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Isolation and characterisation of main olfactory and vomeronasal receptor gene families from the Atlantic salmon (*Salmo salar*)

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Abstract

The Atlantic salmon (*Salmo salar*) has been widely used as a model species in studies of olfactory signal transduction and processing. Here we report the isolation and characterisation of salmon olfactory receptor (*SOR*) and salmon vomeronasal receptor (*SVR*) partial sequences from Atlantic salmon. Six groups of *SOR* sequences (*SORA–F*) and three groups of *SVR* sequences (*SVRA–C*) were identified. All *SORB*, *SORF*, *SVRB* and *SVRC* sequences contained uninterrupted open reading frames. However, all *SORA* sequences and members of the *SVRA* sequence family contained multiple stop codons while *SORC* and *SORE* sequences were truncated in the 3′ region of the sequence. Full length *SORF* and almost complete *SORB* sequences displayed amino acid residues and motifs conserved in fish olfactory receptor genes. In sequence phylogenies, *SOR* sequences fell into the main olfactory receptor (*MOR*) type I clade and were most closely related to either δ or ζ reference sequences, while all *SVR* sequences grouped within a clade of fish type 2 vomeronasal receptor (*V2R*) sequences. A family of sequences (*Sasa CaSR1–6*), isolated using the same degenerate primers that amplified *SVR* sequences, clustered within a group of calcium sensing receptor (*CaSR*) sequences. Analysis of tissue expression patterns of sequences by reverse transcriptase polymerase chain reaction showed that they were transcribed in olfactory epithelium (*SORB*, *SORF*, all *SVR* and *Sasa CaSR* sequences), testis (*SORB*, *SORD* and *Sasa CaSR*) and/or anterior kidney (*SORB* and *Sasa CaSR*). Similar analysis of expression supported the identification of *SORA* sequences as non-transcribed pseudogene(s). Although the level of occurrence of *OR* pseudogenes is within the range found for other, well-characterised vertebrate *OR* genomes, it does not seem to reflect the importance of olfaction in the biology of the Atlantic salmon.

Keywords: Odorant receptors; Molecular evolution; Gene expression; Pseudogenes

1. Introduction

The isolation and characterisation of odorant receptor (*OR*) genes has been a major contribution to our understanding of olfaction (e.g. Buck and Axel, 1991; Mombaerts, 1999; Firestein, 2001). Odour (including pheromone) perception results from binding of odorants to receptors that are expressed on the surface of olfactory sensory neurons. In vertebrates and insects a complex mechanism of gene regulation generally results in each olfactory neuron expressing only one (or a very few) *OR* allele(s) from a potentially wide range of *OR* genes present in each individual (Chess et al., 1994; Serizawa et al., 2003; Hallam and Carlson, 2004). Odour discrimination is then realised through spatial

Abbreviations: bp(s), base pair(s); *CaSR*, calcium sensing receptor gene; cDNA, DNA complementary to RNA; DNase, deoxyribonuclease; dNTP, deoxyribonucleotide; *mGluR*, metabolic glutamate receptor gene; MOE, main olfactory epithelium; *MOR*, main olfactory receptor gene; *OR*, odorant receptor gene; RT-PCR, reverse transcriptase–polymerase chain reaction; *SOR*, salmon olfactory receptor gene; *SVR*, salmon vomeronasal receptor gene; *TASR1*, taste receptor type 1; TM, transmembrane domain; VNO, vomeronasal organ; *VNR*, vomeronasal receptor gene.

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patterning in the olfactory epithelium of neurons expressing particular *OR* loci or alleles and the convergence of their axonal projections to common glomeruli in the olfactory bulb in vertebrates (Korsching, 2001; Luo and Katz, 2004) or antennal lobe in *Drosophila* (Hallam and Carlson, 2004).

In mammals, odours and pheromones are perceived by two organs, both found within each nasal cavity: the main olfactory epithelium (MOE) and the vomeronasal organ (VNO). Cells of the MOE are ciliated and express main olfactory receptor (*MOR*) genes, while the VNO is made up of microvillar cells that express vomeronasal receptors (*VNR*) (Bargmann, 1997). Traditionally, it was thought that the MOR detected only volatile odours while the VNO detected (often non-volatile) pheromones, but this view has recently been challenged and evidence is emerging that both organs may play a role in detecting both classes of odorants (Restrepo et al., 2004). Fish possess only a single olfactory organ within each nasal cavity containing both ciliated and microvillar olfactory sensory neurons and expressing both *MOR* and *VNR* loci (Cao et al., 1998).

Vertebrate *MOR* genes can be classified into two main groups: type I and type II, with several sub-groups within each type (Niimura and Nei, 2005). Two groups of *VNR* genes have been reported in mammals, *V1R*s (Dulac and Axel, 1995) and *V2R*s (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). A third reported group, *V3R*s (Pantages and Dulac, 2000), was subsequently reclassified as a sub-group of *V1R*s (Rodriguez et al., 2002). To date, most *VNR*s isolated from teleost fish show greatest similarity to the *V2R*s (Cao et al., 1998; Naito et al., 1998; Speca et al., 1999), but recently a *V1R* locus has been identified in a range of teleosts (Pfister and Rodriguez, 2005). Here, as previously (Dukes et al., 2004), both main olfactory receptors and vomeronasal receptors are collectively termed odorant receptors.

Olfaction is known to be crucial to accurate homing in anadromous salmonid species. Results from numerous studies indicate that young salmonids imprint on various odours of the homestream and use them as orientation cues in the return migration after a marine feeding phase (Dittman and Quinn, 1996). However, despite the popular use of salmonids as models for studies of olfactory physiology, signal transduction and signal processing for many years (Hara, 1992), the mechanism(s) by which an olfactory memory of the homestream is established are still poorly understood. There is evidence that at least part of this homestream memory is retained in the peripheral nervous system, i.e. within olfactory neurons (Nevitt et al., 1994), which may involve temporal changes in level of expression of *OR* genes during migration to sea (Dukes et al., 2004) or the return spawning migration. The aim of the present study was to isolate as wide a range of *OR* sequences as possible from the Atlantic salmon and to establish patterns of their tissue expression.

2. Materials and methods

2.1. Strategy for amplification of MOR and VNR sequences

PCR primers used previously to isolate *MOR* sequences from zebrafish, *Danio rerio* (Byrd et al., 1996), *Xenopus laevis* (primers X2.4 and OR7.1; Freitag et al., 1995) and catfish, *Ictolarus punctatus* (Ngai et al., 1993) were used in various combinations in an attempt to amplify as diverse a selection of *MOR* sequences as possible from Atlantic salmon genomic DNA from three individuals (one individual each from the rivers Nith, Oykel and Tweed in south-west, northern and south-eastern Scotland, respectively). All primers were degenerate (i.e. containing inosine or ambiguous base positions), and corresponded to amino acid motifs conserved across a wide range of *MOR* molecules (Mombaerts, 1999). For the *VNR*s, published degenerate primers (F1, F2, R1 and R2; Naito et al., 1998), designed from an alignment of human, rat and bovine Ca^{2+} sensing receptor genes (*CaSR*), were used to amplify both genomic DNA and 1st strand cDNA synthesised from testes, brain, olfactory epithelium, liver and anterior kidney (primer pair F1 and R1) and from olfactory epithelium (primer pair F2 and R2) from a single individual.

2.2. PCR amplification, cloning and sequencing

Amplifications were optimised in 25 μl reaction volumes containing 2.5–25 ng genomic DNA in standard buffer conditions [1.2 μM each primer, 0.1 mM each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Nonidet P40, 10 mM Tris–HCl (pH 8.8) and 0.5 U *Taq* polymerase]. Cycling conditions for *MOR* primers were: initial denaturation at 95 °C for 5 min, then 35 cycles of 95 °C for 30 s, 35 °C for 55 s and 72 °C for 45 s, followed by an extension step at 72 °C for 10 min. For the *VNR* primers, 40 cycles of 95 °C for 30 s, 57 °C for 55 s and 72 °C for 45 s were successful in generating sufficient product to use as template for a second round of PCR.

PCR products were excised from agarose gels and purified using either the GeneClean III kit (Amersham) or the QIAquick Gel Extraction Kit before ligation into the pCR2.1 vector and transformation into *E.coli* using the Original TA cloning kit (Invitrogen). DNA sequencing was performed using either the Sequenase 2.0 kit (Amersham) with radio-labelling or the ABI Prism Big Dye Terminator Cycle Sequencing kit with resolution of the fluoro-labelled products on an ABI Prism 377 automated sequencer. Partial sequences from one strand were used as the basis for identification of each insert (through BLAST alignments with Genbank sequences; Altschul et al., 1997), but complete sequences reported here resulted from sequencing of both strands.

2.3. Phylogenetic analysis

The phylogenetic position of the *SOR* sequences was investigated in relation to a number of published teleost *MOR* sequences and the recent classification of fish and tetrapod *MOR*s by Niimura and Nei (2005). Sequences from (1) the channel catfish, *Ictalarus punctatus* (*CORA-I*, L09217–L09225, Ngai et al., 1993); (2) zebrafish, *D. rerio* (*ZR-1, 2, 6, 9, 10* and *13*, U42392 and U42394–U42398, Barth et al., 1996; *ORZF1A, 1L*, and *39*, U44439–U44441, Byrd et al., 1996; *ZOR1–10*, U72683–U72692, Weth et al., 1996); (3) goldfish,

Carassius auratus (*GFA2, 12, 25, 28*, AF083076–AF083079, Cao et al., 1998; *CaOR29–31* and *45*, AJ233785–AJ233788, Freitag et al., 1998); (4) medaka fish, *Oryzias latipes* (*mfOR1–mfOR4*, AB029474–AB029480, Sun et al., 1999; *mfORE1–3 and Y1–3*, AB029474–AB029480, Yasuoka et al., 1999); (5) pufferfish, *Fugu rubripes* (*FOR1.1–1.5, 2*, AB031380–AB031385, Asano-Miyoshi et al., 2000); and (6) Atlantic salmon (*ASOR1*, AY007188, Wickens et al., 2001) were downloaded from Genbank.

Reference sequences for each of the subgroups of *MOR*s identified by Niimura and Nei (2005) were taken from the supporting information for that paper available at http://intl.pnas.org/cgi/content/full/0501922102/DC1 (α sub-group Xt1OR421.1 and Gg2OR1.2; β sub-group Cr3OR9.2, Fr3OR5287.2 and Xt1OR2286.1; γ sub-group Dr3OR5.4, Xt1OR12.1 and Gg2OR1.1; δ sub-group Dr3OR5.1, Fr3OR117.1 and Xt1OR3578.1; ε sub-group Dr3OR10.1, Fr3OR142.6 and Xt1OR4132.1; ζ sub-group Dr3OR10.14, Fr3OR3905.1; η sub-group Dr3OR7.3, Fr3OR59.1 and Xt1OR5329.1; θ sub-group Dr3OR2.1, Fr3OR633.1, Gg2OR9.1 and Xt1OR36957.1; κ sub-group Dr3OR7.2, Fr3OR18.1 and Xt1OR5508.1).

Similarly, the phylogenetic position of *SVR* sequences was assessed with reference to a recent classification of *V2R* sequences from fish and tetrapods by Yang et al. (2005). Pheromone receptor sequences from (1) goldfish (*GFB1*, *2*, *5*, *8*, *9*, *10*, *12*, and *14*, AF083080–AF083088, Cao et al., 1998; *Ca5.24* AF158963, Speca et al., 1999); (2) pufferfish (*Ca1, 2.1, 9, 12, 13* and *15*, AB008858–AB008861 and AB009044, Naito et al., 1998; (3) frog, *X. laevis* (*xV2RA-1–F-1*, AB125669–AB125674, Hagino-Yamagashi et al., 2004) were downloaded from GenBank. Representatives of mouse and rat sequences used to initially classify *V2R*s into groups A–C (Yang et al., 2005) were kindly supplied by Dr. Jianzhi Zhang (University of Michigan) (those included here were—group A, mouse *V2R39*, *60*, *68*, and *69* and rat *V2R104*, *106* and *107*; group B, mouse *V2R20* and *21* and rat *V2R108*; group C, mouse *V2R61* and rat *V2R147* and *148*).

As *V2R* sequences are related to glutamate, calcium sensing and type 1 taste receptors within the Glutamate (or family C) *GPCR*s (Bjarnadottir et al., 2005), representative sequences from these groups were also included in the phylogeny (pufferfish *mGluRA, B, D* and *E*, AB008863–AB008866, Naito et al., 1998), (human *CaSR*, U20760, Garrett et al., 1995; rat *CaSR*, NM_016996, Riccardi et al., 1995; seabream *CaSR*, AJ289717, Flanagan et al., 2002; pufferfish *CaSr* (*Ca1*), AB008857, Naito et al., 1998; tilapia *CaSR*, AY541693, Loretz et al., 2004), (rat T1R3, AAK51601, Nelson et al., 2001; mouse T1R2, AAK39438, Montmayeur et al., 2001).

Amino acid sequences were aligned using the Clustal W (Chenna et al., 2003) option in BioEdit v7.0.4 (Hall, 1999) and the alignment trimmed to the length of the *SOR/SVR* sequences before a neighbour-joining tree was constructed on the basis of mean character difference using Paup v4.0b10 (Swofford, 2002). Support for the nodes in the phylogeny was assessed through bootstrap re-sampling (1000 replicates). Trees were visualised using Treeview v32 (Page, 1996).

2.4. Analysis of tissue expression

Total RNA was extracted from approximately 100 mg of each of a range of Atlantic salmon tissues (olfactory epithelium, brain, anterior kidney, liver, and skeletal muscle) using TRIzol reagent (GibcoBRL). Residual DNA was removed by treatment with DNase I (Pharmacia Biotech), and cDNA produced using an oligo dT primer (1st Strand cDNA Synthesis Kit; Pharmacia Biotech).

Primers for amplification of Atlantic salmon *β-actin* sequences were used to check for presence of cDNA and absence of genomic DNA contamination: the primers span an intron and amplification products from cDNA and genomic DNA are therefore distinguishable on the basis of size. Tissue expression patterns of selected *OR* sequences were then established using PCR primers specific to individual families of salmon olfactory receptor (*SOR*), salmon vomeronasal

Table 1
Primers for amplification of *β-actin*, *SOR* and *SVR* sequences

Target gene	Primer set (5′→3′)	Amplicon length
β-actin	Forward: ATGGAAGATGAAATCGCCGC	~240 bp (cDNA)
	Reverse: TGCCAGATCTTCTCCATGTCG	~560 bp (gDNA)
SORA	Forward: GAGCTTTCCTAATACCTTTGGAC	468 bp
	Reverse: GACCTCTGCATTATCAA ACTGTC	
SORB	Forward: TGCCACCTTCCTGTAGTCTG	470 bp
	Reverse: CCCCTGCGATATTCCATC	
SORD	Forward: AGTGAAACATAATGGACGCC	470 bp
	Reverse: CAAAATGCTGGTGAAGCTG	
SORF	Forward: GGAAGGGGCTGCTTGCTCGA	484 bp
	Reverse: CTGTCTACTGAGGTATAACT	
SVRA	Forward: ATGGCCTTCAGGGCTACGCT	285 bp
	Reverse: AGGCAGCTTCCGAGCCAGAA	
SVRB	Forward: ATAGCTTTCCAGGCCACAAT	282 bp
	Reverse: AGGCAGCTTCCGAGCCAGAA	
SVRC	Forward: GCTGTGTTTAGGACCTCTAA	288 bp
	Reverse: TGGAAGATTCCTGGCCAGGA	
Sasa CaSR	Forward: CTTCTAGTGTTCGAAGCCAA	285 bp
	Reverse: TGGCAGTTTTCGTGATTTAA	

receptor (*SVR*) and Atlantic salmon calcium sensing receptor (*Sasa CaSR*) sequences (Table 1).

To confirm the identity of the PCR products amplified from genomic or cDNA using primers specific to individual *SOR*, *SVR* and *Sasa CaSR* sequence families, the products were Southern blotted from agarose gels onto nylon membrane, and probed by hybridisation to cloned representatives of each of the family of sequences using standard techniques (Sambrook et al., 1989).

2.5. Isolating full length MOR sequences

A cDNA library from the olfactory epithelium of a single adult Atlantic salmon (sampled from a fish farm in western

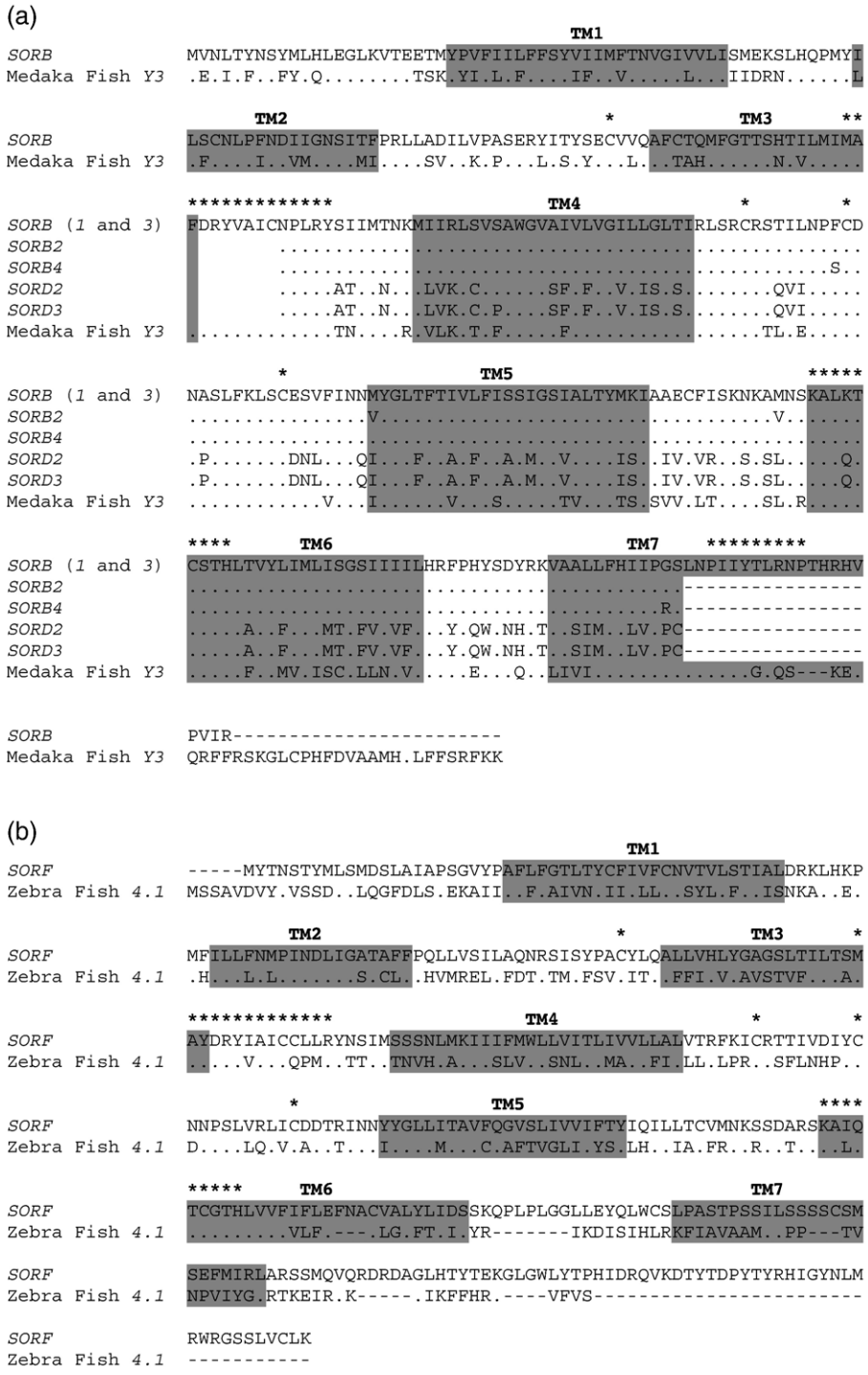

Fig. 1. Deduced amino acid sequences from *SOR* nucleotide sequences aligned with published sequences identified by BLAST search. Areas highlighted in grey represent presumed transmembrane domains. The symbol "-" indicates a gap, "." represents residues identical to the reference sequence and "*" indicates a conserved amino acid residue or motif. The full length *SORB*, *SORB1*, and *SORB3* sequences are identical at the amino acid level, but differ in nucleotide sequence. Medaka fish Y3 = putative medaka fish *MOR*; AB029480 (Yasuoka et al., 1999): zebrafish 4.1 = putative zebra fish *MOR*; AAF27277 (Dugas and Ngai, 2001).

Scotland) was constructed in the Lambda Zap II vector (Stratagene). Overlapping fragments of *MOR* genes were amplified from the library using combinations of *SOR*-specific primers and the T3 and T7 primer sites in the vector, cloned and sequenced as described above. Overlapping sequences were then integrated into single contigs using Sequencher 4.1 (GeneCodes) to produce full length sequences.

3. Results

3.1. MOR sequences

Amplification of PCR products of approximately the expected size was found for the primer pair of Byrd et al. (1996) and primer pairs A/C and B/C of Ngai et al. (1993). The *MOR* sequences obtained were classified into 6 families (*SORA–F*) on the basis of amino acid sequence (>75% identity; Fig. 1) and clustering within a phylogeny of *MOR* sequences (Fig. 2), with between 1–4 members identified within each family (GenBank accession numbers AY005449–AY005460, AY005824–AY005826, DQ375530). *SORA, SORB, SORD* and *SORF* sequences were approximately of the expected length, 468, 470, 470 and 484 bp long, respectively (excluding primer sequences). *SORC* and *SORE* sequences were much smaller in length – 140 and 83 bp long, respectively – and appear to represent truncated sequences homologous to the 5′ end of other *SOR* fragments isolated here.

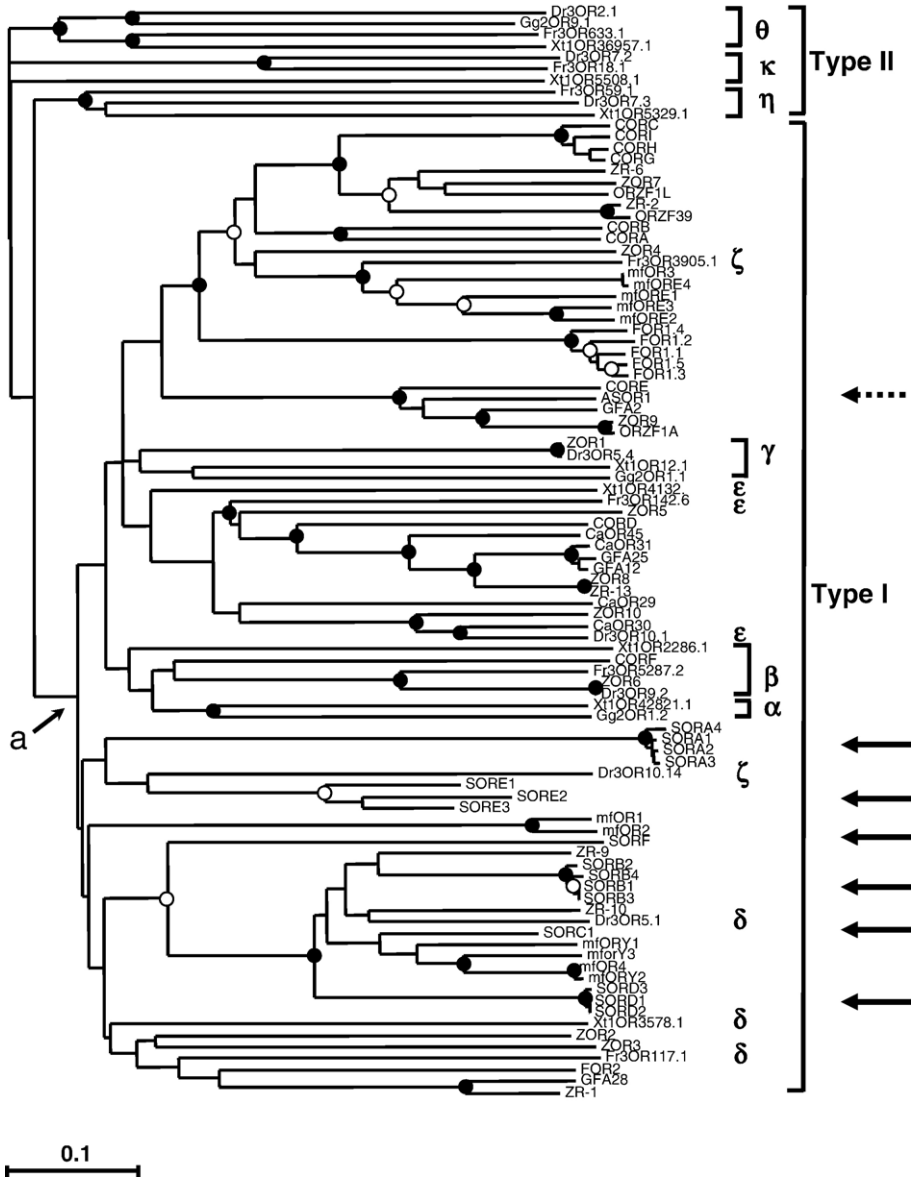

Fig. 2. Sequence phylogeny showing the position of *SOR* sequences in relation to published teleost *MOR* sequences and the classification of fish and tetrapod *MOR*s suggested by Niimura and Nei (2005). The sub-group designation (α, β, γ, δ, ε, ζ, η, θ or κ) of reference sequences or clusters of sequences from the Niimura and Nei (2005) classification are indicated. The tree is rooted using θ sub-group sequences. The positions of *SOR* sequences are shown by a solid arrow—the dashed arrow indicates the position of an Atlantic salmon *MOR* (*ASOR1*) previously isolated in a different study. Nodes with bootstrap support ≥90% (●) and 80–89% (○) and the node (a) which separates type I and type II *MOR* sequences are indicated. The scale bar is an estimate of the number of substitutions per site.

SORA, *SORB* and *SORE* sequences were found in all three individuals used for initial isolation of *SOR* sequences, with *SORD* sequences found in two individuals (from the R. Oykel and R. Tweed) and a *SORF* sequence present in one individual (from the R. Nith—although the presence of *SORF* was confirmed in the cDNA library made from a different individual). The occurrence of more than two sequences from an *SOR* sequence family in an individual (e.g. three different *SORA* and four different *SORB* sequences were amplified from one individual) suggested the presence of more than one locus coding for these sequences, but the number of nucleotide differences between sequences was small and could be due to PCR/sequencing artefacts.

SORB, *SORF, SORD2* and *SORD3* sequences have uninterrupted open reading frames. However, within each *SORA* sequence there are four stop codons, and one stop codon within *SORD1* which were observed in all clones from all individuals sampled. The 2 bp difference in length of *SORA* sequences compared to *SORB/D* sequences also suggests a frameshift deletion in *SORA* sequences, but it is not possible to identify the position of the indel from current data as introduction of indels at numerous possible positions produce equally plausible nucleotide and amino acid alignments. Further work is required to confirm that the stop mutation in *SORD1* is real and not a PCR or sequencing artefact as at present it is known from only a single clone. Similarly, the truncated sequence of *SORC* is also known from only one clone. However, the truncated *SORE* sequences (obtained independently from three individuals) were truncated at the same point in all clones and displayed relatively high levels of polymorphism (data not shown), suggesting that they represent pseudogene sequences.

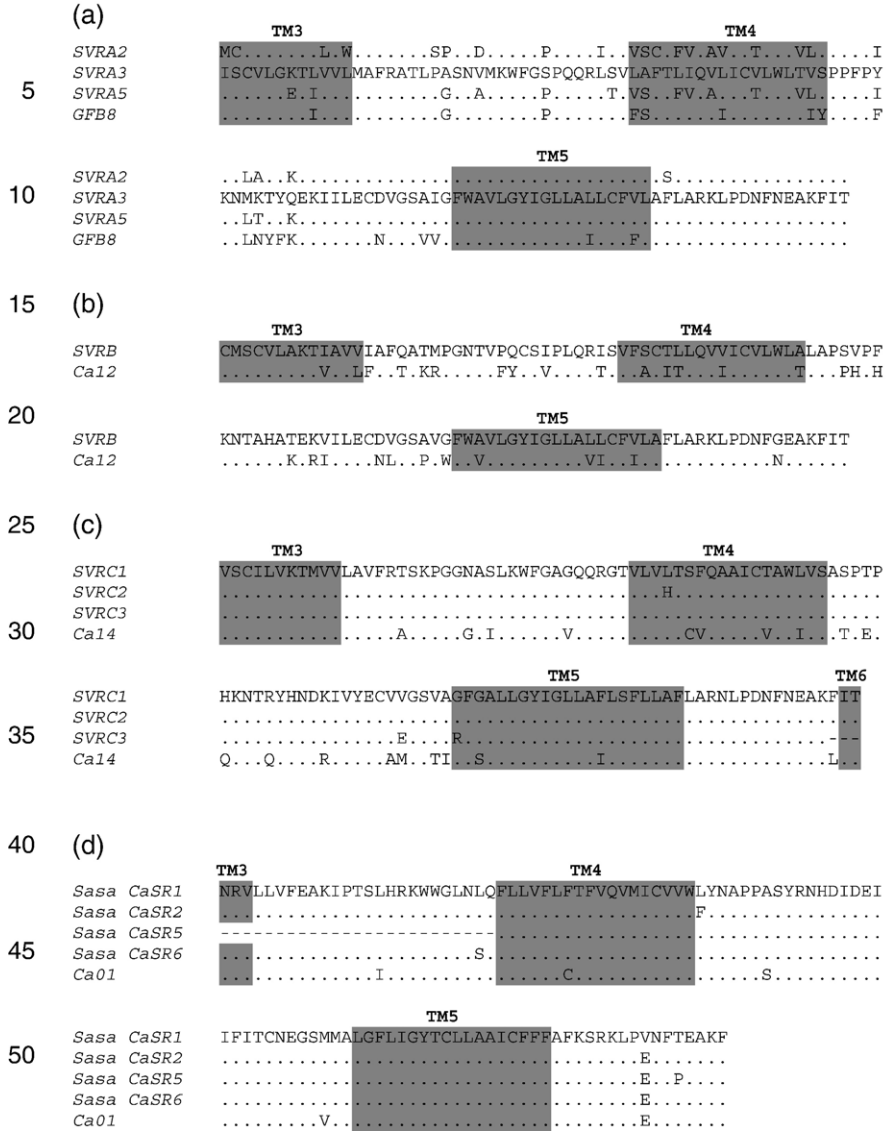

Fig. 3. Deduced amino acid sequences from *SVR* nucleotide sequences aligned with published sequences identified by BLAST search. Areas highlighted in grey represent presumed transmembrane domains. The symbol "-" indicates a gap, while "." represents residues identical to the reference sequence. GFB8 = putative goldfish *VNR*; AF083081 (Cao et al., 1998): *Ca12* and *Ca14* = putative *Fugu VNR*s; AB008860, AB009043: Ca 01 = putative calcium-sensing receptor from *Fugu* (Naito et al., 1998).

Full length and almost full length sequences were obtained from the cDNA library for *SORF* (DQ375530) and *SORB* (DQ375529), respectively (Fig. 1). Both sequences contain amino acid residues and motifs conserved in fish *MOR*s (Irie-Kushiyama et al., 2004): four conserved cysteine residues (one between TM2 and TM3, three between TM4 and TM5), the mayDRyVAICxPlxY motif spanning the C-terminus of TM3 and the N-terminus of the second intracellular loop and the KafsTCxsh motif found at the N-terminus of TM6 (where uppercase indicates highly conserved residue). However, the PPm(l/y)NPilY motif reported to be conserved at the C-terminus of TM7 was poorly conserved in the *SORB* sequence (Fig. 1(a)) and absent from the *SORF* sequence (Fig. 1(b)).

When a phylogeny of teleost and reference vertebrate *MOR* sequences was constructed (Fig. 2), relatively long terminal branches and short internal branches resulted in many deeper nodes being poorly supported. Type I and type II sequences were mutually monophyletic, although the node (node a) defining the groups was not strongly supported (<80% bootstrap support). *SOR* sequences clustered strongly within *SOR* families (all greater than 80% bootstrap support) but the *SOR* families (and a previously reported Atlantic salmon *MOR*, *ASOR1*; Wickens et al., 2001) were dispersed across the type I cluster. The reference sequences most closely related to Atlantic salmon *MOR*s were from sub-groups δ (for *SORB*, *SORC* and *SORD* sequences) and ζ (for *SORA*, *SORE* and *ASOR1* sequences).

3.2. VNR sequences

Ten putative *VNR* sequences were determined from both genomic DNA and cDNA and belonged to 3 distinct groups (*SVRA–C*) on the basis of amino acid sequence (>75%, Fig. 3) and clustering within a phylogeny of *VNR* sequences (Fig. 4)

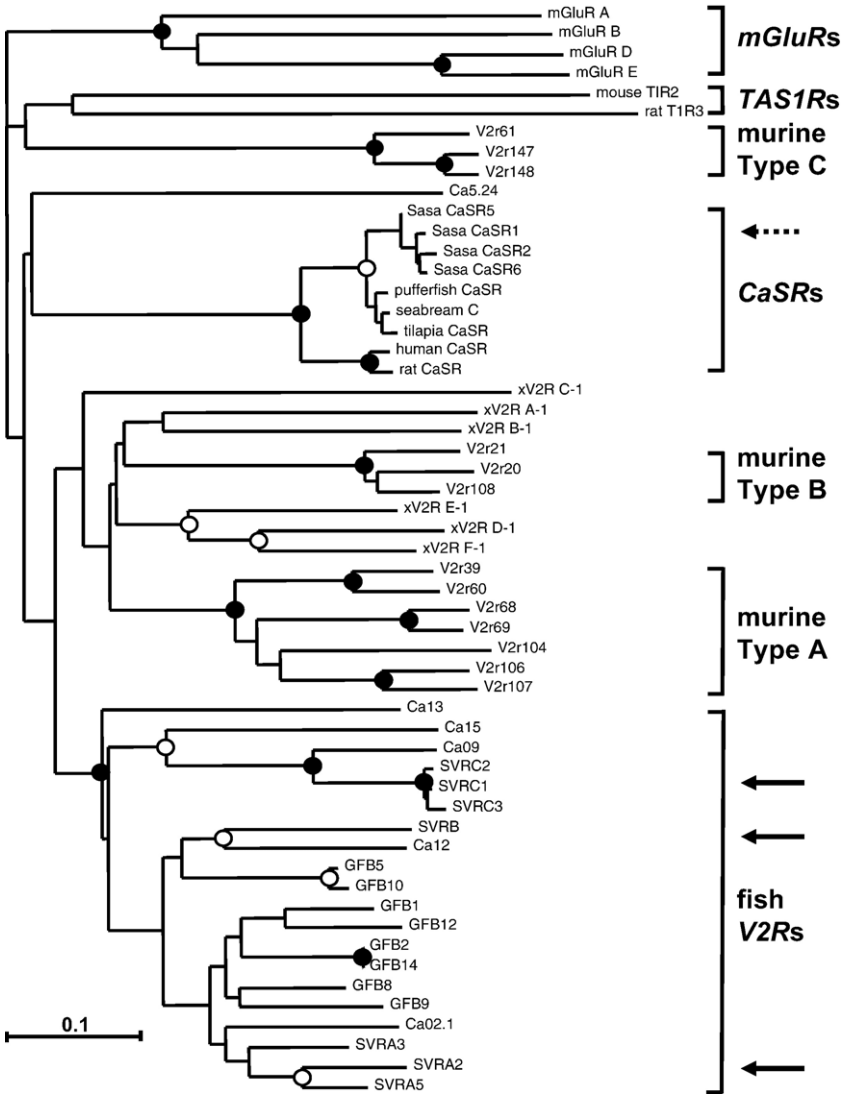

Fig. 4. Sequence phylogeny showing the position of *SVR* sequences in relation to published teleost, *Xenopus* and mammalian *VNR* sequences, mammalian *TAS1R* sequences and teleost and mammalian *CaSR* sequences. The tree is rooted using pufferfish *mGluR* sequences. The murine type A, B and C *V2R* groups identified by Yang et al. (2005) are indicated. The positions of *SVR* sequences are shown by solid arrows and that of *Sasa CaSr* sequences by a dashed arrow. Nodes with bootstrap support ≥90% (●) and 80–89% (○) are indicated.

with 1–5 sequences per group (GenBank accession numbers DQ375531–DQ375540). A separate family of 6 sequences, amplified using the same primers, were more closely related to calcium sensing receptor sequences (Figs. 3 and 4) and were therefore named as *Salmo salar*(*Sasa*) *CaSR1–6* (GenBank accession numbers DQ375541–DQ375546).

Sequences varied in length: *SVRA* sequences were either 353 or 354 bp, except 126 bp for *SVRA6* (truncated at the 5′ end); *SVRB* sequences were 353 bp long; *SVRC* sequences were 357 bp long and *Sasa CaSR* sequences 321 bp, except 249 bp for *Sasa CaSR5* (truncated at the 5′ end). All 16 sequences represented a portion of the molecule from transmembrane domain (TM)3 to TM6 (Fig. 3).

All *SVRC* sequences had uninterrupted reading frames. Within *SVRA* and *Sasa CaSR* sequence families, some members have frameshift mutations, which indicated the presence of pseudogenes. *SVRA1* and *SVRA4* have single base pair deletions at positions 180 and 12, respectively, both resulting in nonsense mutations. Both *Sasa CaSR3* and *Sasa CaSR4* contain in-frame stop codons at the same position in the sequence: at nucleotides 59–61. Both the *SVRA* and the *Sasa CaSR* families are, however, also represented by members with intact reading frames. *SVRB* was identified from a single clone.

As with the *MOR* phylogeny, most basal nodes in the *VNR* sequence phylogeny were not well supported (Fig. 4) but the general topology agrees with previously published phylogenies of Glutamate type *GPCR*s (Bjarnadottir et al., 2005) and *V2R*s (Yang et al., 2005) in terms of individual clusters of functionally distinct genes. All *SVR* sequences fell within a well-supported cluster made up exclusively of *V2R* sequences from teleosts, a sister group to a cluster of tetrapod *V2R*s (including the murine type A and B groups: Yang et al., 2005). As suggested from BLAST searches of GenBank, the *Sasa CaSR* sequences all fell within the well supported *CaSR* cluster, grouping most closely with other teleost *CaSR*s.

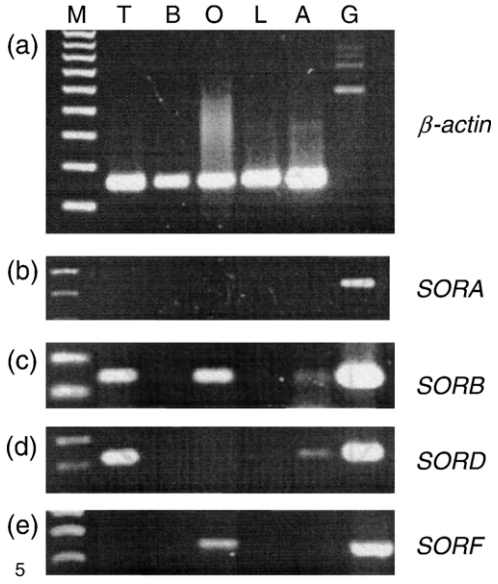

Fig. 5. Patterns of expression of *SOR* sequences in Atlantic salmon tissues from RT-PCR. M, 100 bp molecular size marker; T, testis; B, brain; O, olfactory epithelium; L, liver; A, anterior kidney; G, genomic DNA.

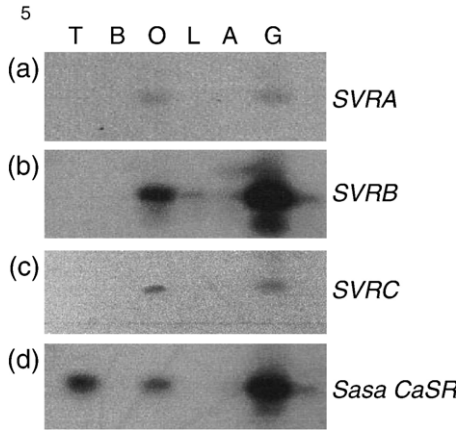

Fig. 6. Patterns of expression of *SVR* sequences in Atlantic salmon tissues from RT-PCR products hybridized with cloned representatives of same sequence family. T, testis; B, brain; O, olfactory epithelium; L, liver; A, anterior kidney; G, genomic DNA.

3.3. Patterns of tissue expression

All tissue samples showed relatively even levels of *β-actin* transcription with no evidence for contamination by genomic DNA (Fig. 5(a))—multiple bands in the genomic sample probably result from a combination of duplicated *β-actin* loci and intron length polymorphism. Amplification with *SORA* primers produced a single product of expected length from genomic DNA only (Fig. 5(b)). In contrast, there was clear evidence of transcription of *SORB* sequences in both testis and olfactory epithelium, with a lower level present in anterior kidney (Fig. 5(c)). Similarly, *SORD* sequences appear to be expressed in testis with relatively weak expression in anterior kidney. However, there was no indication of expression of *SORD* sequences in olfactory epithelium (Fig. 5(d)). *SORF* sequences were transcribed in olfactory epithelium only (Fig. 5(e)).

When amplification products involving *SOR* family-specific primers were Southern blotted and probed consecutively with *SORA*, *SORB*, *SORD* and *SORF* sequences excised from plasmid clones, hybridisation was found only to the PCR products obtained with the appropriate family-specific primers in all cases (data not shown). Similar results were found in an analogous analysis of *SVR* family-specific amplicons. *SVRA*, *SVRB*, *SVRC* and *Sasa CaSR* primers all amplified from olfactory epithelium cDNA and genomic controls (Fig. 6), while the *Sasa CaSR* primer pair also generated a product from testis cDNA (Fig. 6(d)). Tissue expression patterns for *SORB*, *SVRA* and *SVRC* have been reported previously (Dukes et al., 2004).

4. Discussion

4.1. OR pseudogenes

In our analysis of patterns of *SOR* expression we concentrated on the *SORA*, *SORB, SORD* and *SORF* sequences as, unlike *SORC* and *SORE*, these were not extensively truncated in the

clones derived from genomic DNA. The interruption of *SORA* putative coding regions by multiple stop codons suggested that they would not be expressed at the protein level, but it was possible that they could be transcribed. However, there was no evidence of *SORA* transcription in any of the tissues analysed. The presence of a confirmed pseudogene (*SORA*), two putative pseudogenes (*SORC* and *SORE*) and an apparent pseudogene allele (in *SORD* sequences) in a sample of six *MOR* families suggests a relatively high incidence of *MOR* pseudogenes in the Atlantic salmon genome. Furthermore, *SVRA* sequences include potential pseudogene alleles, indicated by the presence of nonsense mutations. Pseudogenes may therefore represent ~50% of the *OR* genes present in Atlantic salmon.

Vertebrate *OR* genomes vary considerably in the level of pseudogene occurrence: from ~25% in zebrafish and mouse, through ~50% in pufferfish, frog and human, to up to 85% in chicken (Niimura and Nei, 2005). However, pseudogenes do not appear to be a feature of the Atlantic salmon genome in general as no pseudogene alleles were found in surveys of the MHC class I and class IIα loci of Irish and Norwegian populations of Atlantic salmon (Consuegra et al., 2005a,b). Loss of multiple *OR* genes in some primate lineages has been hypothesised to be associated with a reduction in the importance of olfactory cues in the biology of those lineages (e.g. development of trichromatic vision, and consequent rise in importance of visual cues, appears coincident with appearance of many *OR* pseudogenes in old world monkeys: Zhang and Webb, 2003; Gilad et al., 2004). However, given the importance of olfactory cues to accurate homing in salmonids, it is perhaps surprising that the species harbours such a high number of *OR* pseudogenes.

4.2. OR expression in non-olfactory tissues

Transcription of *SORD* sequences in testis was not unexpected given that transcription of *SORB* sequences in testis had been reported previously in the Atlantic salmon (Dukes et al., 2004) and that olfactory receptor-like sequences have been found in the male germ cells of a range of mammals (Branscomb et al., 2000) and the channel catfish (Ngai et al., 1993).

The presence of *SORB* and *SORD* transcripts in the anterior kidney was, however, not initially expected and is an intriguing result in the light of evidence for *MOR* sequence transcription in the periarterial lymphatic sheath of rat spleen and the speculation that *OR* molecules might function as chemoreceptors on cells of the immune system (Walensky et al., 1998). The anterior kidney in teleost fish is a lymphomyeloid tissue, and therefore rich in lymphocytes. In addition, the amplification during this study of a sequence not obviously related to *OR* sequences but related to putative chemokine receptors (AY005461) (unpublished data), suggests that certain sequence motifs are conserved (or converged upon) in molecules with a chemotactic function.

4.3. Isolating functional OR genes in Atlantic salmon

Of those isolated in this study, *SORB* and *SORF* sequences appear to be good candidates for genes coding for MOR proteins [adding to the *MOR* sequence previously isolated by Wickens et al. (2001)] and *SVRA–C* represent the most likely *VNR* protein-encoding genes. However, results from this and other studies suggest that both pseudogenes and sequences expressed in tissues other than olfactory epithelium are often closely related to sequences that are expressed on olfactory neurons (Branscomb et al., 2000; Walensky et al., 1998; Spehr et al., 2004). Therefore, it seems likely that sequences from all *SOR* and *SVR* families identified here could be used to isolate additional *OR* sequences expressed in Atlantic salmon olfactory epithelium.

The independent cloning of closely related *MOR* sequences from the same species by different research groups [e.g. *ZR-13*/*ZOR8* and *ZR-2*/*ORZF39* from zebrafish (Barth et al., 1996; Byrd et al., 1996; Weth et al., 1996) and *mfOR4*/*mfORY2* and *mfOR3*/*mfORE4* from medakafish (Sun et al., 1999; Yasuoka et al., 1999)] suggests that currently used PCR-based strategies for isolating *OR* sequences are limited in their ability to isolate a wide range of sequence types as genome sequencing projects have revealed a very diverse range of *MOR* sequences in teleosts (Niimura and Nei, 2005). Indeed, examination of an *MOR* sequence phylogeny shows that type II sequences have only been identified in teleosts (zebrafish and pufferfish) through genome sequencing projects (Niimura and Nei, 2005). Similarly, *V1R* and type C *V2R* sequences in teleosts were identified initially through analysis of data from genome sequencing projects (Pfister and Rodriguez, 2005; Yang et al., 2005). The growing understanding of the extent of diversity in *OR* sub-genomes in model species points towards potentially fruitful areas for identifying further *OR* genes in species where full genome sequences do not currently exist.

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