1Rs were from terrestrials.

Using TBlastN searches with known mammalian V1Rs as query sequences, we identified from the platypus genome sequence 270 intact genes and 579 pseudogenes of the V1R family (table 1). The gene identification procedure is detailed in Methods. To understand the evolution of platypus V1Rs, we reconstructed a gene tree containing all platypus intact V1Rs and 232 representative V1Rs of other vertebrates (fig. 1*A*). These representatives cover all major lineages in the vertebrate V1R tree (Shi and Zhang 2007). The obtained gene tree shows that platypus V1Rs form 3 separate platypus-specific clades, 2 with high bootstrap support, revealing remarkable platypus-specific expansions of 3 V1R lineages after monotremes diverged from the common ancestor of placentals and marsupials (fig. 1*A*). Because the draft platypus genome sequence is organized by contig rather than assembled into chromosomes, it is important to ensure that closely related V1Rs are not allelic variants. To be conservative, V1Rs that are >98% identical in amino acid sequence are considered allelic variants (Zhang et al. 2004) and only one is considered in our study. The lack of chromosomal assembly prevents us from detecting V1R genomic clusters that are prevalent in rodents (Lane et al. 2002; Rodriguez et al. 2002; Grus and Zhang 2004; Lane et al. 2004; Zhang et al. 2004). However, many subclades in figure 1*A* are comprised of V1R genes from a single contig, indicating that there is at least some chromosomal clustering of closely related V1R genes in the platypus genome and that tandem gene duplication was a primary mechanism underlying the platypus V1R repertoire expansion. V1R expansions are thought to be correlated to repetitive element density in the V1R genomic regions (Lane et al. 2002, 2004; Grus et al. 2005). Future analyses based on a more accurate genome assembly will likely reveal if any type of repetitive element has a particularly high density in the platypus genomic regions containing V1Rs.

The platypus V1R repertoire has 83 more intact genes than the largest previously identified mammalian V1R repertoire (187 in mouse, Shi et al. 2005). Hence, the mammalian V1R repertoire has an even larger size variation (~34-fold difference between platypus and dog) than was previously reported (~23-fold between mouse and

[1] Present address: Department of Molecular Genetics, German Institute of Human Nutrition Potsdam-Rehbrucke, Nuthetal 14558, Germany.

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E-mail: jianzhi@umich.edu.

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Table 1
Sizes of Nasal Chemosensory Receptor Gene Repertoires in Vertebrates

Species	VIRs	V2Rs	ORs	TAARs
Human	5 (115)[a]	0 (20)[c]	388 (414)[d]	6 (3)[h]
Mouse	187 (121)[a]	121 (158)[c]	1037 (354)[d]	15 (1)[h]
Rat	106 (66)[a]	79 (142)[c]	1201 (292)[e]	17 (2)[h]
Dog	8 (33)[a]	0 (9)[c]	876 (326)[f]	2 (2)[b]
Cow	40 (45)[a]	0 (16)[c]	970 (1159)[g]	17 (9)[b]
Opossum	98 (30)[a]	86 (79)[c]	901 (618)[f]	22 (0)[b]
Platypus	270 (579)[b]	15 (112)[b]	261 (315)[b]	4 (1)[b]
Chicken	0 (0)[a]	0 (0)[a]	82 (476)[d]	3 (0)[b]
Frog	21 (2)[a]	249 (448)[a]	410 (478)[d]	2 (1)[b]
Zebrafish	2 (0)[a]	44 (8)[a]	102 (35)[d]	57 (40)[i]

NOTE.—Shown are the numbers of intact genes. The numbers of nonintact genes, including potentially intact genes and pseudogenes, are given in parenthesis.

[a] Shi and Zhang (2007).
[b] This study.
[c] Young and Trask (2007).
[d] Niimura and Nei (2006).
[e] Quignon et al. (2005).
[f] Aloni et al. (2006).
[g] Niimura and Nei (2007).
[h] Lindemann et al. (2005).
[i] Gloriam et al. (2005).

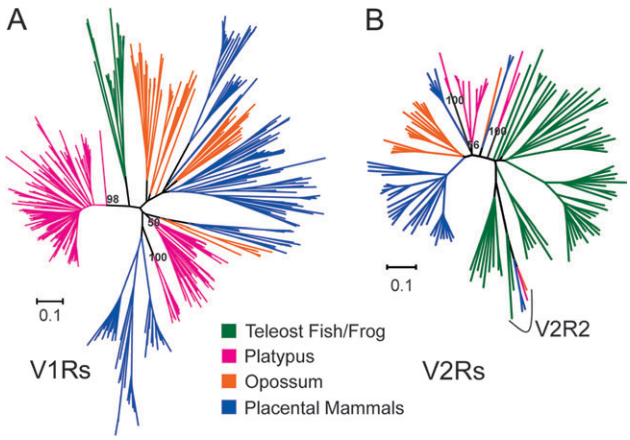

FIG. 1.—Phylogenetic trees of vertebrate intact (*A*) V1Rs and (*B*) V2Rs. The V1R tree includes all 270 platypus receptors and 232 representative receptors from the mouse, rat, dog, cow, opossum, frog, and zebrafish. The V2R tree includes all 15 platypus receptors and 124 representative receptors from the mouse, rat, opossum, frog, and zebrafish, as the dog and cow do not have V2Rs. Pink branches are platypus genes, orange are opossum genes, blue are placental mammal genes, and green are teleost fish and frog genes. Scale bars indicate 0.1 amino acid substitution per site. Bootstrap percentages for clades of platypus receptors are indicated.

dog, Grus et al. 2005). However, the enormous V1R repertoire in platypus is not entirely unexpected. Although previous studies showed the absence or degeneration of MOS and VNS morphological components in some aquatic mammals (Meisami and Bhatnagar 1998), this loss is not observed in the platypus. Instead, VNS complexity in platypus, snakes, and lizards has been ranked highest among all vertebrates (Wysocki 1979). We previously showed a strong positive correlation between the morphological complexity of the vomeronasal organ (VNO) and the number of intact V1R genes across a diverse array of mammals (Grus et al. 2005). The large V1R repertoire in platypus is consistent with this correlation (fig. 2). Although complete V1R repertoires are known for only 12 vertebrates (Shi and Zhang 2007 and this study), we expect that the platypus repertoire will remain one of the largest even when additional vertebrates are examined in the future because of the strong correlation between VNO complexity and V1R repertoire size.

Using an approach similar to Yang et al. (2005), we identified 15 intact genes, 55 potentially intact genes, and 57 pseudogenes of the V2R family from the platypus genome sequence. A potentially intact gene has a partial sequence due to the incompleteness of the genome sequence but has an open reading frame in the available sequence. This "partial sequence" problem did not affect the V1Rs in part because they have a single coding exon. Similarly, we reconstructed the phylogeny of the platypus intact V2Rs with 124 representative V2Rs from other vertebrates (Shi and Zhang 2007). Ten of the 15 platypus V2Rs cluster into a single platypus-specific clade, whereas 4 other V2Rs form 2 clusters and one V2R belongs to the V2R2 clade, which has representatives from all vertebrates that have V2Rs (fig. 1*B*, Yang et al. 2005; Shi and Zhang 2007; Young and Trask 2007). Thus, at least one V2R lineage experienced a relatively large expansion in platypus after monotremes diverged from placentals and marsupials.

In contrast to V1Rs, functional V2R repertoire size is not correlated with VNO complexity (supplementary fig. S1, Supplementary Material online). Based on morphology and immunohistochemistry, Takigami et al. (2004) classified mammalian VNOs into 2 types: segregated and uniform. We recently showed a clear distinction in V2Rs between the 2 types of VNOs (Shi and Zhang 2007): species with segregated VNOs have functional V2Rs, whereas those with uniform VNOs do not. The platypus VNO has not been characterized in this manner, but our result predicts that it belongs to the segregated type.

Previous studies suggested that V1Rs detect airborne volatiles, whereas V2Rs detect water-soluble molecules (Boschat et al. 2002; Emes et al. 2004; Leinders-Zufall et al. 2004; Kimoto et al. 2005). Indeed, comparative genomic analysis identified a shift of vomeronasal receptor types from V2Rs to V1Rs during the vertebrate transition from water to land (Shi and Zhang 2007). Unlike most mammals, which have a nasal VNO opening with a variable amount of oral input into the nasal cavity, the platypus's complex VNO has an exclusively oral opening, similar to a reptilian VNO. Despite this morphological difference, the platypus VNS likely uses the same VNS signal transduction pathway conserved throughout vertebrate evolution (Grus and Zhang 2006), as other components of the signal transduction pathway (e.g., the Trpc2 channel) are also found in the platypus genome sequence (data not shown). With the eyes, ears, and nostrils closed while the platypus is underwater (Griffiths 1978), both types of vomeronasal receptors would be exposed to water-soluble molecules. Thus, one may predict that platypus should have experienced a backward evolutionary change having more V2Rs but fewer V1Rs compared with terrestrial mammals. This, however, is not the case. One potential reason is that the platypus still breaths air and its VNS must still play an important role in recognizing airborne molecules. It is notable that although platypus's V2R repertoire

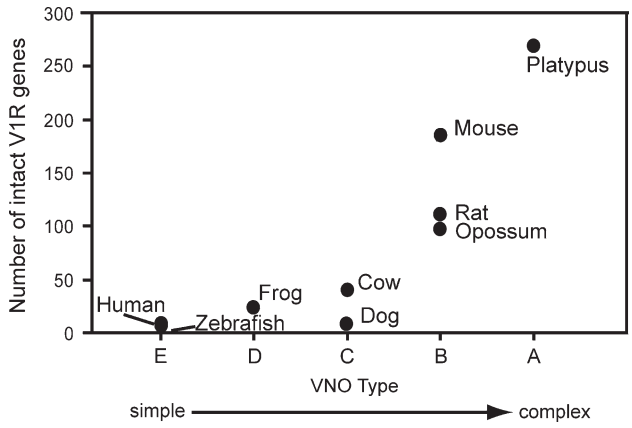

FIG. 2.—Positive correlation of VNO complexity with V1R repertoire size. The number of intact V1Rs is plotted for animals of increasing VNO complexity (E–A) based on morphological categories described in Takami (2002). The 270 platypus V1Rs are described in this paper, whereas the repertoire sizes for human (5), zebrafish (2), frog (21), dog (8), cow (40), opossum (98), rat (106), and mouse (187) were previously described (Shi and Zhang 2007).

is not large, the total number of intact V1Rs and V2Rs in platypus is 285, nearly highest among all vertebrates surveyed (table 1). If at least half of the potentially intact platypus V2Rs are actually intact, which is quite likely given the current quality of the genome sequence and the length of each partial V2R sequence identified ($\geq$234 nt), the total number of platypus V1Rs and V2Rs becomes highest among all vertebrates (table 1).

Given the enormous repertoire size of platypus's VNS receptors, it is interesting to examine whether the opposite is true for its MOS receptors. We raise this possibility for 3 related reasons. First, previous studies showed that morphological and/or genetic components of the MOS have been lost or reduced independently in aquatic mammals, such as cetaceans, manatees, and seals (Oelschlager 1992; Freitag et al. 1998; Meisami and Bhatnagar 1998). Characterization of the monotreme brain also found that the platypus has a less complex olfactory bulb and olfactory tubercle than terrestrial monotremes (Ashwell 2006a, 2006b). Second, in most snakes, particularly marine snakes, the VNS is the dominant chemosensory system and the MOS is very reduced (Evans 2003). In other words, the complex VNS might compensate for a reduced MOS. Indeed, morphological characterization of the platypus brain shows that its accessory olfactory bulb, which is part of the VNS, is larger than its main olfactory bulb, part of the MOS (Ashwell KW, personal communication). Third, whereas terrestrial monotremes rely on their MOS to detect food, platypuses forage for their food underwater with eyes, ears, and nostrils closed (Griffiths 1978). In some aquatic mammals, the loss of olfactory ability is compensated by the enhancement of a different sense, such as audition. Indeed, the monotreme sense of electroreception appears to be more defined in the platypus than in terrestrial monotremes (Pettigrew 1999), and platypuses are thought to use electroreception to locate prey underwater (Scheich et al. 1986).

Applying the same method used for identifying V1Rs, we detected 261 intact genes, 94 potentially intact genes, and 221 pseudogenes of the OR family from the platypus genome sequence. The number of intact ORs in platypus is much smaller than that in human, mouse, rat, dog, and frog, although it is greater than that in chicken and fish (reviewed in Niimura and Nei 2006), suggesting that as a mammal, platypus has a relatively small OR repertoire (table 1). It is notable that the proportion of intact genes in the platypus OR family (45%) is similar to that found in humans, which have a degenerating MOS, and much lower than that (75–80%) in mouse, rat, and dog (Niimura and Nei 2006). Whereas the human OR repertoire is thought to be degenerating due to acquisition of trichromatic vision in catarrhine primates ~23 MYA (Gilad et al. 2004), fossil evidence suggests that platypuses are a specialized lineage of monotremes that have been semiaquatic since the Mesozoic (Musser 2003), leaving at least 65 Myr for platypus olfactory receptor genes to degenerate with increased aquatic specialization. Furthermore, a fossil platypus from the Miocene shares the extant platypus's reduced morphology of main olfactory brain regions (Macrini et al. 2006). Because the platypus lineage became aquatic a long time ago, many platypus OR pseudogenes may have degenerated beyond detection, leading to an overestimate of the proportion of intact OR genes.

Previous studies of vertebrate ORs describe 2 classes of ORs: class I is more prevalent in aquatic vertebrates, whereas class II is dominant in terrestrial vertebrates (Freitag et al. 1998; Shi and Zhang 2007). Despite the return to an aquatic life by the platypus, only 11.5% (30 of 261) of the OR genes are the class I aquatic type, which is only slightly higher than that in mouse (11%, Niimura and Nei 2006). This finding may not be unexpected because the platypus nostrils are closed underwater (Griffiths 1978) and the main olfactory epithelium is not exposed to the water. We also examined TAARs, the second class of MOS chemoreceptors (Liberles and Buck 2006), and identified 4 intact genes and one pseudogene from the platypus genome. The TAAR repertoire size is also smaller in platypus than in artiodactyls, rodents, and primates (table 1). Because the platypus VNO opens into the oral cavity, the vomeronasal receptors might also compensate for taste receptors. Preliminary screening of the platypus genome sequence for the T1R sweet and umami receptor family and T2R bitter receptor family reveals a substantively reduced T2R repertoire size in platypus (Shi P, Zhang J, unpublished data) than in other mammals (Shi and Zhang 2006).

In sum, the investigation of the third major lineage of mammals identified an unexpectedly large repertoire of vomeronasal receptors in the semiaquatic platypus. This finding challenges the current view that olfaction is unimportant to aquatic mammals and calls for study of the role of vomeronasal reception in platypus physiology and behavior as no study has investigated vomeronasal-mediated behavior in platypus. The surprising diversity of vomeronasal sensitivity across vertebrates provides an invaluable resource for us to learn how nature solves different types of sensory tasks in drastically different environments.

Methods

Platypus V1Rs were identified first using TBlastN searches on the high-quality platypus genome sequence

(6× coverage) available from the Ensembl Database (http://www.ensembl.org/Ornithorhynchus_anatinus/index.html). Previously described mammalian V1R genes were used as query sequences. Next, BlastN searches were done on the same platypus genome sequence with the platypus V1R nucleotide sequences from the previous step as query sequences. A receptor having a complete open reading frame across the middle 13 protein domains (7 transmembrane, 3 extracellular, and 3 intracellular) was considered intact and is most likely functional. V1R pseudogenes were identified by premature stop codons or incomplete sequence across the 13 middle protein domains of the 15 domains. The N-terminal extracellular and C-terminal intracellular domains were not considered in our criterion because they are highly variable in sequence length. A similar 2-step procedure was used to identify ORs from platypus and TAAR genes from frog, chicken, platypus, opossum, cow, and dog. Platypus V2Rs were identified using TBlastN searches on the platypus genome sequences. The more complex structure of the V2Rs requires a few additional steps in gene identification. The computational pipeline for identifying V2Rs from genomic sequence has been outlined in a previous study (Yang et al. 2005). To ensure that the sequences represented independent loci rather than allelic variation, we required that 2 sequences be at least 2% different at the protein sequence level to be considered as 2 genes. Protein sequences of the newly identified platypus V1Rs, V2Rs, ORs, and TARRs are supplied in Supplementary data sets 1–4 (Supplementary Material online), respectively. The V1Rs and V2Rs were aligned by ClustalX (Thompson et al. 1997) with manual adjustment. Phylogenetic trees were constructed using the Neighbor-Joining method (Saitou and Nei 1987) with Poisson corrected protein distances and 1000 bootstrap replications (Felsenstein 1985). MEGA3.1 (Kumar et al. 2004) was used for these evolutionary analyses.

Supplementary Material

Supplementary data sets 1–4 and Supplementary figure S1 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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