Olfactory Receptor Related to Class A, Type 2 (V1r-Like Ora2) Genes Are Conserved between Distantly Related Rockfishes (Genus Sebastes)

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V1r-like Ora genes express putative chemoreceptors that may function as pheromone receptors in fishes. We used a candidate gene approach to test whether V1r-like Ora2 genes show evidence of positive selection that could suggest a role in mate recognition and the avoidance of hybridization between closely related rockfishes. We amplified a 492-bp fragment of a single V1r-like Ora2 gene from each of 5 species of rockfish. Despite separation of up to 7.8 My, the sequence of V1r-like Ora2 is highly conserved. Genetic distances are small, and all our study species shared at least one sequence with another species. Sequence comparisons suggested that, although most amino acids were subject to purifying selection, 9 amino acids showed evidence of positive selection. Because many of these amino acids were not associated with the areas of the protein suggested to be involved in ligand binding based on structural similarity to other olfactory receptors, this signal may reflect an echo of the relaxation of selection associated with the speciation events that separate these species. Strong sequence conservation suggests that this gene is of functional significance. However, because of shared alleles among species, the V1r-like Ora2 gene, in isolation, would be unlikely to differentiate species during mating season.

Key words: olfaction, pheromone receptor gene, V1r, mate choice, vomeronasal, sensory systems

The vertebrate olfactory sense provides key information on the environment, predators and prey, and potential mates. In fishes, olfaction is mediated by 4 olfactory receptor (OR) families: ORs, trace amine–associated receptors (TAARs), vomeronasal type 2 receptors (V2r-like OlfCs), and OR class A–related receptors (V1r-like Oras) (Pfister and Rodriguez 2005; Saraiva and Korsching 2007). Olfactory receptors are present in large numbers in fishes (143 genes in zebra fish) (Alioto and Ngai 2005), as are TAARs (>100 genes in zebra fish) (Gloriam et al. 2005; Liberles and Buck 2006; Hussain et al. 2009). Vomeronasal type 2 receptors (>50 genes in zebra fish) (Alioto and Ngai 2006) sense amino acids in teleosts and, along with V1r-like Oras (~6 genes in fishes), are thought to be involved in pheromone reception in mammals, although direct evidence is currently lacking for V2r-like OlfC receptors having such a role (Speca et al. 1999; Pfister and Rodriguez 2005; Saraiva and Korsching 2007).

The OR related to class A (V1r-like Ora) gene family, first described as V1r genes in rodents (Buck and Axel 1991), is one of 2 families of G protein–coupled receptors used to detect pheromones in mammals (Grus et al. 2005). Although fishes lack a vomeronasal organ, for which the gene family was originally named, numerous species have been shown to possess and express complete V1r-like Ora genes (Pfister and Rodriguez 2005; Pfister et al. 2007; Saraiva and Korsching 2007; Shi and Zhang 2007). In contrast to the mouse and the rat, however, which contain ~100–150 functional receptors, no fish species has yet been found to have more than a handful (5–6) (Saraiva and Korsching 2007). Additionally, in fishes, some research has shown evidence of positive Darwinian selection on these genes, suggesting that some neofunctionalization may have occurred (Pfister et al. 2007). Other researchers have suggested that these genes are highly conserved, finding no evidence for positive selection (Saraiva and Korsching 2007). In this project, we examine one member of the V1r-like Ora gene family in 5 species of the genus Sebastes, testing the hypothesis that this gene may be experiencing positive selection, putatively due to a role in pheromonal communication and mate recognition.

Rockfishes of the genus Sebastes comprise an extremely diverse and successful species complex. With an estimated 110 species worldwide (Love et al. 2002) and numerous examples of convergent evolution for similar lifestyles
(Hyde and Vetter 2007), rockfish present unique opportunities to use genetic studies to examine the process of speciation. Changes in mate recognition processes may provide one mechanism for the formation of reproductive isolation in rockfishes. Mate pairing in rockfishes is discrete because they engage in internal fertilization and require complex courtship rituals before copulation can take place (Love et al. 2002). In general, male rockfish align themselves next to a female before swimming forward and placing their urogenital papilla near her snout (Hallacher 1974; Helvey 1982; Shinomiya and Ezaki 1991; Gingras et al. 1998). It has been suggested that this behavior allows the male to release a courtship pheromone as near as possible to the olfactory rosette of the female (Love et al. 2002). In this study, we describe V1r-like Ora genes for 5 species of rockfishes (genus Sebastes), representing a broad sampling of rockfish phylogeny and ecology. Sebastes caurinus and S. maliger are separated by approximately 1.6 My, co-occur in rocky reef habitats in the Northeast Pacific, and have been shown to hybridize in Puget Sound, WA (Seeb 1998; Love et al. 2002). Sebastes melanops occurs around the same rocky habitats as S. maliger and S. caurinus but is separated by approximately 6.3 My of evolution (Love et al. 2002; Hyde and Vetter 2007). Sebastes ruberrimus and S. crenieri occupy a similar geographic range and similar habitats but may be found somewhat deeper and are separated from the other species in our set by approximately 7.1 and 7.8 My, respectively (Love et al. 2002; Hyde and Vetter 2007). Complete life-history data are lacking for several of these species, but it is known that some portion of the larval release period overlaps between all 5 species, as all release larvae during the spring and early summer (Love et al. 2002). It is thus conceivable that the mating season overlaps for the sample set as well and that they could be exposed to reproductive members of other species. We also test for a signal of positive and purifying selection on individual amino acids in our sequences using a Bayesian approach to test for deviations of the rate of nonsynonymous to synonymous nucleotide substitutions from neutral expectations (dN/dS = 1). If genes are involved in mate recognition or have played a role in speciation, we might expect to find a signal of positive selection at amino acids in the transmembrane domains of the protein, which are predicted to function in ligand binding based on structural analogy with related ORs. Additionally, we would anticipate finding evidence for divergence between species, particularly associated with these putatively functional domains.

Materials and Methods

To isolate and identify V1r-like Ora genes from rockfishes, we first retrieved nucleotide sequences for Ora genes from Botia macracantha, Danio albolineatus, D. frankei, D. malabaricus, D. rerio, Cyprinus carpio, Epalzeorhynchos frenatum, and Tetraodon nigroviridis from GenBank. These sequences were aligned and degenerate polymerase chain reaction (PCR) primers designed using the program FastPCR (Kalendar 2009). Genomic DNA was extracted from fin-clip tissue samples using a glass fiber plate protocol (Ivanova et al. 2006). PCR was performed on 8–24 individuals per species using primers 1F235 (5′-TGC-ATC-ACC-ATG-CTG-AGC-GT-3′) and R3BEM (5′-GGC-ACC-TGA-GGC-ACT-GTC-AGC-ATG-ATG-3′), which amplify a 495-bp portion of the V1r-like Ora type 2 gene. PCR was performed using the Promega GoTaq PCR reagent system (Promega) following the manufacturer’s protocols and optimized for high fidelity (low MgCl2 and low dNTP concentration, high annealing temperature, and short extension time). Thermal cycling conditions were an initial denaturing step at 94 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1:30, and a final extension step of 72 °C for 4 min. PCR products were separated via agarose gel electrophoresis and purified using a QIAquick gel extraction kit (Qiagen Inc., Valencia, CA). Purified PCR products were direct sequenced using the 1F235 and R3BEM PCR primers. To test for the existence of multiple V1r-like Ora2 genes or amplification of other members of the V1r-like Ora gene family, PCR products were also cloned using the TOPO TA cloning kit (Invitrogen Inc., Grand Island, NY) and 6 colonies sequenced per individual. Clones were sequenced on an ABI 3730XL automated sequencer using M13 forward and reverse primers. Forward and reverse sequence reads were aligned and edited using Sequencer (Gene Codes Inc., Ann Arbor, MI). Unique sequences have been deposited in GenBank (Accession nos GU589587–GU589842).

jModelTest v. 0.1.1 (Guindon and Gascuel 2003; Posada 2008) was used to calculate the most likely model of evolution in our sequences. Based on these jModelTest results, pairwise genetic distances (HKY85+G) (Hasegawa et al. 1985) were calculated using PAUP* v4.0b10 (Sinauer Associates, Sunderland, MA), Bioedit v.6.0.7 (Hall 1999) was used to convert DNA to amino acid sequence. We used the WebLogo 3 (Crooks et al. 2004) online server (http://threeplusone.com/weblogo/) to generate an amino acid sequence logo. We then used the TMHMM server v.2.0 ( Krogh et al. 2001; http://www.cbs.dtu.dk/TMHMM) and TMpred (Hofmann and Stoffel 1993; http://www.ch.embnet.org/software/TMPRED_form.html) to predict the transmembrane coding domains for the sequences.

To assess the hypothesis that positive selection may have caused the V1r-like Ora2 genes in different species to diverge in their functional domains, we used the SELECTON server v.2.4 (Stern et al. 2007; http://selecton.tau.ac.il/index.html) to identify amino acid sites under positive or purifying selection. In general, a dN/dS ratio (ω) > 1 at an amino acid site indicates positive selection on that site, whereas a ω < 1 suggests purifying selection at that site. We tested the significance of the selection estimates by calculating a 95% confidence interval for the ω value at each amino acid position and by performing a likelihood ratio test on the whole gene comparing the M8a null model (does not allow for positive selection) with the M8 model (does account for sites under positive selection).
Results

We successfully isolated a 492-bp fragment of a single V1r-like Ora type 2 gene from each of our 5 species of rockfish. None of our cloning work suggested the presence of additional V1r-like Ora2 genes or amplification of additional V1r-like Ora genes. We found 16 different sequences in 24 individual S. caurinus, 17 sequences in 24 S. maliger, 3 sequences in 8 S. crameri, 5 sequences in 8 S. ruberrimus, and 8 sequences in 8 S. melanops. Our sequences included most of transmembrane domain 3 and ran through the first few amino acids of extracellular domain 3 (Figure 1). The overall picture of V1r-like Ora type 2 diversity in our 5 rockfish species is that these genes are highly conserved, as evidenced by the strong amino acid conservation in our sequence logo (Figure 1) and by the short genetic distances (HKY85+G) we measured between species (Table 1). Additionally, we observed a number of shared sequences between species in our data set (Table 1).

Although most amino acids in the V1r-like Ora sequences are characterized by purifying selection when tested with SELECTON, we found significant evidence for strong positive selection in 9 amino acids in the complete data set of V1r-like Ora type 2 sequences (Figures 1 and 2). When we

Figure 1. Amino acid sequence logo for V1r-like Ora2 genes from 5 species of rockfish. Letter height indicates the relative frequency that a particular amino acid appears at that position. Labeled features include transmembrane domains (TMD) 3–6 as predicted by TMpred (gray bars) and TMHMM (black bars), intracellular loops (IC), extracellular loops (EC), and sites putatively under positive selection (Δ).

Table 1  Minimum and maximum genetic distances (HKY85+G) between species (below the diagonal), maximum genetic distance within species (on the diagonal), and average dN/dS ratio for between-species sequence comparisons (above the diagonal)

<table>
<thead>
<tr>
<th></th>
<th>Sebastes caurinus</th>
<th>S. maliger</th>
<th>S. melanops</th>
<th>S. ruberrimus</th>
<th>S. crameri</th>
</tr>
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<td>S. caurinus</td>
<td>0.01023</td>
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<td>0.2932</td>
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<td>0.4232</td>
<td>0.4051</td>
<td>0.3054</td>
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<tr>
<td>S. melanops</td>
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<td>0–0.01648</td>
<td>0.01852</td>
<td>0.6027</td>
<td>0.2614</td>
</tr>
<tr>
<td>S. ruberrimus</td>
<td>0–0.01645</td>
<td>0–0.01645</td>
<td>0–0.01643</td>
<td>0.01643</td>
<td>0.5139</td>
</tr>
<tr>
<td>S. crameri</td>
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<td>0.00203–0.01439</td>
<td>0–0.01025</td>
<td>0.00203–0.01439</td>
<td>0.00818</td>
</tr>
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most recent common ancestor by as much as 7.1 Ma (Hyde and Vetter 2007), and sequences in common. However, in a few cases, sequences were shared between species that are separated by much greater lengths of time: S. caurinus shares sequences with S. ruberrimus, separated by 7.8 My (Hyde and Vetter 2007), share a sequence in our data set. Additionally, most of the amino acids in the sequence show evidence of moderate to strong purifying selection. As such, it seems unlikely that sequence differences function to differentiate species at mating time. Visual, auditory, or lateral line cues, along with differences in reproductive timing and location, may function to prevent hybridization in related rockfishes (Love et al. 2002). We found 9 amino acid residues that showed a significant pattern of positive selection based on testing with SELECTON, which might be interpreted as consistent with selection causing pheromone receptor genes to diverge in close relatives. These positively selected sites are not isolated to the putatively functional transmembrane domains (Figure 1), which are associated with ligand binding in related ORs. However, at least one was found within each transmembrane domain, and those outside could conceivably be associated with conformational or other changes that affect ligand binding as well.

The overall high level of sequence conservation found in V1r-like Ora type 2 genes in rockfishes likely reflects their strong functional significance. Pfister et al. (2007) found a similar pattern of overall purifying selection in V1r genes, with evidence of positive selection on selected amino acid residues across 5 related species of teleost fishes. In comparisons between orthologous V1r-like Ora genes using a similar set of fish species, Saraiva and Korschning (2007) found low dN/dS ratios, indicating strong negative selection. The totality of evidence thus suggests that the V1r-like Ora gene family is very ancient and slowly evolving, that orthologous V1r-like Ora genes may have a common role across species, and that these receptors may be focused to recognize only a single or a very small number of molecules (Pfister and Rodriguez 2005; Pfister et al. 2007; Saraiva and Korschning 2007). The actual ligands recognized by fish V1r-like Ora genes are currently unknown (Pfister et al. 2007; Saraiva and Korschning 2007), but if these genes recognize pheromones, as has been suggested for the related V1r genes in mammals (Boschat et al. 2002), then they would be expected to have little species specificity, given their high level of sequence conservation (Saraiva and Korschning 2007). We hypothesize instead that these genes are involved in assessing mate condition, gender, or reproductive status or other qualities that are important across species and regardless of species identity.

**Discussion**

Overall, V1r-like Ora type 2 genes seem to be highly conserved across the sample species, which represent a broad sampling of the Sebastes phylogeny. Several sequences were shared among species, most commonly in close relatives: S. maliger and S. caurinus, separated by approximately 1.6 My (Hyde and Vetter 2007), had 8 sequences in common. However, in a few cases, sequences were shared between species that are separated by much greater lengths of time: S. ruberrinus shares sequences with S. caurinus, S. maliger, and S. melanops, although evidence suggests that ancestors of these species diverged from their most recent common ancestor by as much as 7.1 Ma (Hyde and Vetter 2007), and S. crameri and S. melanops, separated by 7.8 My (Hyde and Vetter 2007), share a sequence in our data set. Additionally, most of the amino acids in the sequence show evidence of moderate to strong purifying selection. As such, it seems unlikely that sequence differences function to differentiate species at mating time. Visual, auditory, or lateral line cues, along with differences in reproductive timing and location, may function to prevent hybridization in related rockfishes (Love et al. 2002). We found 9 amino acid residues that showed a significant pattern of positive selection based on testing with SELECTON, which might be interpreted as consistent with selection causing pheromone receptor genes to diverge in close relatives. These positively selected sites are not isolated to the putatively functional transmembrane domains (Figure 1), which are associated with ligand binding in related ORs. However, at least one was found within each transmembrane domain, and those outside could conceivably be associated with conformational or other changes that affect ligand binding as well.

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**References**


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