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ISOLATION AND CHARACTERIZATION OF ATLANTIC SALMON, *SALMO SALAR*, OLFACTORY RECEPTOR GENES

By

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF
ATLANTIC SALMON, *SALMO SALAR*, OLFACTORY RECEPTOR GENES

By

JILL ELIZABETH THORPE

Olfactory cues mediate Atlantic salmon behavior during different life history stages. To obtain an understanding of mechanisms influencing salmon behavior, we have addressed olfactory discrimination at the molecular level by cloning and characterizing 8 olfactory receptor gene fragments from the olfactory epithelium of the Atlantic salmon, *Salmo salar*. Gene fragments were extracted from salmon representing two life stages, 4 genes from mature parr and 4 genes from smolt. Gene sequence and structural analysis reveals shared motifs with published putative odorant and pheromone receptors of other vertebrates. Reverse Transcriptase-Polymerase Chain Reaction performed on the olfactory tissues of mature parr and smolt yielded gene products. No products were amplified from non-olfactory tissues. Phylogenetic and Southern blot analysis suggest the 8 salmon gene fragments represent four distinct subfamilies. Northern blot assays indicate that gene fragments from smolt are expressed solely in the olfactory organ of smolts, and that mature parr OR genes are primarily expressed in the tissues of mature parr. The findings represent the first identification of olfactory receptor genes in the Salmonidae family and demonstrate differences in OR gene expression between the mature parr and smolt. The results provide a foundation for understanding olfaction and olfactory mediated behaviors in the mature parr and smolt characteristic life histories.

DEDICATION

To Ken, my family, and Christine for all of their love and support.

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CHAPTER ONE: A Brief Review of Olfactory Receptor Genes

Olfactory systems of vertebrates detect and differentiate a large variety of odorants. The molecular mechanisms leading to the versatility and specificity of olfactory function and discrimination are poorly understood. Odor recognition begins when chemical cues interact with specialized, peripheral receptors expressed within the sensory epithelia (Lancet 1986). This primary discrimination mechanism may be elucidated using molecular cloning techniques to characterize olfactory receptor (OR) genes. The intronless, olfactory receptor genes represent the largest family of 7 transmembrane domain (TMD), G-Protein coupled receptors (Buck and Axel 1991). Excellent reviews on the recent developments of OR genes are provided by Mombaerts (1999), Hildebrand and Shepard (1997), and Buck (1996). Although significant advances in understanding the transduction mechanisms, characterization, and genetic composition of olfactory receptors have emerged (Bargmann et al. 1993; Barth et al. 1997; Raming et al. 1993), questions regarding olfactory evolution, odorant recognition, and olfactory impact on physiology and behavior have yet to be answered.

Molecular research presents an approach to deciphering olfactory function. Identification of olfactory receptor genes provides insight into the evolution of stimulus discrimination, reinforces the understanding of inter-species relationships, and confirms the olfactory receptor 7 transmembrane domain characteristic (Freitag et al. 1998; Buck and Axel 1991; Ngai et al. 1993; Byrd et al. 1996). In addition, characterization of OR genes allows the identification of functional domains, offers the opportunity to associate a particular gene with a specific odorant molecule, and furthers an understanding of olfactory mediated behaviors.

In 1991, Buck and Axel's study on rat olfactory receptor genes gave great impetus to the genetic basis of olfactory research (reference thereafter). The investigators reported the presence of a large, multigene family encoding hundreds of putative olfactory receptor proteins. The findings were based on three assumptions. First, OR's have the characteristic of seven transmembrane domain, G-protein coupled receptors. Secondly, olfactory receptor genes may represent a multi-gene family due to the olfactory system's ability to discriminate a wide range of chemical structures. Finally, OR gene expression should be localized to sensory epithelium. Using degenerate primers prepared from transmembrane domains II, III, and VII, cDNA from rat olfactory epithelia was subjected to the polymerase chain reaction (PCR). Amplified PCR products, approximately 600-1300 base pairs in length, were cloned and sequenced. These DNA fragments were treated with restriction enzymes. The restriction digest revealed that the sum of the fragment lengths exceeded that of the original PCR product suggesting the presence of a gene family. Sequence analysis of the cDNA clones implied introns were not contained within the OR gene coding region. Intronless genes consist only of coding regions and can not be differentially spliced. The deduced amino acid residues consisted of seven hydrophobic domains, supporting the 7 transmembrane domain characteristic. Unlike other 7 TMD proteins that exhibit 80% sequence conservation, olfactory proteins show considerable sequence divergence within the III, IV, and V domains. These variant domains may reflect binding sites for different odorant structures. Northern blot assays indicated that the members of the multi-gene family are expressed solely in the olfactory epithelia of the rat. Lastly, Buck and Axel (1991) estimated that the rat genome contains 500-1000 OR genes, corresponding to 0.8-1.6% of the 60,000 or so mammalian genes.

Recent research indicates that the OR gene family consists of smaller sub-families as suggested by sequence and Southern blot analysis (Ngai et al. 1993, Ressler et al. 1993; Berghard and Dryer 1998). OR genes contain variant sequences and share sequence conservation with a sub group of related genes. Therefore, subfamilies exhibit a greater sequence similarity amongst themselves than with other gene subfamilies. Subfamily definition may be dependent upon researcher discretion. Buck and Axel considered related gene sequences that share 91% identity to be of the same subfamily, whereas Barth, et al. (1997) set the criterion at 60%. Southern blot assays provide additional support of the subfamily characteristic. Southern analysis of an organism's OR genes reveal a number of hybridization bands that do not appear to cross hybridize. The degree of cross hybridization may be related to the degree of gene similarity. Thus, variations in Southern assay hybridization patterns may suggest the presence of subfamilies. The significance of subfamilies may be inherent to their structural and functional properties. It has been proposed that genes possessing similar structural properties bind compounds with a specific structural motif (Kobilka et al. 1988).

Since the discovery of OR genes, researchers have employed similar approaches to identify receptor genes that may detect pheromones (Ryba et al. 1997; Cao et al. 1998). It is hypothesized some vertebrates maintain a vomeronasal organ (VNO), an accessory olfactory organ, to detect pheromones and odorants involved in foraging and reproductive behaviors (Eisthen 1997). Tetrapods appear to possess the VNO (Doving and Trotier 1998), while fish do not. The presence and function of the VNO is still under investigation. Therefore, for simplicity purposes, all receptors capable of detecting chemical stimuli will be referred to as olfactory receptors.

OR genes have been cloned from various vertebrates, including rats, mice, dogs, cows, pigs, chicken, river lamprey, chimpanzees, orangutans, goldfish, zebrafish, catfish, and frogs (Cf. Buck and Axel 1991; Ngai et al. 1993; Byrd et al. 1996; Blache et al. 1998; Mombaerts 1999). The cloning strategies of Buck and Axel have continued to be used, but the primer sequences have been refined to reflect changes in similarity and degeneracy across species. Confirmation of OR genes is supported by sequence similarity to known OR genes, Northern blot analysis, and *in situ* hybridization experiments.

Subsequent research has provided information on spatial expression patterns of OR genes. *In situ* hybridization experiments on rats and mice reveal specific OR genes correspond to distinct zones in the olfactory epithelia (Ressler et al. 1993; Vassar et al. 1993; Barth et al. 1997). Topographical patterns appear to consist of three (Ressler et al. 1993) or four (Vassar et al. 1993) expression zones. A distinctive pattern of odorant receptor expression on olfactory sensory neurons has yet to be firmly established in fish (Byrd et al. 1996; Ngai et al. 1993a; Weth et al. 1996). Researchers suggest zones may correspond to distinct subfamilies involved in the detection of particular odorants.

In addition, olfactory receptor genes are not isolated to the olfactory epithelium. OR gene expression has been documented in the testis of rat, mouse, human, and dog (Parmentier et al. 1992; Mombaerts 1999). The presence of OR's in reproductive organs implies a link between olfaction and reproduction. Ultimately, isolating olfactory receptor genes will be an important component in comprehending the impact of olfaction on an organism's behavior and reproductive physiology.

Our approach to comprehending olfactory stimulus discrimination involves the molecular characterization of OR genes and their expression in the olfactory organ of the Atlantic salmon, *Salmo salar*. Atlantic salmon offer an efficient model for studying olfactory function at the molecular level. In comparison to tetrapods, fish possess a smaller repertoire of OR genes (Ngai et al. 1996; Barth et al.1997). Additionally, information on salmon behavior, olfactory anatomy, and physiology is readily available (Hasler and Scholz 1978; Hara 1994). The olfactory sense is vital to the survival and behavior of salmon. In addition to migratory behavior and spawning stream selection (Hasler and Scholz 1978), chemical cues are critical to sexual maturation, reproductive behavior, and food recognition (Moore and Scott 1991; Moore and Waring 1996; Sorensen et al. 1988; Mearns 1985). Therefore, investigating salmon olfactory function at the molecular level presents an opportunity to understand the role of chemical cues in mediating salmonid behaviors.

Olfaction plays a critical role of sensory discrimination in migratory behavior. Salmon, an anadromous fish, spend early life stages, as alevin, fry, and parr, in fresh water. After spending one to two years in the natal stream, some fish undergo smoltification in the spring and migrate to sea in order to feed and grow. When the fish are sexually mature and capable of reproducing, the salmon undergo upstream migration to spawn. The salmon are directed to their natal streams by chemical cues (Sutterlin et al. 1973; Hasler and Scholz 1978). Understanding the role of olfactory receptors in this process may lead to ascertaining the specific odorants that guide a fish to its place of birth. In addition to being the first documentation of odorant receptor gene sequences in

a salmonid species, identifying salmon OR genes will provide a basis for determining stimuli that mediate salmon migration.

Furthermore, salmon exhibit high sensitivity to odorants during specific months of the year and particular periods of physiological development (Moore & Scott 1991; Dittman et al. 1997). By screening tissues of sexually mature and immature salmon for OR genes, differences in patterns of gene expression between two stages of maturity can be deciphered. The relationship and interaction between the reproductive and olfactory sensory systems may be linked as implied by OR gene expression in the sex organs of vertebrates. Overall, cloning salmon olfactory receptor genes will help advance knowledge of olfactory reception and provide a basis for further studies in olfactory function.

CHAPTER TWO: Isolation and Characterization of Atlantic salmon, *Salmo salar*, Olfactory Receptor Genes

INTRODUCTION

Olfaction plays a primary role in the survival and life history of Atlantic salmon, *Salmo salar*. Chemical cues are critical to migratory behavior, spawning stream selection, sexual maturation, reproductive behavior, and food recognition (Hasler et al. 1978; Moore and Scott 1991; Moore and Waring, 1996; Mearns 1985). Although the electrical properties and signal transduction cascades of Pacific salmon olfactory receptor cells have been elucidated (Nevitt and Moody 1992; Nevitt 1987; Dittman et al. 1997), knowledge of the genetic sequence of Pacific and Atlantic salmon olfactory receptors is minimal. Furthermore, the molecular mechanisms whereby odorants mediate characteristic behavior of salmon life histories have yet to be established.

Atlantic salmon provide an efficient model for investigating olfactory function at the molecular level. In comparison to tetrapods, fish possess a smaller repertoire of OR genes (Ngai et al. 1996; Barth et al. 1997). Furthermore, due to its large role in aquaculture industry (Anderson 1997), commercial and recreational importance (Myers 1984), and suitability as a model in behavioral and ecological studies, the Atlantic salmon species is intensely studied. For these reasons, information on salmon behavior, physiology, and olfactory anatomy is readily available. Moreover, studying Atlantic salmon sensory discrimination presents the opportunity to ascertain the role of olfaction in mature parr and smolt life history behaviors.

Among vertebrates, Atlantic salmon maintain unique life history strategies. Anadromous salmon, hatched in streams, spend 20-50% of their life in fresh water (Hutchings and Jones 1998). Depending on growth opportunities, genetic variables, and physiological drives, a subset of male fish, the mature parr, mature as yearlings in the autumn and reproduce with adult females (Thorpe et al. 1992). Another subset, sexually immature parr, undergo smoltification in spring and migrate to seawater as smolts. When smolts become sexually mature and capable of reproduction, the fish, now termed salmon, guided by chemical cues, enter their natal stream to spawn (Sutterlin et al. 1973; Hasler and Scholz 1978). Mature parr may smoltify after they have completed their reproductive cycle. Understanding the variation in mature parr and smolt life history behaviors involves identifying distinctive characteristics in their physiology, behavior, and anatomy (Thorpe et al. 1992; Lundqvist et al. 1986; Saunders et al. 1982; Jakobsson et al. 1997). Differences of olfactory sensitivity in Atlantic salmon life stages have been established (Moore and Scott, 1991). Therefore, mature parr and smolt behaviors may be distinguished further by variations in sensory discrimination.

The first step in transduction of odorants is stimulus interaction with olfactory receptors. One approach to comprehend the mechanism of Atlantic salmon odorant discrimination is the molecular characterization of olfactory receptor genes. Buck and Axel (1991) discovered a family of genes they proposed to encode putative olfactory receptors in the rat olfactory epithelium. The intronless genes represent the largest family of 7 trans-membrane, G-protein coupled receptors. Identification of OR genes provides insight into the evolution of stimulus discrimination, reinforces the understanding of inter-species relationships (Freitag et al. 1998), and confirms the olfactory receptor 7

transmembrane domain (TMD) characteristic (Cf. Buck and Axel 1991; Ngai et al. 1993; Byrd et al. 1996). In addition, OR gene characterization allows identification of functional domains (Buck and Axel 1991), offers the opportunity to associate a particular gene with a specific odorant (Raming et al. 1993; Krautwurst et al. 1998), and provides a foundation for understanding olfactory mediated behaviors. Since the findings of Buck and Axel (1991), olfactory receptor genes have been cloned in over 25 species, including mammalian, fish, amphibian, avian, and invertebrate organisms (Cf. Buck and Axel 1991; Ngai et al. 1993; Freitag et al. 1995; Byrd et al. 1996; Barth et al. 1996; Blache 1998; Berghard and Dryer 1998; Mombaerts 1999; Bargmann et al. 1993; ORBD 1999). Although significant advances in the understanding of transduction mechanisms, characterization, and genetic composition of olfactory receptors have emerged (Goulding et al. 1992; Bargmann et al. 1993; Barth et al. 1997; Raming et al. 1993), questions regarding the molecular strategy by which OR function mediates the distinctive behavior of the Atlantic salmon have yet to be established.

This study aims to isolate and identify OR genes in the Atlantic salmon. In addition to illustrating olfactory receptor gene expression in various salmon tissues, differential expression of OR genes at two critical life stages, mature parr and smolt, was investigated. The findings represent the first documentation of OR gene expression in two salmon life history stages.

MATERIALS AND METHODS

Isolation of Nucleic Acids

Atlantic salmon, *Salmo salar*, were obtained from the USGS Conti Anadromous Fish Lab, Turners Falls, MA and Lake Superior State University (LSSU) Hatchery, Saulte Ste. Marie, Michigan. 6 and 10 mature parr were obtained through electro-fishing in the Sawmill (Fall, 1998) and Four Mile Brook (Fall, 1999) rivers, respectively. 10 and 15 hatchery smolts were obtained from the USGS Conti Anadromous Fish Lab during the fall of 1998 and 1999, respectively. 30 hatchery smolts were obtained from LSSU during the spring of 1999. Tissue samples from all mature parr and smolt salmon were collected and preserved on dry ice or in RNA*later* (Ambion, Austin, TX) for total RNA extraction. In the fall of 1999, muscle tissue from 2 mature parr and 2 smolt salmon was preserved in 95% ethanol for genomic DNA extraction. Sexual maturity of fish was determined by the developmental stage of the gonads. Mature parr had sexually mature testes, while smolts did not (Saunders et al. 1982). RNA was prepared from the olfactory organ, gills, brain, liver, muscle, and gonads of all mature parr and smolts according to the Trizol protocol (Gibco, Rockville, MD) and quantified by spectrophotometry (Ausubel et al. 1995). Due to the small olfactory organ size and amount of RNA required for molecular applications, olfactory organs from three like fish were combined into one sample (mature parr and smolts were pooled separately). Genomic DNA was extracted in accordance with the PureGene protocol (Gentra, Minneapolis, Minnesota) and quantified by fluorimetry (Hoefer: DNA Quant 200).

First Strand cDNA Synthesis

Total RNA from the olfactory organ, gills, brain, liver, muscle and gonads of mature parr and smolts was used to generate cDNA. RNA samples were treated with DNase I (0.1 U per μg of RNA). First strand cDNA synthesis was carried out in 20 μl reaction volumes. 1 μg of total RNA was incubated with 1 μl oligo dT 18 bases (500 μg/ml) at 70°C for 10 minutes. The RNA, oligo dT, 0.1 M DTT, 10 mM each dATP, dGTP, dCTP, and dTTP, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$ were incubated at 37°C with 200 units of M-MLV enzyme (Gibco, Rockville, MD) for 50 minutes. Inactivation of the reaction was accomplished by heating at 70°C for 15 minutes.

PCR and RT-PCR

cDNA from mature parr and smolt olfactory organ, gills, brain, liver, muscle, and gonads was used as a template for amplification in the Polymerase Chain Reaction (PCR) (Mullis 1990). Degenerate primers, designed from conserved regions of transmembrane domains II, III, and VII of published vertebrate OR and vomeronasal (VNO) gene sequences, were utilized (Buck and Axel 1991; Ngai et al. 1993; Byrd et al. 1996; Cao et al. 1998). Primers were chosen at random. PCR reactions of 50 μl total volume contained 100 ng of template, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM dNTP's, 1U Taq Polymerase (Gibco, Rockville, MD), and 100 pmol each primer. Thermal cycling parameters (MJ Research PTC-200) for the goldfish VNO primer pair, 5' primer: 5'-ACNCCNATHGTNAARGCNAAYAA-3' and the 3' primer: 5'-YTTNGCYTCRTTRAANGYRTC-3', (Cao et al. 1998), were as follows: 1 cycle- 94° C

for 2 min.; 30 cycles- 94° C for 1 min., 38° C for 1 min., 72° C for 2 min.; 1 cycle- 72° C for 10 min. Aliquots of PCR products were analyzed by agarose gel electrophoresis.

Cloning and sequencing

PCR products were excised from a 1.2 % agarose gel, purified using the Wizard Purification System (Promega, Madison, WI), ligated into the pGEM Easy Vector System (Promega, Madison, WI), transformed into heat shock JM109 competent cells (Promega, Madison, WI), and cultured with SOC media (Sambrook et al. 1989). After incubation of plates for 24 hours, colonies were screened for vector inserts. Prospective colonies were picked and grown in 4 ml of LB media with 30 µl of ampicillin. Plasmid DNA was subjected to PCR to amplify the gene insert and obtain clone size using T7 and SP6 primers. PCR reactions of 50 µl total volume contained 100 ng of DNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.0 mM MgCl2, 0.2 mM dNTP's, 1 U Taq Polymerase, and 50 pmol each primer. Thermal cycling (MJ Research PTC-200) parameters were: 1 cycle- 94° C for 4 min.; 30 cycles- 94° C for 45 sec., 50° C for 45 sec., 72° C for 2 min.; 1 cycle- 72° C for 10 min. Plasmid DNA was prepared and purified according to the 5'-3' Mini-Prep kit protocol (5Prime-3Prime, Boulder, Co).

Cloned DNA was sequenced using Hex labeled T7 and SP6 primers from Integrated DNA Technologies, Inc. (Coralville, IA). Sequencing reactions followed the Epicentre Biotechnologies cycle sequencing protocol (Epicentre Technologies, Madison, WI). Sequencing reactions of 6 µl contained 50 ng of plasmid DNA, 2.5 pmol of primer, 2 µl of dNTPs/ddNTPs termination mix (G, A, T, C), 1.8 µl SequiTherm EXCEL II sequencing buffer, and .25 µl SequiTherm EXCEL II DNA Polymerase (5 U/µl). Thermal cycling parameters: 1 cycle- 95° C for 2 min; 30 cycles- 95° C for 45 sec., 55° C

for 45 sec., 72° C for 2 min. Reaction products were separated on a 6% polyacrylamide gel, scanned on a Hitachi FMBio II Multi-view System, read using FMBio102 Read Image v1.1 Software, and scored by FMBio Analysis 8.0 Software. Gene specific primers were designed to walk across the gene to obtain the complete sequence of insert DNA. Complementary strands of the insert were sequenced.

Sequence Analysis

The cloned fragments were compared to sequences in the National Center for Biotechnology Information (NCBI) database using BLAST software in order to receive homologous gene sequences (see: http://www.ncbi.nlm.nih.gov/). Nucleotide and deduced amino acid sequences were aligned using the CLUSTAL-W algorithm (The Baylor College of Medicine Search Launcher). Representatives of goldfish, *Carassius auratus,* (Acc: AF083081) and pufferfish, *Fugu rubripes*, (Acc: AB009040) odorant receptor gene sequences were included for comparison. The goldfish and pufferfish OR genes were chosen randomly. A phylogeny was created using PAUP 4.0 (Swofford 1998) with salmon gene fragments for the ingroup and representatives of goldfish (Acc: AJ233788; AJ233787), pufferfish (Acc: AB009032; AB009034), zebrafish (Acc: U72691; U72692), catfish (Acc: L09219; L09218), medaka (Acc: AB029476; AB029478), frog (Acc: AJ011429; AJ011430), rat (Acc: AF016178; AF053992), river lamprey (Acc: AF069553; AF069554), and mouse (Acc: NM_013618; NM_013619) OR genes as outgroups. Two OR genes from each outgroup species were chosen randomly and utilized in the phylogeny. Each gene spanned TMD III-V and was aligned with the Atlantic salmon clones prior to phylogenetic analysis to ensure gene homology.

RACE experiments

In order to identify additional characteristics of gene fragments, Rapid Amplification of cDNA Ends (RACE) was performed according to the SMART RACE 5' and 3' Kit (Clontech, Palo Alto, CA). Nested primers were generated from the sequences of interest. The gene specific primer (GSP) for 5' RACE, the anti-sense primer, is 5'-TCTGCATCTCTTGTGTTCTGGGG- 3'. The GSP for 3' RACE, the sense primer, is 5'-GCTGAACCCACATCACACTCTAG- 3'. All procedures followed the protocol outlined in the Clontech SMART RACE manual, except for thermal cycling parameters. The appropriate adjustments for using the MJ Research PCR thermocycler were as follows: 5 cycles: 94° C for 10sec., 70° C for 30 sec., 72° C for 3 min; 10 cycles: 94° C for 20sec., 68° C for 30 sec., 72° C for 3 min.; 20 cycles: 94° C for 20sec., 65° C for 30 sec., 72° C for 3 min. RACE products were analyzed by electrophoresis on 1.2 % agarose gels. cDNA fragments over 300 bases were excised from the gel, purified using the Wizard Purification kit (Promega, Madison, WI), and cloned according to the procedure outlined above.

dsDNA Probe Synthesis

Biotinylated probes were generated according to the protocol provided in the BrightStar Psoralen-Biotin non-isotopic labeling kit (Ambion, Austin, Texas). Based on sequence identity, genes were grouped into subfamilies whereby members exhibit over 85% nucleic acid similarity. Assuming gene fragments with a high degree of similarity would reveal similar hybridization patterns, only one representative clone of each subfamily was generated into a probe to be used in blotting assays (ASOR16, ASOR11, ASOR17, and ASOR55). Each representative clone was chosen randomly. Double

stranded DNA was obtained from plasmid PCR using SP6 and T7 primers with recombinant pGEM vectors as templates. The Plasmid PCR products were run on a 1.2% low-melting temperature agarose gel. Target fragments were excised and subjected to Phenol/Chloroform extraction. Gene products were exposed to a restriction digest using NdeI and NcoI to eliminate flanking primer regions. This dsDNA (0.5 μg) template was labeled according to the protocol outlined in the BrightStar Psoralen-Biotin instruction manual. Probes ranged in size from 200 bases to 500 bases.

Southern Blotting

Atlantic salmon genomic DNA, isolated from muscle tissue from 2 hatchery smolts obtained from LSSU, was digested with EcoRV and HindIII, electrophoresed on a 1% agarose gel, and blotted, through downward capillary transfer, onto a positively charged nylon membrane (Ambion, Austin, Texas). Blots were hybridized with Psoralen-Biotin labeled probes corresponding to gene fragments ASOR16, ASOR11, ASOR17, and ASOR55. Hybridizations were carried out at 45^0 C. Low stringency washes, using 0.1 standard saline citrate (SSC), were performed at 42^0 C. Labeled DNA probes were detected using the BrightStar BioDetect Nonisotopic Detection Kit (Ambion, Austin, Texas). Detection was carried out using chemoluminescence generated from enzymatic reactions of conjugated biotinylated probes.

Northern Blotting

Probes representing ASOR16, ASOR11, ASOR17, and ASOR55 were used in Northern Blotting experiments to characterize OR gene expression in olfactory and non-olfactory tissues of smolt and mature parr. Total RNA extracted from the olfactory organ, gills, liver, and testes of mature parr and smolts obtained from Turners Falls and

LSSU was utilized in assays. Hybridization and washing conditions were as follows: 45^0 C for hybridization; 45^0 C for washing. Detection of biotinylated probes followed the chemoluminescent detection procedure employed in Southern Blot Analysis.

RESULTS

Isolation of Atlantic salmon odorant receptor genes was based upon their expected homology to published OR genes of aquatic and terrestrial organisms (Buck and Axel 1991; Cao 1998). Degenerate oligonucleotide primers, designed from conserved regions of vomeronasal receptor gene sequences (Cao et al. 1998), yielded PCR products of approximately 500 base pairs from olfactory tissue cDNA templates RNA (Figure 1). PCR using cDNA templates synthesized from the mRNA of mature parr and smolt gill, heart, liver, muscle, and gonadal tissues did not yield results.

Cloning and sequencing of PCR products generated six gene fragments that may encode for olfactory receptors. Six plasmid PCR reactions contained gene fragments of the expected length (samples 2, 5, 6, 16,17, and 18). Samples 2, 5, 6, 16, and 18 were received from reactions using cDNA based on mature parr olfactory mRNA. Sample 17 was obtained from reactions using cDNA based on smolt olfactory mRNA. Plasmid DNA was subjected to DNA sequencing. Five of the six DNA sequences (ASOR2, ASOR5, ASOR16, ASOR17, and ASOR18) share over 75% sequence similarity to goldfish and *Fugu* odorant receptor genes at the III, IV, and V TMD regions. Another gene sequence, OR55, identified through additional PCR experiments using template cDNA derived from smolt mRNA, is homologous to published OR genes.

RACE performed on cDNA derived from smolt olfactory mRNA yielded PCR products of 200, 300, 400, and 1000 base pairs (Figure 2). According to calculations based on the size of the cloned insert and OR gene sequences, the 5' end fragment was expected to range from 350-500 base pairs. The 300 and 400 base pair 5' RACE PCR products were cloned and sequenced. Two clones shared 80% sequence similarity with goldfish and pufferfish OR gene families between transmembrane domains III and V. The 3' cDNA end of the clones, ASOR6 and ASOR11, were aligned with the 5' cDNA end of the previous six OR genes. Overlapping sequences were not apparent in either gene. The two clones may represent partial OR gene sequences different from the gene fragments previously identified. The Gene Specific Primer (GSP) designed for the 5' RACE may correspond to a highly conserved region in the OR gene family.

Nucleotide and amino acid alignments revealed regions of inter- and intra- species identity (Figure 3 and Figure 4). The deduced amino acid sequences of salmon OR gene fragments were approximately 200 residues long. Clones appear to represent TMD III, IV and V. Sequence comparisons exhibited similar OR motifs among the Atlantic salmon, Goldfish, and Pufferfish: SELSFLL in TMIII, LCISCVL at the interface of intracellular loop 3 and TMIV, and NVMK in TMIV.

Alignment data demonstrated Atlantic salmon genes possess sequence and structural properties unique to the Atlantic salmon species. Clones share short motifs, HWPA and VSP, that are not shared by goldfish or pufferfish OR genes (Cao et al. 1998; Naito et al. 1998). Pairwise comparisons were carried out using PAUP 4.0 to predict the percent identity amongst salmon nucleic acid sequences (Swofford 1998). Percent nucleic acid identity is represented in Table 1. Four clones (ASOR2, ASOR5, ASOR16,

and ASOR18) exhibit over 90% nucleic acid identity. ASOR6 and ASOR 11 share 95% sequence identity, but only 45% nucleic acid identity with other gene fragments. ASOR17 and ASOR55 possess distinct sequences, exhibiting less than 60% identity with the other Atlantic salmon clones.

A phylogeny was created using PAUP 4.0 (Swofford 1998) with Atlantic salmon clones for the ingroup and representatives of teleost, mammalian, and amphibian OR genes as outgroups. Bootstrap analysis supports the presence of distinct Atlantic salmon gene families (Figure 5). Moreover, partitioning of the total genetic variance revealed the majority of genetic variation resides across subfamilies rather than within.

Southern blot analysis revealed separate hybridization patterns for each Atlantic salmon OR gene. Three cDNA clones, ASOR55, ASOR11, and ASOR17, displayed 3-6 hybridization bands in genomic blots (Figure 6). Probe 16 revealed one hybridization band in the HindIII digested DNA and two hybridization bands in the EcoRV digestion. Patterns of hybridization for each probe appear to be different.

Northern blot analysis demonstrated that OR genes are expressed in salmon tissues. The expected size of teleost olfactory receptor mRNA ranges from 800 bp- 5.0 kb (see: http://www.ncbi.nlm.nih.gov/). Assays were performed on smolt and mature parr olfactory and non-olfactory tRNA samples. Four probes, ASOR16, ASOR11, ASOR17, and ASOR55, were utilized individually in all experiments. Northern Analysis performed with ASOR11, ASOR17, and ASOR55 revealed hybridization to approximately 1.3 kb, 2.2 kb, and 1.8 kb, respectively, in RNA from smolt olfactory tissue (Figure 7), but not to RNA from mature parr olfactory organ. ASOR16 did not appear to hybridize with RNA from mature parr gill and liver or RNA from smolt

olfactory and non-olfactory tissue. Analysis with ASOR16 revealed one band at 1.0 kb in mature parr testes.

DISCUSSION

Olfactory cues mediate Atlantic salmon behavior during different life history stages. One approach to determine the significance of odorants in salmon behavior involves identifying and characterizing olfactory receptor genes. We have cloned a novel family of genes likely to encode for odorant receptors in Atlantic salmon, *Salmo salar*. Our data signify the first record of olfactory receptor gene sequences in the Salmoniforme order. Differential expression of olfactory receptor genes in tissues of the mature parr and smolt was illustrated representing the first correlation between olfactory receptor gene expression and salmonid life histories.

As a result of sequence and Northern analysis, we suggest that the Atlantic salmon gene fragments encode for olfactory receptors, based on three lines of evidence. First, gene products were observed exclusively in PCR's utilizing template cDNA from the olfactory organ. These findings suggest genes are localized to olfactory tissue. Secondly, deduced amino acid sequences of salmon cDNA's identify a family of proteins that share structural and sequence properties with other seven trans-membrane domain, olfactory receptors. BLAST analysis reveals salmon gene sequences display over 75% nucleic acid similarity to published goldfish and pufferfish OR genes. Finally, Northern blot assays verified that gene fragments were expressed primarily in the olfactory organ.

Phylogenetic and amino acid alignment studies suggest that the Atlantic salmon OR genes form a distinct ancestry in the olfactory receptor gene lineage. Phylogeny of

salmon sequences performed with representative teleost, mammalian, and amphibian OR genes as outgroups support the novel salmonid gene characteristic. Bootstrap analysis supports the presence of distinct Atlantic salmon gene families. Additionally, salmon gene fragments exhibit motifs distinctive for Atlantic salmon. The salmon OR gene lineage may have emerged as an adaptation to odorants mediating salmon specific life history behaviors. For example, spawning salmon migration is guided by chemical cues particular to an individual's natal stream (Hasler et al. 1978).

Nucleic acid analysis and Southern blot results suggest salmon OR genes are organized into subfamilies. Nucleic acid comparisons indicate that eight salmon gene fragments represent four OR subfamilies in which members exhibit at least 85% sequence similarity (Berghard and Dryer 1998; Ngai et al. 1993). Representative gene fragments of the olfactory receptor subfamilies reveal distinct hybridization patterns in Southern blot analysis. Southern blot analysis confirms the presence of separate OR gene lineages. Probe hybridization pattern variations indicate genes may be clustered on different loci of the genome. Therefore, results support the organization of genes into 4 subfamilies based on sequence similarity and hybridization patterns.

The existence of Atlantic salmon OR gene subfamilies may reflect an adaptation of the olfactory system to various classes of odorants. In general, G-protein coupled receptors possess structural domains that confer binding specificity onto the proteins (Kobilka et al. 1988). Therefore, OR gene subfamilies, sharing similar structural properties within subfamilies, may detect a particular class of chemicals. Sequence divergence between subfamilies may lead to variations in receptor/ligand relationships. One distinguishing feature of odorant receptor proteins is the diversity exhibited in

several of the transmembrane domains. Although OR genes maintain maximal conservation in the second intracellular and extracellular loops and within transmembrane domains II, VI, and VII (Buck and Axel 1991), hypervariable regions, associated with transmembrane domains III, IV, and V, exist (Ngai et al. 1993). The pronounced variable regions may correspond to ligand binding domains, accounting for the olfactory systems ability to discriminate a large variety of chemical structures. Our Atlantic salmon gene fragments extend from TMDIII to TMDV, the most variable of domains in these G-protein coupled receptors. Therefore, the variability in structural properties exhibited by the salmon subfamilies may confer binding specificity upon each lineage of proteins. Further testing of subfamily specificity requires functional data.

Our data suggest mature parr and smolts maintain distinct olfactory receptor gene sequences and expression patterns. Sequence analysis reveals mature parr genes retain distinct sequences from smolt clones. The four clones obtained from mature parr tissue represent one subfamily separate from the three subfamilies containing genes solely extracted from smolt tissue. Additionally, Northern assays suggest smolt genes hybridize solely to RNA from the olfactory organ of smolt. No hybridization with smolt gene probes was detected in olfactory or non-olfactory tissues of the mature parr. Sequence variation and differential expression of olfactory receptor genes may reflect variations in olfactory sensitivity of Atlantic salmon at different stages in their life history. Olfactory receptor expression confers the ability upon an organism to detect stimuli (Amoore 1977). Consequently, a fish at one stage of development, expressing a particular OR, may detect chemicals not discernible by a fish, in a different life stage, which does not appear to exhibit the same receptor gene. Olfactory sensitivity discrepancies are evident

in mature parr and smolt. For instance, Scott and Moore (1991) reported an increased olfactory sensitivity to testosterone in mature parr compared to immature individuals. Identification of OR genes provides a foundation for deciphering chemical cues driving variations in salmon behavior and offer a basis for understanding the impact of odorants on salmon. Ultimately, these biochemical and molecular data assist not only in the furthering of basic olfactory knowledge, but also offer important considerations for natural variables influencing Atlantic salmon life histories and strategies.

Our findings have many implications for understanding and conserving the threatened Atlantic salmon species. Through molecular characterization of odorant receptor genes, we have provided a basis for comprehending olfactory homing, a behavior essential for Atlantic salmon survival. Salmon rely on olfactory discrimination to detect natal homing stimuli. If the chemical cues are not detected, salmon may not locate adequate spawning ground for successful reproduction. Therefore, ensuring species survival through olfactory homing requires a comprehensive understanding of olfactory discrimination at the molecular level. Through identifying the genetic sequence of olfactory receptors, information on olfactory discrimination is obtained. Moreover, identification of Atlantic salmon OR genes will greatly assist management practices of the salmonid species. Utilizing OR genes in functional assays may identify critical odorants involved in olfactory imprinting, the process of forming olfactory memories for homing. Understanding imprinting would provide for the development of techniques used to detect its occurrence. Thus, it can be determined if hatchery raised fish have been imprinted for their environment, thereby increasing the chances of successful homing and reproduction. Additionally, it may be possible to imprint hatchery fish to

streams other than those utilized by wild salmon populations, preventing genetic pollution of wild Atlantic salmon populations. Our results provide a basis for comprehending molecular aspects of olfactory discrimination in salmon, in turn, contributing to the efforts put forth to preserve this valuable resource.

Gene Accession Numbers

AF191680, ASOR2; AF191681, ASOR5; AF191682, ASOR16; AF191683, ASOR18; AF191684, ASOR17; AF191685, ASOR6; AF191686, ASOR11; AF206035, ASOR55

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		1	2	3	4	5	6	7	8
1	**ASOR6**	-							
2	**ASOR11**	0.0408	-						
3	**ASOR55**	0.4544	0.4350	-					
4	**ASOR16**	0.5987	0.6013	0.5694	-				
5	**ASOR16**	0.6022	0.5962	0.5912	0.0734	-			
6	**ASOR18**	0.5996	0.5933	0.5680	0.0488	0.1042	-		
7	**ASOR2**	0.5950	0.5896	0.5751	0.0612	0.0926	0.0859	-	
8	**ASOR5**	0.6280	0.6284	0.6284	0.3452	0.3659	0.3561	0.3643	-
9	**ASOR17**	0.6942	0.6762	0.6674	0.5634	0.5555	0.5618	0.5684	0.6294

Table 1: Pearson Distance Matrix

Atlantic salmon sequences were analyzed using PAUP 4.0 (Swofford 1998).
Values represent the difference between Atlantic salmon sequences.
Example: 0.04 represents 4% difference between nucleic acid sequences.

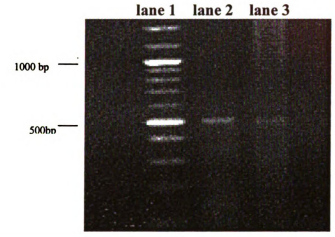

Figure 1: Electrophoresis Analysis of PCR products using VNO primers
cDNA prepared from mature parr and smolt olfactory organ RNA was subjected to PCR amplification with degenerate oligonucleotide primers. PCR yielded products of 500 base pairs using Atlantic salmon olfactory organ cDNA as templates. Lane 1 contains a 100 base pair marker. Lane 2 contains a 500 base pair PCR product amplified from cDNA from smolt olfactory organ RNA. Lane 3 contains a PCR product of 500 base pairs amplified from cDNA of mature parr olfactory organ RNA.

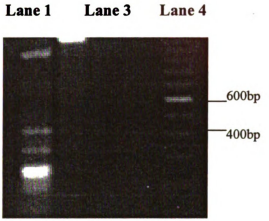

Figure 2: Electrophoresis Analysis of 5'Rapid Amplification of cDNA Ends (RACE) using cDNA from smolt olfactory organ RNA
Using the SMART RACE kit from Clontech, 5' RACE ready cDNA prepared from Atlantic salmon olfactory organ RNA was subjected to PCR using Gene specific primers. Lane 1 contains PCR products of 200, 300, 400, and 1000 base pairs using smolt olfactory organ cDNA as a template. Lane 3 contains a PCR negative control in which no template was utilized. Lane 4 contains a 100 base pair marker.

Figure 3: Gene Sequence Alignments

The nucleic acid sequences of 8 Atlantic salmon cDNA's were aligned with representatives of Goldfish (CAOR) and Pufferfish (FRPR) odorant receptor genes (AF083081 and AB009040, respectively) using the Clustal-W algorithm. Inter-species sequence identity is indicated in the dark shaded region. Atlantic salmon intra-species sequence identity is represented in the light shaded regions.

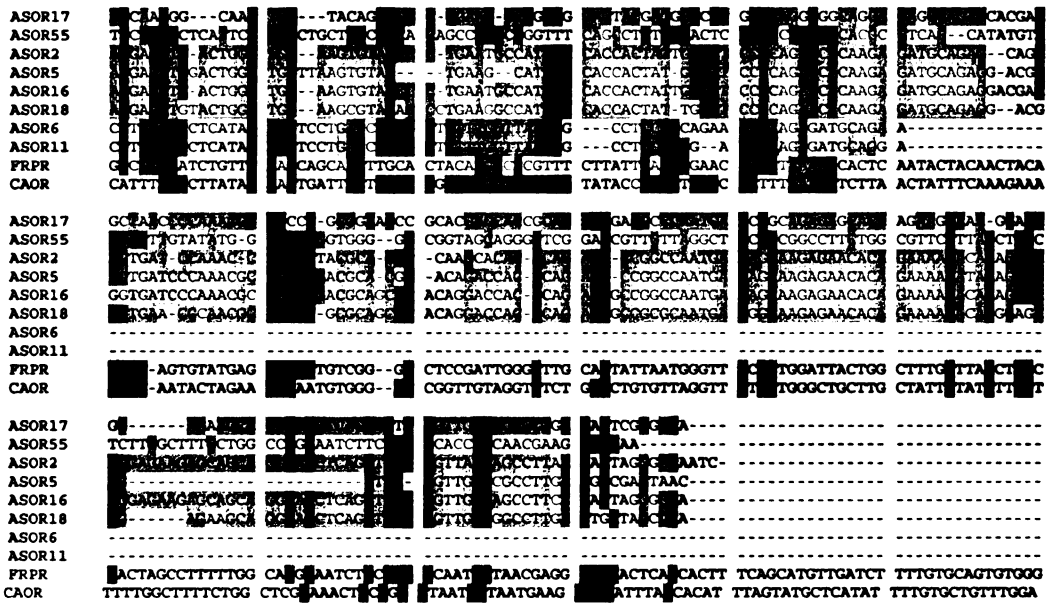

Figure 3 (continued): Gene Sequence Alignments

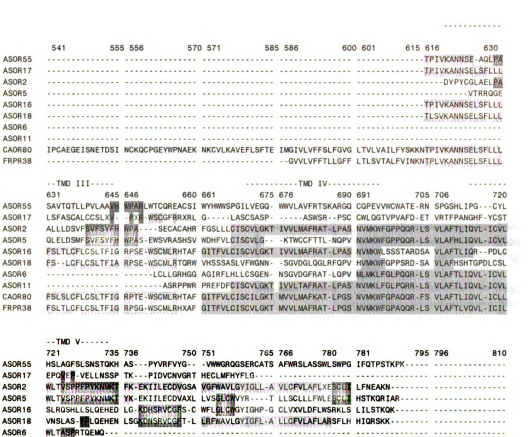

Figure 4: Comparison of Deduced Amino Acid Sequences of Atlantic salmon, Goldfish, and Fugu cDNA's.

The deduced amino acid sequences of 8 Atlantic salmon cDNA's were aligned with representatives of goldfish (CAOR80) and fugu (FRPR380) olfactory receptor gene sequences (AF083080 and AB009038, respectively). Amino acid residues were aligned with the Clustal- W algorithm. Identical sequences of Atlantic salmon, fugu, and goldfish are shown in the shaded, bold type region. Identical salmon sequences are revealed in darker shaded regions with bold typeface.

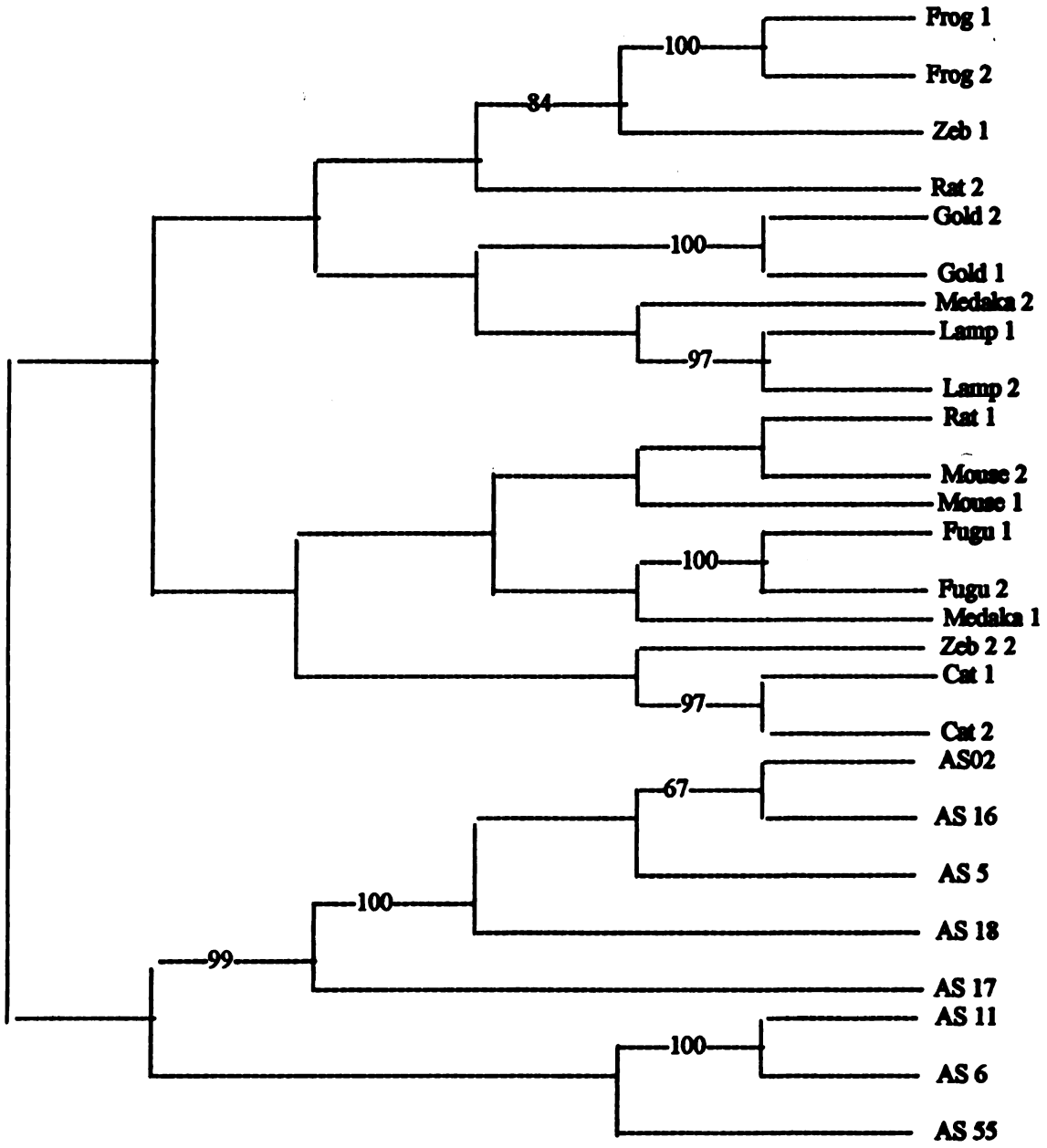

Figure 5: Phylogenetic Tree of Gene Sequences
A phylogenetic tree was created with PAUP 4.0 (Swofford, 1998) using Atlantic Salmon (AS), Goldfish (Gold), Zebrafish, (Zeb), Frog (Frog), Catfish (Cat), Medaka (Medaka), Pufferfish (Fugu), River Lamprey (Lamp), Rat (Rat), and Mouse (Mouse) OR gene sequences. Accession numbers are provided in the Materials and Methods section and are listed as gene 1 and 2, respectively. Values represent the proportion of 100 bootstrap replicates supporting particular nodes. Values under 60 are not shown. Atlantic salmon clones remain separate from other species.

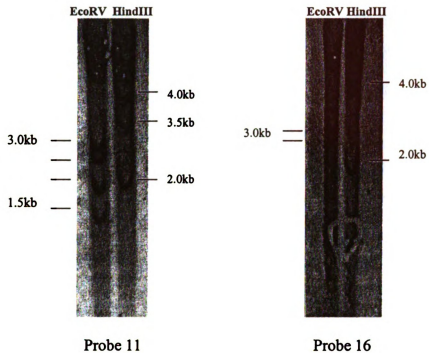

Figure 6: Southern Blot Analysis with Four Atlantic Salmon cDNA's

Atlantic salmon genomic DNA, isolated from muscle tissue, was digested with EcoRV and HindIII, electrophoresed on a 1% agarose gel, and transferred to a positively charged nylon membrane. The membrane was cut into four separate membranes and probed with individual Psoralen Biotin labeled gene probes, ASOR11, ASOR16, ASOR17, and ASOR55.

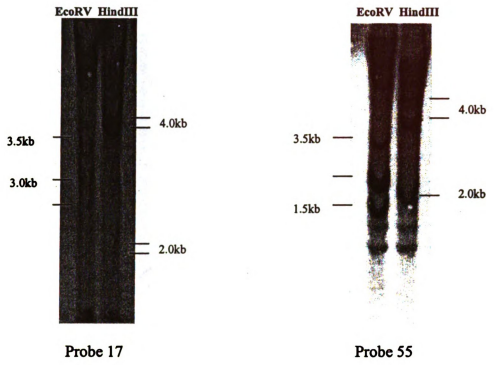

Figure 6 (continued): Southern Blot Analysis with Four Atlantic Salmon cDNA's

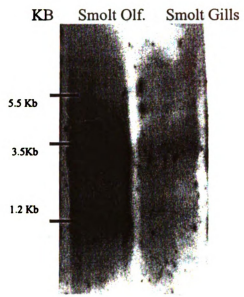

Figure 7: Expression of Olfactory Receptor RNAs in Atlantic salmon tissues Psoralen Biotin labeled DNA probes were annealed to 30ug tRNA isolated from smolt olfactory tissues and gills. Hybridization was performed with probe 11, representing ASOR11 received from smolt tissue. Bands appear in smolt olfactory tissue at the 1.2 Kb marker. No hybridization of this size is detected in tRNA from smolt gills.

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