Origin of the Genetic Components of the Vomeronasal System in the Common Ancestor of all Extant Vertebrates

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Comparative genomics provides a valuable tool for inferring the evolutionary history of physiological systems, particularly when this information is difficult to ascertain by morphological traits. One such example is the vomeronasal system (VNS), a vertebrate nasal chemosensory system that is responsible for detecting intraspecific pheromonal cues as well as environmental odorants. The morphological components of the VNS are found only in tetrapods, but the genetic components of the system have been found in teleost fish, in addition to tetrapods. To determine when the genetic components of the VNS originated, we searched for the VNS-specific genes in the genomes of two early diverging vertebrate lineages: the sea lamprey from jawless fishes and the elephant shark from cartilaginous fishes. Genes encoding vomeronasal type 1 receptors (V1Rs) and Trpc2, two components of the vomeronasal signaling pathway, are present in the sea lamprey genome, and both are expressed in the olfactory organ, revealing that the genetic components of the present-day VNS existed in the common ancestor of all extant vertebrates. Additionally, all three VNS genes, Trpc2, V1Rs, and vomeronasal type 2 receptors (V2Rs), are found in the elephant shark genome. Because V1Rs and V2Rs are related to two families of taste receptors, we also searched the early diverging vertebrate genomes for taste system genes and found them in the shark genome but not in the lamprey. Coupled with known distributions of the genetic components of the vertebrate main olfactory system, our results suggest staggered origins of vertebrate sensory systems. These findings are important for understanding the evolution of vertebrate sensory systems and illustrate the utility of the genome sequences of early diverging vertebrates for uncovering the evolution of vertebrate-specific traits.

Introduction

Comparative genomics is a valuable tool for understanding the evolution of physiological systems (Arendt 2003; Zhang and Webb 2003; Okabe and Graham 2004; Go et al. 2005; Serb and Oakley 2005) because it provides information about the phylogenetic distribution of system-specific genetic components (Wray and Abouheif 1998; Serb and Oakley 2005). This approach allows a more complete understanding of the evolution of physiological systems than was previously available with only morphological characterization. For example, this approach has been used to identify a common origin for the invertebrate and vertebrate visual systems, two systems that were thought to have distinct origins (Gehring 1996; Arendt et al. 2004) and to identify how lepidopteran scales and the tetrapod parathyroid gland evolved in their respective lineages (Galant et al. 1998; Okabe and Graham 2004). Using this approach, we here investigate the evolutionary history of the vertebrate vomeronasal system (VNS) through determining when the VNS-specific genes arose in chordate evolution.

The VNS is one of the two nasal chemosensory systems found in many vertebrates (the other being the main olfactory system or MOS), and it is responsible for detecting intraspecific pheromonal cues as well as some environmental odorants (Grus and Zhang 2006). Prior to the identification of the VNS genetic components, the evolutionary history of the VNS was inferred based on the phylogenetic distribution of VNS morphological characteristics, the vomeronasal organ (VNO), an organ in the nasal cavity with sensory neurons expressing vomeronasal receptors, and the accessory olfactory bulb, which is the part of the brain excited by the VNO sensory neurons (Bertmar 1981; Eisthen 1992, 1997; Dulka 1993). Because these morphological characters are found only in tetrapods, the VNS was thought to have evolved in the common ancestor of tetrapods as an adaptation to terrestrial living (Bertmar 1981). However, finer-scale studies of the morphological components, such as sensory neuron morphology, gave hints that the VNS might exist in an unrecognized form in teleost fish (Eisthen 1992; Dulka 1993). Furthermore, evidence of VNS development in larval amphibians and neotenic salamanders suggests that the VNS is not an adaptation to terrestrial life (Eisthen 2000; Jermakowicz et al. 2004). Therefore, an additional type of character became necessary to address the lingering possibility of the presence of the VNS in teleosts.

The solution came with the identification of VNS-specific genes, which are defined as the genetic components of the vomeronasal signal transduction pathway that are not used for other functions and include two receptor gene families and a channel protein gene. The two families of VNS-specific receptors, vomeronasal type 1 receptors (V1Rs) and type 2 receptors (V2Rs), were first identified from rodents (Dulac and Axel 1995; Herrada and Dulac 1997; Matsunami and Buck 1997; Ryba and Tirindelli 1997). Both families are 7-transmembrane-domain G protein-coupled receptors (GPCRs), but they are evolutionarily unrelated. Each type of receptor is coexpressed with a unique G protein (Dulac and Axel 1995; Ryba and Tirindelli 1997), and their expression is spatially segregated in tetrapods (Hagino-Yamagishi et al. 2004; Takigami et al. 2004; Date-Ito et al. 2008). It has been proposed that V1Rs and V2Rs are specialized for binding to air-borne molecules and water-soluble peptides, respectively (Boschat et al. 2002; Leinders-Zufall et al. 2004; Kimoto et al. 2005), although this functional separation may not be complete (Shi and Zhang 2007). In addition to the receptors, a calcium channel protein, Trpc2, was identified with specific expression in the VNO (Liman et al. 1999). The indispensable and sole function of Trpc2 in vomeronasal signal transduction is supported by studies of Trpc2-deficient mice, which show altered intraspecific interactions such as sex discrimination and male–male aggression (Leypold et al. 2002; Stowers et al. 2002;

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Kimchi et al. 2007), and by evolutionary studies that found catarrhine primates, which do not have a VNO, have a nonfunctional Trpc2 gene (Liman and Innan 2003; Zhang and Webb 2003).

Interestingly, despite the fact that the morphological traits of the VNS are found only in tetrapods, VNS-specific genes are present in teleost fish and exhibit expression patterns consistent with their involvement in a distinct teleost olfactory system (Cao et al. 1998; Naito et al. 1998; Dukes et al. 2004; Hansen et al. 2004; Pfister and Rodriguez 2005; Sato et al. 2005). These findings suggest that the genetic components of the VNS and possibly a primordial VNS existed in the common ancestor of teleosts and tetrapods (Grus and Zhang 2006). Because earlier diverging vertebrates, such as jawless fish and cartilaginous fish, have a single olfactory organ, one may not expect the presence of VNS-specific genes in these lineages. However, based on the findings that teleost fish have one organ but two distinct olfactory signaling pathways, it is possible that even earlier diverging vertebrates may possess the genetic components of the VNS. With the newly available genome sequences of a jawless fish (sea lamprey) and a cartilaginous fish (elephant shark), we explore the evolutionary history of the VNS-specific genes, hoping to shed light on the origin of the VNS.

Furthermore, because the genetic components of several chemosensory systems are homologous to each other, we can also study the relative timing of the origins of genes specific to different chemosensory systems. It is known that odorant receptors (ORs) and trace amine-associated receptors (TAARs), the two chemoreceptor families expressed in the teleost and tetrapod MOS (Buck and Axel 1991; Berghard and Dryer 1998; Liberles and Buck 2006), are not closely related to the V1Rs and V2Rs. Rather, both vomeronasal receptor families have closely related homologs in the vertebrate taste system: V1Rs are closely related to T2R bitter taste receptors (Adler et al. 2000) and V2Rs are closely related to T1R sweet and umami taste receptors (Hoon et al. 1999). We thus explore how the taste receptor families evolved in relation to the VNS receptors in earlier diverging vertebrates.

Materials and Methods

Computational Identification of VNS and Taste System Genes

TBlastN searches for V1R, V2R, Trpc2, T1R, T2R, and Trpm5 genes were conducted on the sea lamprey (*Petromyzon marinus*), elephant shark (*Callorhinchus milii*), tunicate (*Ciona intestinalis*), and amphioxus (*Branchiostoma floridae*) genome sequences. The 5.9× coverage sea lamprey genome sequence is available from Ensembl (http://pre.ensembl.org/Petromyzon_marinus/index.html). The 1.4× elephant shark genome sequence is available from the Institute of Molecular and Cellular Biology (http://esharkgenome.imcb.a-star.edu.sg/) (Venkatesh et al. 2007). The 11× tunicate genome sequence is available from the Joint Genome Institute (http://genome.jgi-psf.org/Cioin2/Cioin2.home.html). The 8.1× amphioxus genome sequence is available from the Joint Genome Institute (http://genome.jgi-psf.org/Brafl1/Brafl1.home.html). Zebrafish V1Rs, V2Rs, and Trpc2 (Pfister and Rodriguez 2005; Sato et al. 2005; Shi and Zhang 2007) were used as query sequences. Two zebrafish T1Rs (GenBank NP_001034614 and NP_001077325), two zebrafish T2Rs (GenBank NP_001034717 and NP_001018341), and mouse Trpm5 (GenBank AAI33713) were used as query sequences. Because the early diverging vertebrate and invertebrate chordate taxa are distantly related to the source species of the query sequences, we used an *E*-value cutoff of 10^{-1} in the TBlastN searches. The hits from these searches were then used as queries to Blast the GenBank protein database. A putative V1R gene was considered to be real if its best hit was a previously known V1R, and similar criteria were used for the other VNS and taste system genes.

Sequence Alignment and Phylogenetic Analysis

Gene sequences were aligned following protein sequence alignment by ClustalX (Thompson et al. 1997) and PRANK (Loytynoja and Goldman 2008) with manual adjustment. Phylogenetic trees were reconstructed using the Neighbor-Joining (NJ) method (Saitou and Nei 1987) with Poisson-corrected distances (Nei and Kumar 2000) and the maximum likelihood (ML) method implemented in PhyML (Guindon and Gascuel 2003), evaluated by 1,000 bootstrap replications (Felsenstein 1985).

Assessing Genome Sequence Coverage

Because the sea lamprey and elephant shark genome sequences are incomplete, we did a coverage quality analysis for each genome. To determine how complete the coverage is, we randomly picked 22 full-length nuclear sea lamprey mRNAs (supplementary data file 1, Supplementary Material online) from 132 available entries in GenBank. We Blasted these full-length mRNAs against the sea lamprey trace archives at NCBI (http://www.ncbi.nlm.nih.gov/Traces/home/). Based on a minimum identity match of 98%, we identified exons and estimated the number of missing exons from the sea lamprey genome for each gene. A similar quality assessment was done for the elephant shark genome. Twenty-three mRNAs from four chaemeriformes species (*C. milii*, *C. callorhynchus*, *Hydrolagus colliei*, and *Chimaera phantasma*; supplementary data file 1, Supplementary Material online) were Blasted against the elephant shark genome assembly. We identified exons and estimated the number of missing exons from the elephant shark genome. The number of missing exons was estimated by summing up the number of sequence gaps in the comparison between the genome sequence and the mRNA sequence. In the instances when these gaps are large, it is likely that the number of missing exons is underestimated.

Expression Patterns of Sea Lamprey Vomeronasal System Genes

Adult sea lampreys were obtained from Hammond Bay Biological Station (Millersberg, MI). They were

euthanized according to university animal care procedures (UCUCA #09470). Genomic DNA was isolated from fresh tissue with a DNeasy Tissue Kit (Qiagen, Valencia, CA). Sea lamprey tissues (testis, heart, tongue, and olfactory organ) were dissected out and frozen in an ethanol–dry ice bath. RNAqueous-4PCR (Ambion, Austin, TX) was used to extract RNA from the tissues, and RetroScript (Ambion) was used for cDNA synthesis.

Putative sea lamprey Trpc2 and V1R genes were amplified from both genomic DNA and cDNA. Based on an alignment of Trpc2 from zebrafish, frog, mouse, and dog, nested degenerate primers for Trpc2 were designed using CODEHOP (Rose et al. 1998, 2003). These primers amplify a portion corresponding to mammalian Trpc2 exons 13–15. With primers 1315F1 and 1315R1 (supplementary table S1, Supplementary Material online), the following cycling conditions were used: 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, followed by a final elongation for 5 min at 72 °C. The nested polymerase chain reaction (PCR) with primers 1315F2 and 1315R2 (supplementary table S1, Supplementary Material online) was conducted with the same cycling conditions except that the annealing temperature was increased to 53 °C.

Most V1Rs have a single coding exon, making it difficult to distinguish true V1R expression from contamination of the cDNA by genomic DNA. To investigate V1R expression patterns unambiguously, we performed 3′ rapid amplification of cDNA ends (RACE) with a FirstChoice RLM RACE kit (Ambion) with primers V1RRACEOUT and V1RRACEIN (supplementary table S1, Supplementary Material online). The following cycling conditions were used for the outer PCR: 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by a final elongation for 3 min at 72 °C. The conditions were the same for the inner PCR except that there were only 30 cycles. All PCRs were carried out in a 25-μl volume with the following final concentrations: 40 nM of each primer, 200 μM dNTPs, and 1.5 mM $MgCl_2$. PCR amplicons were confirmed on an agarose gel. PCR products from genomic DNA and olfactory and tongue cDNA were sequenced by the University of Michigan DNA Sequencing Core.

Results

Coverage Quality of Early Diverging Vertebrate Genome Sequences

Before searching for VNS-specific genes in the lamprey and shark genome sequences, it is important to first know the coverage of these incomplete genome sequences. We used full-length mRNA sequences previously reported for the lamprey and chaemeriformes (sharks and related species) to test whether the corresponding genes can be found in the incomplete genome sequences. Only 3 of the 22 lamprey mRNA sequences tested had all of their exons in the genome sequence trace archives, and these three complete mRNAs were short (<1,000 nucleotides). The remaining 19 mRNAs each had at least three exons and had matches for at least two exons from the trace archives (supplementary data file 1, Supplementary Material online). From this analysis, one may roughly estimate the false negative rate or the probability (P) that a gene is not found in the genome sequence when it actually exists in the species. Because our evaluation of the lamprey genome showed that all of the 22 tested genes are found, the probability of this event must be greater than 0.05. In other words, $(1 - P)^{22} > 0.05$. This gives $P < 0.13$. Thus, the probability that an existing gene is missing from the lamprey genome sequence is smaller than 13%. Note that this is a conservative estimate. The ML estimate of P is 0.

Thirteen (including five single-exon mRNAs) of the 23 chaemeriformes mRNAs tested had all their exons covered in the shark genome sequence. These 13 mRNAs ranged in size from 109 to 1,416 nucleotides. Four additional mRNAs had >80% coverage. The remaining six mRNAs had 0 or 1 hit in the shark genome sequence. Four of these six mRNAs were from the elephant shark's congener *C. callorynchus*, including both mRNAs with no hits. Thus, sequence divergence among the four chaemeriformes species used is not the reason for the poor results of this coverage assessment. Similarly, the conservative estimate of P is smaller than 0.25, whereas the ML estimate of P is 0.087. Together, these findings suggest that gene sequences identified from the lamprey genome may often be partial, but it is unlikely that an entire gene will be missing from the genome sequence. By contrast, some genes may be missing from the shark genome.

V1Rs from Early Diverging Vertebrates

We were able to identify three and two putatively functional V1R genes from the sea lamprey and elephant shark genomes, respectively. Given the relatively high coverage of the sea lamprey genome (5.9×) and the result from the above section, this set likely represents the majority of the lamprey V1R repertoire. However, given the extreme protein distance between the known teleost V1Rs and the putative lamprey V1Rs and the rapid evolution characteristic of this gene family in mammals (Grus and Zhang 2006), we cannot completely exclude the possibility that some lamprey V1Rs were too divergent to detect by our methods. The low coverage of the elephant shark genome (1.4×) suggests that the V1R repertoire might be incomplete. The small V1R repertoire size in lamprey and shark is similar to what is observed in teleost fish (Saraiva and Korsching 2007), but contrasts to that in most tetrapod lineages (Shi and Zhang 2007). An alignment of the three lamprey V1Rs, two shark V1Rs, and representative V1Rs from zebrafish, frog, and mouse revealed that all 10 residues conserved in teleost and tetrapod V1Rs are conserved in the early diverging vertebrate V1Rs (Pfister and Rodriguez 2005) (supplementary fig. S1, Supplementary Material online). Furthermore, these V1Rs contained the conserved N-linked glycosylation site in the second extracellular domain (supplementary fig. S1, Supplementary Material online).

To understand the evolution of the V1Rs in early diverging vertebrates, we used the NJ method to reconstruct a protein-sequence-based V1R gene tree with the 3 putatively functional sea lamprey V1Rs, 2 putatively functional elephant shark V1Rs, 6 putatively functional zebrafish

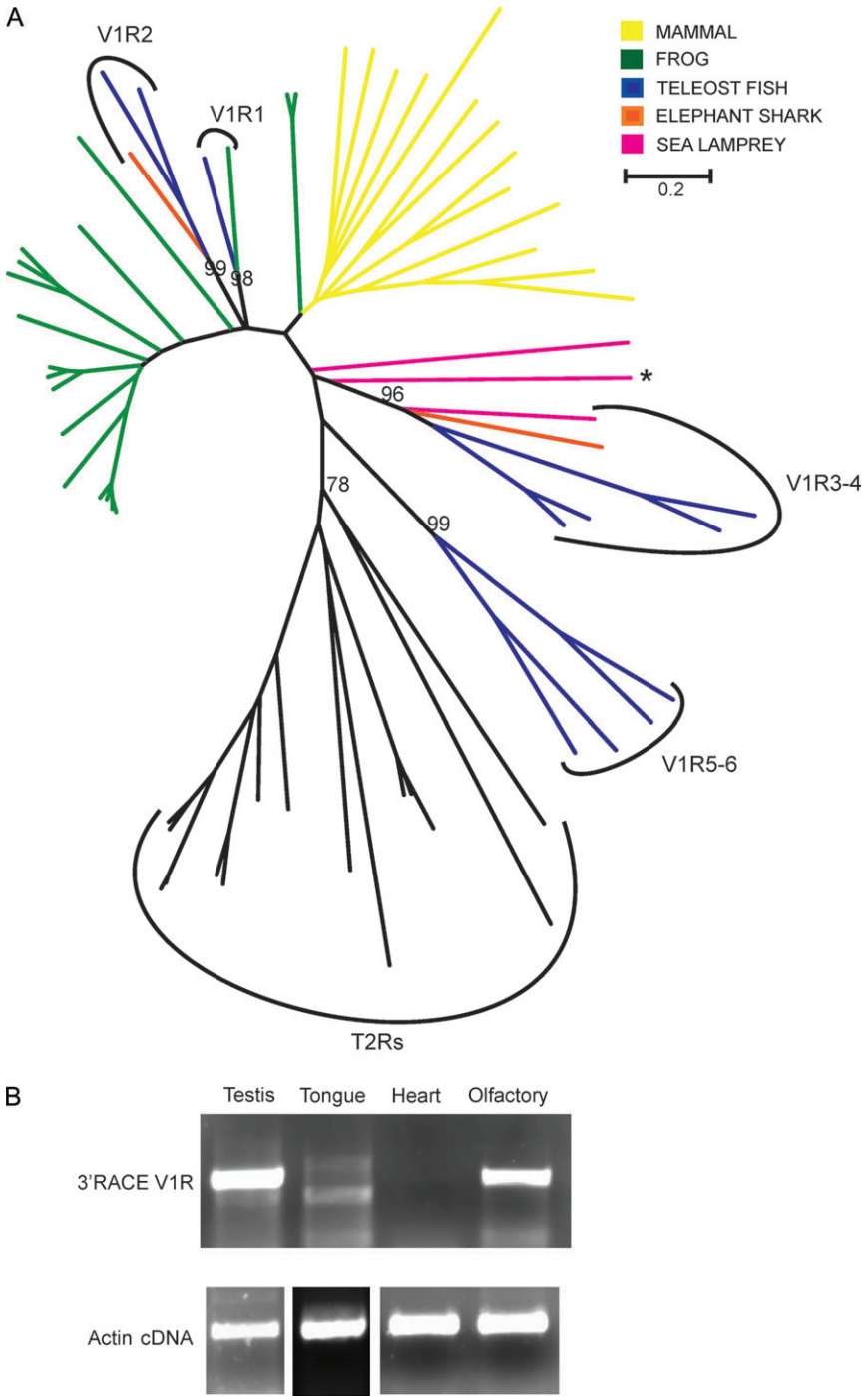

FIG. 1.—V1Rs are found in both the sea lamprey and elephant shark genomes and are expressed in the sea lamprey olfactory epithelium. (*A*) Unrooted NJ tree of 3 sea lamprey, 2 elephant shark, 7 teleost fish (zebrafish and fugu), 21 frog, and 13 mammalian V1Rs. If the T2R taste receptors (black) are assumed to be the outgroup, all V1Rs cluster together. Two zebrafish T2Rs (GenBank BAE78480 and BAE80435) and 13 *X. tropicalis* T2Rs (GenBank BAE80422-34) are included. The distinction between T2Rs and V1Rs remains if mammalian T2Rs are also included (data not shown). Bootstrap support for each of the four teleost V1R families and for the clade of T2Rs is given. Scale bar shows 0.2 amino acid substitutions per site. The lamprey V1R gene indicated by * is examined for expression in panel *B*. (*B*) Sea lamprey V1R expression in the olfactory organ. Presence of cDNAs in all samples is verified by amplification of the actin cDNA. The lack of amplification in 3′RACE of olfactory organ RNA without reverse transcriptase confirmed that the band was not from genomic contamination.

V1Rs (Saraiva and Korsching 2007), 5 putatively functional Tetraodon (*Tetraodon nigroviridis*) V1Rs (Saraiva and Korsching 2007), 21 putatively functional frog (*Xenopus tropicalis*) V1Rs (Shi and Zhang 2007), and 13 representative mammalian V1Rs (Grus and Zhang 2004; Shi et al. 2005) (fig. 1*A*). These 13 mammalian genes represent 13 V1R gene groups present in the mouse and rat genomes (Grus et al. 2005). Although other placental mammal V1R

families likely exist and marsupial and monotreme V1R families are unique to their lineages, previous studies of V1Rs from nonmammalian vertebrates have shown that all mammalian V1Rs cluster together in relation to nonmammalian V1Rs (Grus et al. 2007; Saraiva and Korsching 2007). One lamprey V1R and one shark V1R cluster with a teleost V1R clade that was lost in mammals: teleost V1R3-4. These teleost V1Rs are unique in that their coding regions contain introns (Saraiva and Korsching 2007). However, according to the Blast searches, the lamprey and shark V1R3-4 homologs contain a single coding exon, suggesting that these teleost V1Rs acquired the introns after the teleosts diverged from cartilaginous fish. The other elephant shark V1R clusters with teleost V1R2, which has orthologs in all teleost fish thus far studied (Pfister et al. 2007; Saraiva and Korsching 2007). The remaining lamprey V1Rs fall within the V1R clade but not with a specific teleost V1R gene. Neither shark nor lamprey V1Rs cluster with the teleost-specific V1R5-6 genes or the V1R1 gene, which has homologs in tetrapods (Shi and Zhang 2007). We also reconstructed an ML tree, which has a similar topology as the NJ tree. A major difference between the two trees is that in the ML tree, the V1R5-6 clade and V1R3-4 clade joined and formed a single clade with three lamprey and one shark sequences, although high bootstrap values are found in neither the NJ nor the ML tree for this part (supplementary fig. S2, Supplementary Material online). If we assume that the V1R tree can be rooted with T2Rs (bitter taste receptors) (Shi and Zhang 2007), the phylogenetic reconstruction (fig. 1*A*) strongly supports the clustering of both lamprey and shark genes with other vertebrate V1Rs. The tree also presents strong evidence that the ancestral vertebrate genome had at least one V1R, whereas the ancestral jawed vertebrate genome likely had at least two V1Rs.

Using 3′RACE and subsequent DNA sequencing, we amplified 415 nucleotides from one of the sea lamprey V1Rs (Genbank FJ209301; * in fig. 1*A*) from olfactory organ cDNA (fig. 1*B*). Although there is also 3′RACE V1R product from the testis cDNA, this tissue does not express Trpc2 (see below). The apparent 3′RACE products from the tongue were found by DNA sequencing to be non-V1R sequences. Because one of the two primers used in 3′RACE does not match genomic DNA, our results cannot be due to genomic DNA contamination in the RNA samples. Indeed, when no reverse transcriptase was used in 3′RACE, no amplification was observed (data not shown).

V2Rs from Early Diverging Vertebrates

No V2Rs were found in the sea lamprey genome. However, V2Rs were present in the elephant shark genome. Because of their complex multiexon structure and because the elephant shark genome was not completely assembled or sequenced, no full-lengthV2Rs were identified. However, both the 7-transmembrane domain and extracellular domain of the V2R have multiple hits, and three shark V2Rs contain multiple exons and cover over 600 amino acids each (supplementary fig. S3, Supplementary Material online). These three shark V2Rs covered four or five of the six total exons in the canonical V2R structure and shared exon boundaries (supplementary fig. S3, Supplementary Material online). To recover unique V2Rs from the shark, we required that a hit be at least 200 amino acids long in the 7-transmembrane region and share no more than 99% amino acid sequence identity with another elephant shark V2R in this region. Although these criteria appear overly stringent, the 7-transmembrane region of V2Rs is highly conserved and sequences with $<$99% identity are most likely unique. For example, many unique frog V2Rs are even identical in this region (supplementary fig. S3, Supplementary Material online). Based on these criteria, we found 32 unique shark V2Rs, which is likely a conservative estimate for the total number of shark V2Rs (supplementary data file 2, Supplementary Material online). The 25 V2Rs with the most complete sequence in the 7-transmembrane region, ranging in size from 222 to 684 amino acids, were used for phylogenetic analysis. We made an NJ tree with these 25 V2Rs and the 7-transmembrane region of 43 zebrafish V2Rs, 18 fugu V2Rs, and 4 tetraodon V2Rs, 249 frog V2Rs, and 5 representative mammalian V2Rs (Shi and Zhang 2007). The five mammalian V2R genes were chosen from diverse mammalian V2R clades including the V2R2 clade known to have distinct expression, function, and origin from other V2Rs (Yang et al. 2005; Shi and Zhang 2007; Young and Trask 2007). As in the V1Rs, teleost V2Rs form distinct clades from tetrapod V2Rs, aside from the V2R2 group (Shi and Zhang 2007). Among the shark V2Rs was an ortholog to the V2R2 clade (fig. 2, and supplementary fig. S4, Supplementary Material online). One other shark V2R appeared to be orthologous to a fugu V2R. All remaining shark V2Rs form a single cluster that is sister to some teleost V2Rs (fig. 2). We also made an ML tree and found that the relationships among shark V2Rs to be unchanged. The same relationships were also found in an NJ tree including all V2Rs from shark, zebrafish, fugu, xenopus, platypus, opossum, mouse, and rat (data not shown).

Early Diverging Vertebrate Trpc2

From the sea lamprey genome sequence and trace archives, we could identify four Trpc2 exons (7, 12, 14, and 15) (supplementary figs. S5*A*, S5*D*, and S5*E*, Supplementary Material online). Additionally, we identified a second copy of Trpc2 exons 14 and 15 on a different contig (supplementary figs. S5*A* and S5*C*, Supplementary Material online). The two copies of exons 14 and 15 were not allelic variants because the two versions of the intron between exons 14 and 15 diverge by an indel and many nucleotide substitutions. Furthermore, the second copy had a nonsense mutation in exon 14 and appears to be a pseudogene whereas the first copy had an open reading frame across this region (supplementary fig. S5*C*, Supplementary Material online). From the elephant shark genome, we identified one sequence that corresponded to vertebrate Trpc2 exon 8 (supplementary fig. S5*F*, Supplementary Material online).

We amplified a 303-nucleotide cDNA sequence corresponding to part of exon 13, exon 14, and part of exon 15 of the sea lamprey Trpc2 (Genbank FJ209300), including sequence not present in the genome assembly or the trace

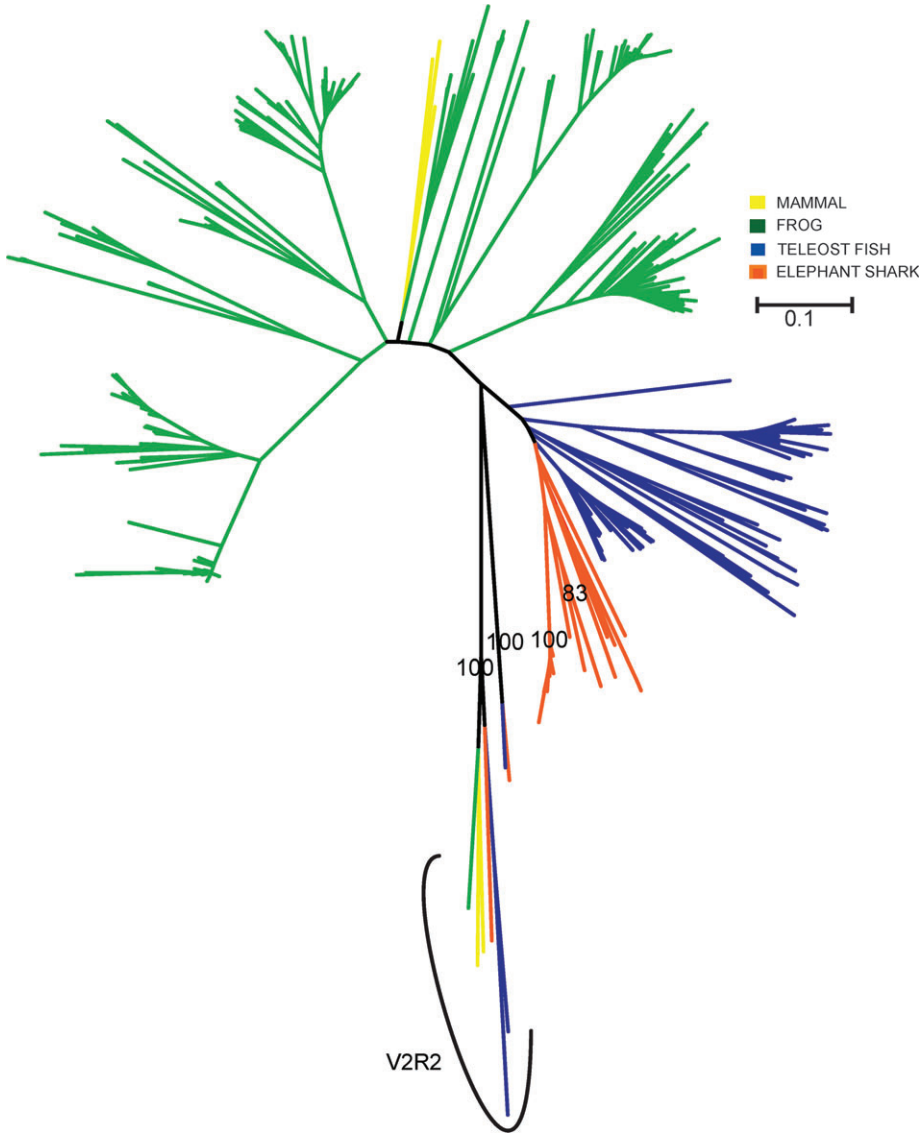

FIG. 2.—V2Rs are present in the elephant shark genome. An unrooted phylogenetic tree of the 7-transmembrane-domain region from 249 frog, 43 zebrafish, 4 tetraodon, 18 fugu, 5 mammalian, and 25 elephant shark V2Rs. The scale bar shows 0.1 amino acid substitutions per site. The unique subfamily of V2R2 is indicated. Bootstrap percentages >70 are shown for clades containing shark V2Rs.

archives. The 3′ end of this sequence overlapped the contig-containing exons 14 and 15 with the open reading frame. The cDNA sequence was combined with the contig sequence to make a 396-nucleotide sequence, including part of exon 13, exon 14, and exon 15 (supplementary fig. S5*B*, Supplementary Material online). This sequence was conceptually translated into a 132 amino acid sequence. Exons 13–15 of sea lamprey Trpc2 correspond to part of the fifth transmembrane domain, the channel pore, and the complete sixth transmembrane domain (Vannier et al. 1999) (supplementary fig. S5*B*, Supplementary Material online). To confirm that this sequence was from the Trpc2 gene, we reconstructed an NJ tree with sea lamprey Trpc2 exons 13–15, the homologous regions from tetrapod and teleost Trpc2 protein sequences, and other mouse members of the Trpc gene family (fig. 3*A*). The sea lamprey Trpc2 sequence clustered with high bootstrap support with the other vertebrate Trpc2 genes, strongly suggesting that this sequence comes from the sea lamprey ortholog of Trpc2. When the noninterrupted portion of the sea lamprey Trpc2 pseudogene copy was included in the tree, the two sea lamprey Trpc2 copies cluster together (data not shown). Although sea lamprey Trpc2 exons 7 and 12 were excluded from the analysis because they could come from either Trpc2 copy, alignments including these exons produce the same tree topology (data not shown).

Because we could not identify the same exons of Trpc2 from both the lamprey and shark genomes, we could not include both species in the same tree. However, a separate phylogenetic reconstruction using Trpc2 exon 8 sequences confirms that the elephant shark partial Trpc2 sequence is indeed Trpc2 (fig. 3*B*). Note that the gene tree including the elephant shark Trpc2 exon 8 is not completely concordant with the known species tree, as the shark appears sister to the frog in the gene tree (fig. 3*B*). This discordance may have resulted from the use of a short

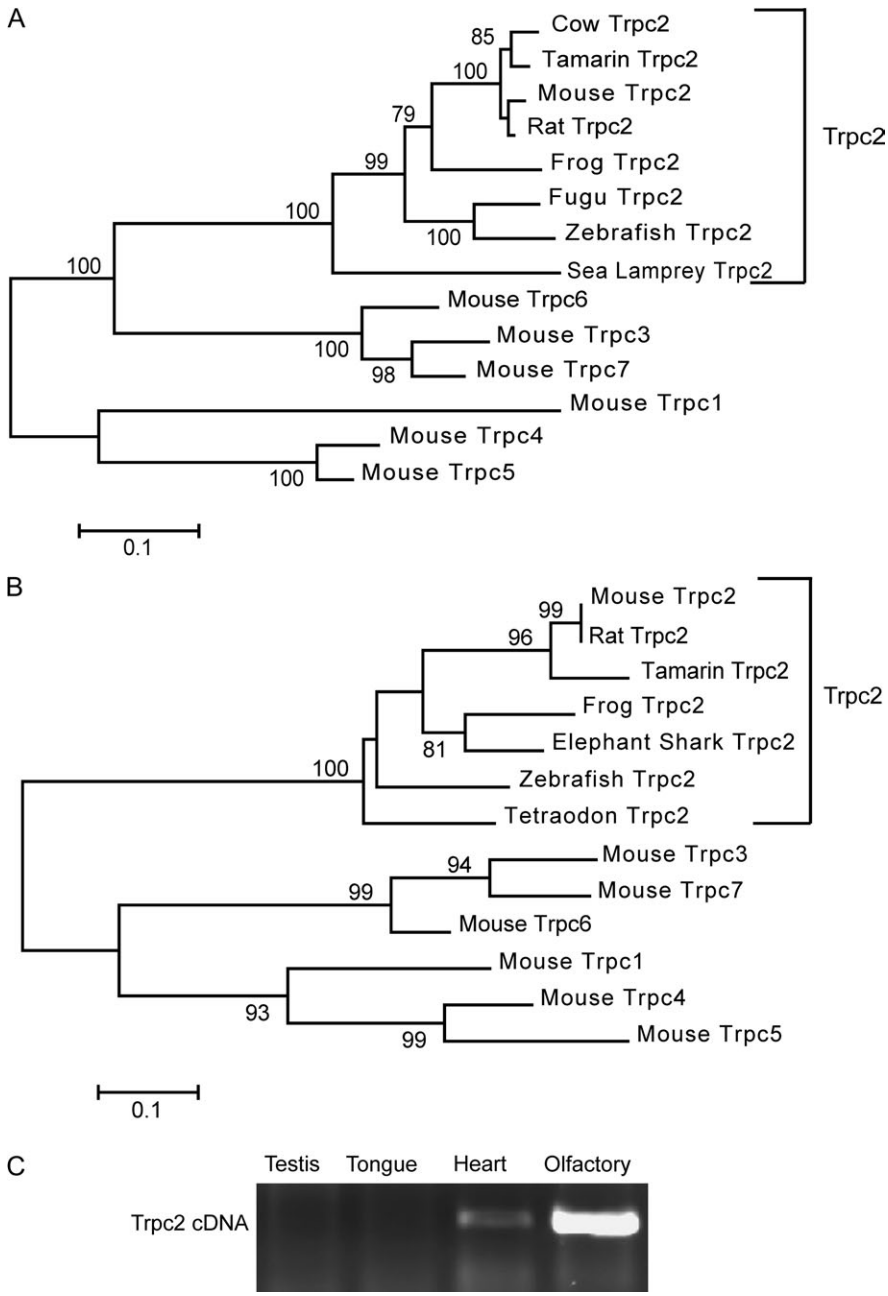

FIG. 3.—Trpc2 is present in the sea lamprey and elephant shark genomes. Phylogenetic reconstruction of (*A*) sea lamprey Trpc2 exons 13–15 and (*B*) elephant shark Trpc2 exon 8 with corresponding regions of the Trpc2 from several other vertebrates and other Trpc genes from mouse. The scale bar shows 0.1 amino acid substitutions per site. Bootstrap percentages greater than 70 are shown. (*C*) Trpc2 is expressed in the sea lamprey olfactory organ. Presence of cDNA in all samples is verified by amplification of sea lamprey actin as shown in figure 1*B*. The size of the amplified fragment indicates that it is not from genomic contamination of RNA samples. Further evidence came from the observation of no cDNA amplification when reverse transcriptase is absent.

sequence (67 amino acids) in a region that has high sequence identity between teleosts and tetrapods. Regardless of the cause of the discordance, the shark sequence clusters with the Trpc2 gene of other vertebrates, rather than with other Trpc genes.

To determine if the lamprey Trpc2 functions in chemoreception, we examined the expression pattern of Trpc2 in sea lamprey using RT-PCR. We found that the sea lamprey Trpc2 is expressed in the olfactory organ and has low expression in the heart, but it is not expressed in the testis or the tongue (fig. 3*C*). Because the amplified fragment spans more than one exon, it is clear from the fragment size that the amplification must be from cDNA rather than genomic DNA.

Taste System Genes in Early Diverging Vertebrate Genomes

To further investigate the relationship between the taste system and the VNS, we also searched for the genetic

components of the taste system (T1Rs, T2Rs, and Trpm5) in the sea lamprey and elephant shark genomes. None of these genes was found in the sea lamprey genome. Because the probability that an existing gene is missing from the lamprey genome sequence is lower than 0.13, it is extremely unlikely that all taste genes would be missing from the genome sequence if they actually exist. In the elephant shark genome, T1Rs had two distinct hits to the C-terminus and two distinct hits to the N-terminus (supplementary figs. S6 and S7, Supplementary Material online). Because the elephant shark genome is not completely assembled, we do not know if these partial sequences represent two, three, or four T1Rs. Six elephant shark Trpm5 exons were identified (supplementary fig. S8, Supplementary Material online). To confirm that the sequence we identified is from the Trpm5 gene, we reconstructed an NJ tree using elephant shark Trpm5 and the homologous regions from tetrapod and teleost Trpm5 protein sequences and other mouse members of the Trpm gene family (supplementary fig. S6, Supplementary Material online). The elephant shark Trpm5 clusters with high bootstrap support with other vertebrate Trpm5 orthologs and not with the other Trpm paralogs, suggesting that the shark sequence is from Trpm5.

VNS and Taste System Genes are Absent from Invertebrate Chordate Genomes

To investigate the origin of VNS and taste system genes, we searched for them in the genome sequences of a tunicate (*C. intestinalis*) and an amphioxus (*B. floridae*). None of the three VNS-specific genes or the three taste system genes was found in either genome, suggesting that these sensory systems and their unique genetic components originated within the vertebrate lineage.

Discussion

Previous studies have established that the primordial VNS likely existed in the common ancestor of bony vertebrates (Grus and Zhang 2006). To examine if the genes specific to the system originated even earlier, we examined the elephant shark and sea lamprey, which represent cartilaginous fish and jawless vertebrates, respectively. With both computational and experimental methods, we identified two of the three VNS-specific genes from the sea lamprey (V1Rs and partial Trpc2) and all three VNS-specific genes (V1Rs, partial V2Rs, and partial Trpc2) from the elephant shark. In addition, we showed that lamprey V1Rs and Trpc2 are both expressed in the olfactory organ but not coexpressed in several other tissues examined (tongue, testis, and heart), suggesting an ancestral role for these genes in nasal chemoreception. Although only partial sequences were identified for the sea lamprey and elephant shark Trpc2 orthologs, we determined that the incompleteness is within the bounds expected from these incomplete genome sequences. Furthermore, these partial sequences are found on short contigs (<1 kb each), suggesting that the incompleteness is due to lack of sequencing rather than absence from the genome.

Although V2Rs were identified from the elephant shark genome, they were not found in the sea lamprey genome. Three scenarios could cause this result. First, V2Rs might not be present in the sea lamprey genome, either because of gene loss in the lamprey lineage or because this gene superfamily did not originate until after the divergence of jawed and jawless vertebrates. Second, V2Rs might be present in the sea lamprey genome but absent in the genome assembly because the current assembly is incomplete. From the analysis of the sea lamprey genome coverage, multiexon genes present in the sea lamprey genome tend to have at least two exons in the genome assembly. Given that V2Rs have six exons, they should have been found (at least partially) if they are present. Also, our assessment of the lamprey genome showed that each of the 22 tested genes can be found from the lamprey genome sequence. Thus, if there are multiple V2R genes in the lamprey, they should not have been missing from the genome assembly. However, because V2R genes are clustered in vertebrate genomes (Yang et al. 2005), it still remains possible, although unlikely, that V2Rs are present in the species but are not found because of lack of genomic sequence coverage. Even if V2Rs are present in the sea lamprey genome, the gene family would likely be very small because the elephant shark genome sequence with lower coverage has over 30 V2Rs. Finally, V2Rs in the sea lamprey genome could be so divergent from the other vertebrate V2Rs that they are not hit in our Blast search. Our attempts to amplify V2Rs from the sea lamprey genomic DNA or olfactory cDNA with degenerate V2R primers (Cao et al. 1998) were also unsuccessful. However, the divergent sequence explanation does not fit our Blast results, because when we Blasted the sea lamprey genome with mammalian or zebrafish V2Rs, we were able to identify more distantly related non-V2R sea lamprey genes, such as calcium-sensing receptors (CaSRs) and metabotropic glutamate receptors. Thus, our search was sensitive enough to identify distantly related sequences and should have been able to identify divergent V2Rs. Although we are unsure of the exact time of origin of the V2R superfamily, it arose at least before the divergence of cartilaginous fish and bony vertebrates. Identifying these genes from the other extant jawless vertebrate lineage, the hagfishes (Takezaki et al. 2003), would clarify if this family was present in the ancestor of all extant vertebrates and was subsequently lost in the lamprey lineage.

Although the evolutionary history of V2Rs remains ambiguous, the two other VNS-specific genes, V1Rs and Trpc2, are clearly present in the sea lamprey genome and must have been present in the common ancestor of all extant vertebrates. Note that some mammals with vomeronasal sensitivity do not have V2Rs because of secondary losses (e.g., dog and cow; Shi and Zhang 2007; Young and Trask 2007). Thus, the ancestral vertebrates, having only V1Rs and Trpc2, potentially had this type of VNS. Additionally, among the tissues sampled, the only sea lamprey tissue that expresses both V1Rs and Trpc2 is the olfactory organ. It remains possible that this pathway functions in other tissues, and a more in-depth expression analysis would reveal the specificity of this pathway. Further support for the origin of the VNS in the ancestor of vertebrates would come from identifying the exact expression location

within the sea lamprey olfactory organ. As in teleost fish, olfactory sensory neurons of sea lamprey are polymorphic, with three morphologies: ciliated, microvillar, and crypt (Laframboise et al. 2007). In teleost fish, the VNS-specific genes are expressed in the apical layer of microvillar sensory neurons, whereas the MOS-specific genes are expressed in a more basal layer of ciliated sensory neurons (Hansen et al. 2004; Sato et al. 2005). If the two olfactory systems are distinct in the sea lamprey, we would predict that such spatial distinction is seen in the expression of sea lamprey V1Rs and Trpc2 compared with MOS genes, which will be interesting to confirm in the future by in situ hybridization.

Given that the VNS-specific genetic components arose in the ancestor of vertebrates, how did the system originate? Because of their related functions, it was hypothesized that the VNS arose via duplication of the MOS (Eisthen 1992, 1997). Although the two systems are morphologically similar and, in tetrapods, the two distinct organs both develop from the olfactory placode (Taniguchi et al. 1996; Taniguchi K and Taniguchi K 2008), the genetic components of their signaling pathways are distinct and nonhomologous (Dulac and Axel 1995). Instead, V1Rs are closely related to T2Rs, and V2Rs are closely related to T1Rs. However, we did not detect T1Rs or T2Rs in the sea lamprey genome. Additionally, it appears that the sea lamprey genome does not have Trpm5, a channel protein necessary for bitter, sweet, and umami taste signal transduction (Perez et al. 2002; Zhang et al. 2003). In contrast, the elephant shark genome has T1Rs and Trpm5 orthologs (supplementary figs. S6–S8, Supplementary Material online). The initial descriptions of the two taste receptor families recognized their relationships to V1Rs and V2Rs, but they could not provide any information on the possible timing of the origin of any of these receptor families. Our results suggest that the VNS arose prior to the taste system, and it does not appear that the VNS evolved as the result of duplication of the taste system, although the converse remains an intriguing possibility. Interestingly, Trpm5 and Trpc2 are not closely related, suggesting that in addition to duplication, co-option may have played a role in the evolution of vertebrate sensory signal transduction pathways (Plachetzki and Oakley 2007). Alternatively, the ancestral chemosensory system could have features and functions of both systems, or receptors from both systems could have evolved from a third unknown class of receptors, although the likelihood of the latter scenario appears minute. Depending on how the tree in figure 1 is rooted, it might suggest that the divergence between T2Rs and V1Rs predated the separation between the lamprey and jawed vertebrates. Thus, it remains possible that T2Rs originated in the common ancestor of all vertebrates but were secondarily lost in lampreys.

If the genetic components of the taste and vomeronasal systems had a common origin, the ancestral system could have dual chemosensory functions. It is interesting to speculate on the function of the ancestral system. Based on the expression patterns of the sea lamprey VNS-specific genes, the ancestral system likely had a role in nasal chemoreception. Sea lampreys produce unique bile acids that act as pheromones both in migration and mate finding (Li et al. 1995, 2002; Siefkes and Li 2004). However, bile acids in teleost fish are known to require components of the main olfactory signal transduction pathway (Hansen et al. 2003), and interruption of the VNS signal transduction pathway had no effect on bile acid olfactory response (Hansen et al. 2003). In contrast to the large V1R gene repertoire and rapid gene turnover in many mammals (Grus WE and Zhang J 2008), the small repertoire size and strict orthologous relationships in this gene family in teleosts (Pfister et al. 2007; Saraiva and Korsching 2007) suggest that V1R chemoreception may play different physiological roles in teleosts than it does in mammals. The evolutionary patterns observed in the early diverging vertebrate V1Rs are similar to what is seen in teleosts, suggesting that the lamprey and shark V1Rs might have a function similar to those in teleosts. In teleost fish, V2Rs are thought to be amino acid receptors and T1Rs are known to be amino acid receptors (Alioto and Ngai 2006; Oike et al. 2007). The shark V2Rs share the amino acid binding signature in the C-terminal end (Alioto and Ngai 2006), suggesting that shark V2Rs are amino acid receptors (supplementary fig. S3, Supplementary Material online).

In contrast to the vertebrate-specific VNS and taste system, the vertebrate MOS appears to have evolved earlier in chordate evolution. The evolution of the two MOS-specific receptor families, ORs and TAARs, has been studied extensively in teleosts and tetrapods (Niimura and Nei 2005; Hashiguchi and Nishida 2007). Both families have also been identified with olfactory epithelium expression in lamprey (Berghard and Dryer 1998; Freitag et al. 1999). The lamprey TAARs were nominally identified as "ancient vertebrate odorant receptors" before mammalian TAARs were first described (Berghard and Dryer 1998). However, these lamprey receptors are clearly homologous to mammalian TAARs (data not shown). Additionally, a closely related receptor has been identified in the amphioxus genome and shows expression in amphioxus primary sensory neurons (Satoh 2005). Further Blast searches on the amphioxus genome sequence identify that this gene is part of a large receptor family that is homologous to vertebrate ORs (Grus and Zhang, unpublished data), suggesting that ORs originated in invertebrate chordates. Interestingly, this gene family is absent from the tunicate genome (Satoh 2005). Our combined analysis of vertebrate chemosensory signaling genes from available chordate genomes suggests a staggered pattern for the timing of vertebrate sensory system origins (fig. 4). The two MOS chemoreceptor families, ORs and TAARs, arose in the common ancestor of cephalochordates and vertebrates (Satoh 2005) and in the ancestor of vertebrates (Berghard and Dryer 1998), respectively. Our finding of VNS and taste system genes in early diverging vertebrates suggested that the two VNS chemoreceptor families, V1Rs and V2Rs, evolved in the ancestor of vertebrates and jawed vertebrates, respectively, whereas the two taste chemoreceptor families, T1Rs and T2Rs, evolved in the ancestor of jawed vertebrates and bony vertebrates, respectively. Searching for T2Rs in nonteleost ray-finned fish, such as the sturgeon or the bichir, would give a clearer picture of the T2R origin. It should be noted, however, that there is also a possibility that the divergence of V1Rs and

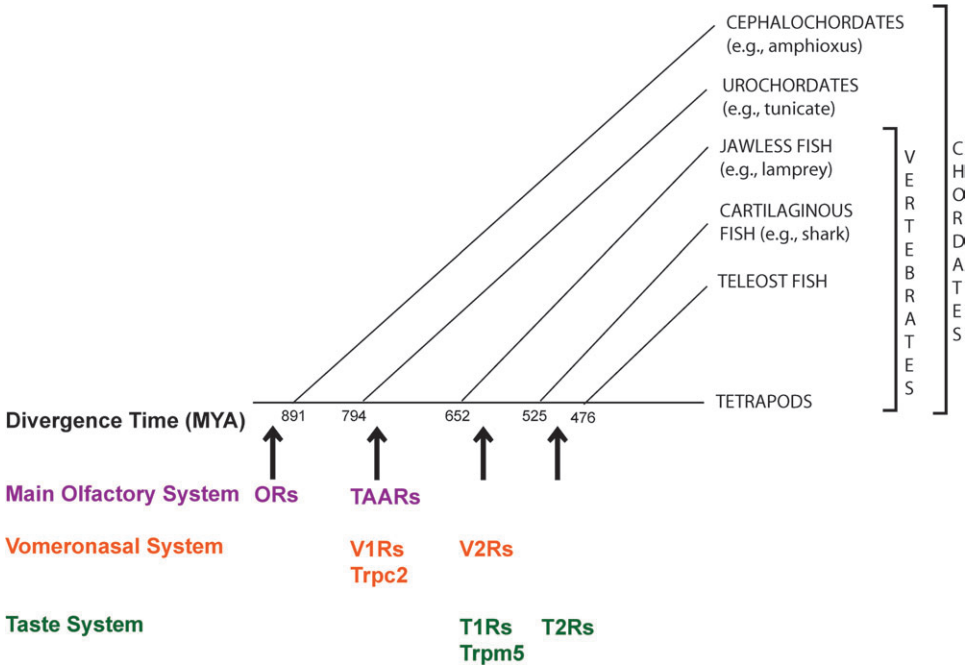

FIG. 4.—Staggered origins of vertebrate chemosensory systems viewed from the origins of their genetic components, based on the currently available genome sequences. Divergence times are based on Blair and Hedges (2005).

T2Rs was earlier than the separation of jawless and jawed vertebrates (fig. 1). If confirmed with further genome analysis, the staggered pattern could reflect changes in environment, social structure, ecological exploitations, or alternative sensory systems, such as electroreception or the lateral line system, that appear in different lineages throughout vertebrate evolution.

Aside from ORs, none of the chemoreceptor families has homologs outside of vertebrates. The lack of chemoreceptor homologs might stem from gene loss in the examined invertebrate chordates rather than an origin within vertebrates. Recent molecular studies showed that urochordates (tunicates) are the sister group to vertebrates and that the second most closely related group is cephalochordates (amphioxus) (Bourlat et al. 2006; Dunn et al. 2008). Although VNS and taste system genetic components are absent from the tunicate genome, this genome is known to have substantial gene loss compared with the ancestral chordate (Hughes and Friedman 2005; Putnam et al. 2008). Over 20% of 3,921 ancestral chordate gene families are absent from the tunicate genome (Hughes and Friedman 2005). Additionally, Putnam et al. (2008) identified 9,975 ancestral chordate gene sets, of which 2,427 (24.3%) were lost or were highly divergent in the tunicate genome, including 1,350 (13.5%) that are clearly lost from the tunicate genome. By contrast, only 859 (8.6%) of the 9,957 ancestral chordate gene sets were lost or highly diverged from the amphioxus genome (Putnam et al. 2008). Thus, the gene content of the amphioxus is more similar to the potential ancestral chordate genome (Putnam et al. 2008). However, we could not find VNS or taste system genes in the amphioxus genome. Hence, the most likely scenario is that these sensory genes did not originate till the appearance of vertebrates.

Comparative analysis of vertebrate and invertebrate genomes suggests that 22% of vertebrate genes have no homologs in invertebrates (Prachumwat and Li 2008). However, because this study included only representative vertebrate genomes from tetrapods and teleosts, it does not reveal when in vertebrate evolution these vertebrate-specific genes arose. Although the current sea lamprey genome assembly has limited coverage and the shark genome sequence has low coverage, we have shown that these genome sequences provide a more complete picture of the evolution of the VNS and other chemosensory systems. We expect that these genome sequences, particularly when the coverage is improved, will offer vital information about the origin of vertebrates in general.

Supplementary Material

Supplementary data file 1, table S1, and supplementary figures S1–S8 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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