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Two Subfamilies of Olfactory Receptor Genes in Medaka Fish, *Oryzias latipes*: Genomic Organization and Differential Expression in Olfactory Epithelium[1]

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We cloned and characterized two subfamilies of olfatory receptor (OR) genes from medaka fish (*Oryzias latipes*). Southern blot analysis showed that each of the two subfamilies, designated as subfamilies Y and E, consists of about five members, as usually observed for other vertebrate ORs. Analyses of the genomic clones encoding these members revealed that two members of subfamily Y and four members of subfamily E are tandemly reiterated in 15 and 22 kbp regions of the medaka genome, respectively. The members of each subfamily show very similar amino acid sequences, with similarities greater than 70%. However, the similarities to the sequences of other vertebrate ORs are lower. Members of subfamily Y show amino acid sequence similarities of *ca.* 30% to other fish ORs, including subfamily E members, as well as to mammalian ORs. On the other hand, members of subfamily E show sequence similarities of *ca.* 50% to other fish ORs and *ca.* 30% to mammalian ORs. Phylogenic analyses of various fish ORs, including medaka, catfish, and zebrafish ORs, indicate that the primary structures of fish ORs are diverse compared with those of mammalian ORs, which consist of much larger numbers of members. The expression patterns of subfamilies Y and E genes in the olfactory epithelium of adult medaka fish were examined by *in situ* hybridization, showing that the frequency of positive signals is different between the two subfamilies: about 2% of the olfactory neurons are positive to probes for members of subfamily Y, while less than 1% are positive to probes for members of subfamily E. These results indicate that each subfamily is under different transcriptional control.

Key words: gene cluster, medaka fish, olfactory receptor.

Vertebrates are able to discriminate and respond to various kinds of odorants, resulting in appropriate behaviors such as eating, escaping, and mating. Odor recognition begins with the binding of odorants to the cillia of olfactory neurons, which leads to the activation of an inner cellular signaling pathway, transmitting neuronal excitation to the central nervous system. The mechanisms underlying this process were brought to light by the molecular cloning of olfactory receptor (OR) genes (*1, 2*) and other related genes encoding G proteins (*3, 4*) and ion channels (*5, 6*) from the olfactory epithelium of several vertebrates. In rat, ORs are encoded by a large gene family comprising about 1,000 members, and only one member in the genome is expressed in an olfactory neuron (*7*). Neurons expressing a certain OR gene are scattered on the OE, but their axons converge into a few glomeruli where the olfactory information is processed (*8-10*). Experiments using *in vivo* and heterologous expression systems revealed that a given OR possesses a certain spectrum of responsiveness to chemical compounds (*11-13*), a finding that matches the physiological observation. That is, single olfactory neurons can be activated by a limited number of chemical compounds that share common features, such as functional groups and length of the carbohydrate chain (*13, 14*). It is thus assumed that the modality and the large number of OR molecules tune the vertebrate olfactory system and, as a result, enable it to discriminate among the huge number of odorants existing in the environment.

In physiological and behavioral studies of olfaction, fishes are often recognized as suitable systems because of their high sensitivity to odorants and the small number of chemical compounds to which they can respond (*15, 16*). In addition, some fishes, such as zebrafish (*Danio rerio*),

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Abbreviations: ORF, open reading frame; OR, olfactory receptor; OE, olfactory epithelium; OMP, olfactory marker protein.

pufferfish (*Fugu rubripes*), and medaka fish (*Oryzias latipes*), have been established as models for genome studies in vertebrates (*17-19*). It is thus expected that gene manipulation of the olfactory system would be available in these systems. Fish ORs were first cloned from catfish (*2*), and then from zebrafish (*20*) and goldfish (*21*). One of the characteristics of fish ORs is their smaller number of genes per genome (~100 genes) than in mammals (~1,000 genes), which may reflect the smaller number of chemicals detectable by fish. However, the similarities among cloned fish ORs are not always greater than those among mammalian ORs, although all the fishes described above belong to the superorder that includes carp, *Ostariophysi*. Accordingly, the fish ORs cloned so far might form only a fraction of the fish OR families, and the diversity of fish ORs might be even greater than in mammals.

In this study, we chose medaka fish, which belongs to the superorder that includes sardines, *Acanthopterygii*, to investigate the diversity of ORs among fish species. We cloned two subfamilies of ORs, one of which is similar to both mammalian ORs and fish ORs and the other similar to known fish ORs. We also found that these OR genes are expressed in different manners in the olfactory epithelium of adult medaka fish.

MATERIALS AND METHODS

Cloning of Olfactory Receptor Genes—The nucleotide sequences of the degenerate oligonucleotide primers used for PCR are as follows 5′-GC(CT)TACGACAG(AG)GT-(CG)ATCGC(CT)ATCTG-3′ (TM3Y primer), 5′-GCI(TC)TIGCITA(TC)GA(TC)(AC)G-3′ (TM3E primer), 5′-TAG-ATGAT(AG)GGGTT(CG)AGCAT(AG)GGNGG-3′ (TM7Y primer), and 5′-TA(AGT)AT(AGT)ATIGG(AG)TTIA(A-G)CAT-3′ (TM7E primer) and (where N denotes any of the four nucleotides). These sequences correspond to the amino acid sequences AYDRVIAIC (TM3Y primer), ALAYDR (TM3E primer), PPMLNPIIY (TM7Y primer), and MLN-PIIY (TM7E primer), respectively, observed in the third and seventh transmembrane domains of catfish ORs. Chromosomal DNA was extracted from medaka fish by a standard SDS-phenol method (*22*). Aliquots were dispensed and stored at 4°C until used. The PCR conditions were as follows. Reaction solutions containing 0.01 μg of genomic DNA, 25 pmol of each primer, and thermostable DNA polymerase (EX Taq, Takara Shuzo) were reacted for 50 cycles of 1 min at 94°C, 2 min at 52°C, and 3 min at 72°C. The products were fractionated by agarose gel electrophoresis and DNA fragments with the expected lengths were recovered from the gel and subcloned into pUC 18 vector. Two kinds of clones, named clones Y and E, encoding partial ORs were isolated and subjected to further analyses.

Isolation of Genomic Clones—Genomic libraries of medaka fish were constructed as described previously (*23*). About 3×10^5 pfu of recombinant phage clones were screened using DNA inserts of clones Y and E as probes. The conditions for plaque hybridization were the same as described previously (*23*) except that the hybridization and washing temperatures were each 55°C. Clones that gave strong signals were isolated and subjected to restriction mapping analysis. Five clones positive to clone Y probe were isolated. Among them, two clones, λ208-1 and λ208-3, overlapped and covered a 15 kbp genomic region containing Y1 ORF. The other three clones, λ201-1, λ202-1, and λ205-1, covered a 18 kbp region containing Y2 ORF and Y3 ORF. Three clones positive to the clone E probe, λ202, λ203, and λ206, were isolated. They collectively covered a region of about 22 kbp containing the four E1 to E4 ORFs. Restriction fragments around the ORFs were subcloned and sequenced using a sequencing reaction mix (Amersham) and a DNA sequencing system (Li-Cor).

Isolation of Medaka OMP Genes—DNA fragments encoding olfactory marker proteins (OMPs) of medaka fish were obtained by PCR amplification from genomic DNA. Two degenerate primers were used: 5′-TGGACCCCNGA-CCT(CG)ACCAAC(CT)TNATG-3′ and 5′-AA(AG)TACA-TNAC(TC)TTNC(TG)(ATG)AT(TC)TTNGC-3′ (where N

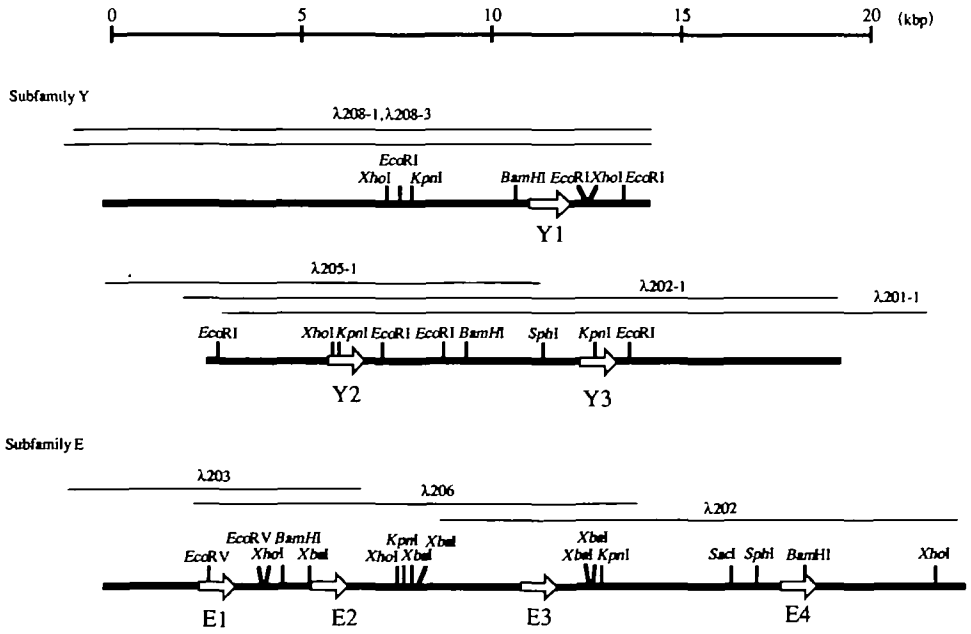

Fig. 1. **Genomic organization of medaka OR subfamilies Y and E.** Genomic regions containing OR genes are represented by solid lines. The λ-phage clones covering these regions are indicated by lines above. A nucleotide sequence length scale is at the top of the figure. Restriction sites are indicated by the vertical lines and the letters above them. Open arrows indicate the positions of open reading frames (ORFs) and their directions of translation (nucleotide and amino acid sequence information is available in the DDBJ/EMBL/GenBank databases with accession numbers AB029474 to AB029480).

denotes any of the four nucleotides), corresponding to the amino acid sequences WTPDLTNLM and AKIRKVMYF, respectively, that are conserved in mammalian and *Xenopus laevis* OMPs (*24-26*). Two kinds of 170 bp DNA fragments were obtained and sequenced.

Genomic Southern Analysis—Ten micrograms of medaka chromosomal DNA was completely digested with appropriate restriction enzymes (see Fig. 2, A and B), electrophoresed in 0.7% agarose gels, and blotted onto nylon membranes (Hybond-N, Amersham). Hybridization was carried out under the same conditions as used for library screening using probes corresponding to the coding regions of medaka ORs.

In Situ Hybridization—*In situ* hybridization was performed basically as described previously (*27, 28*). In detail, the head of an adult medaka fish was frozen in O.C.T. compound bathed with liquid nitrogen, and sectioned (see Fig. 5A) into 10 μm slices at -18°C. The sections were fixed with 4% paraformaldehyde in PBS for 10 min, treated twice with 0.1% diethylpyrocarbonate in PBS for 15 min, washed once in 5×SSC for 15 min, and prehybridized in a solution containing 50% formamide, 5×SSC, and 40 μg/ml herring sperm DNA for 2 h at 58°C. The solution was changed to hybridization solution containing 50% formamide, 5×SSC, 5×Denhardt's solution, 500 μg/ml herring sperm DNA, 250 μg/ml torula tRNA, 1 mM DTT, and 1-10 ng/μl of digoxigenin-labeled RNA probes corresponding to the coding regions of medaka ORs or DNA fragments encoding medaka OMPs. The sections were covered with siliconized coverslips and hybridized for 48 h at 58°C. After hybridization, the sections were gently soaked in 5×SSC to remove the coverslips and washed once in 5×SSC for 5 min, twice in 0.2×SSC for 30 min at 58°C, and once in 0.2×SSC for 5 min at room temperature. Subsequently, the sections were immersed in TBS containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl for 5 min, blocked with 0.5% blocking reagent (Boehringer) in TBS for 1 h, and incubated with anti-digoxygenin Fab fragment-conjugated with alkaline phosphatase (1:1,000 dilution) in blocking solution for 1 h at room temperature. The sections were washed three times with 0.3% Tween20 in TBS for 15 min and immersed once in AP buffer containing 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 50 mM $MgCl_2$ for 5 min. The color reaction was performed by placing a solution containing nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, 0.3 μg/μl each in AP buffer, onto a slide, and incubating the slide for 2 h at room temperature or for 18 h at 4°C.

Cell Counting—Sections from several individual fish were used. Cells in the apical half region of the tissue (presumptive mfOMP-positive cells) were counted. The number of cells was about five hundred in the middle of the serial sections (see Fig. 5). Then cells positive to each OR probe were counted, and the frequency was represented as the percentage of OR-positive cells *vs.* presumptive mfOMP-positive cells. The values were averaged among three samples spaced 50 μm apart in the serial sections.

RESULTS

Cloning of Olfactory Receptor Genes from Medaka Fish—Degenerate oligonucleotide primers encoding consensus sequences in transmembrane domains 3 and 7 of several fish ORs were synthesized and used to amplify the DNA fragments encoding ORs from the genomic DNA of adult medaka fishes. Two kinds of DNA fragment were obtained. The encoded amino acid sequences showed significant similarities (30-50%) to known vertebrate ORs and a lower similarity (28%) to each other. In addition, preliminary genomic Southern analysis using the inserts as probes showed that medaka fish have several copies of genes related to these clones and, consequently, each of them is likely to constitute a gene subfamily, which is a characteristic of OR gene families (*29*). Thus, we named these clones clone Y and clone E of medaka OR genes. Next, we screened a genomic library of medaka fish using these clones as probes to obtain total coding sequences and additional OR genes that might constitute gene subfamilies.

Genomic Organization of Medaka OR Subfamilies Y and E—From low stringency plaque hybridization experiments, we isolated two groups of phage clones that gave strong signals to clones Y and E probes. These clones were subjected to restriction mapping analysis, and were shown

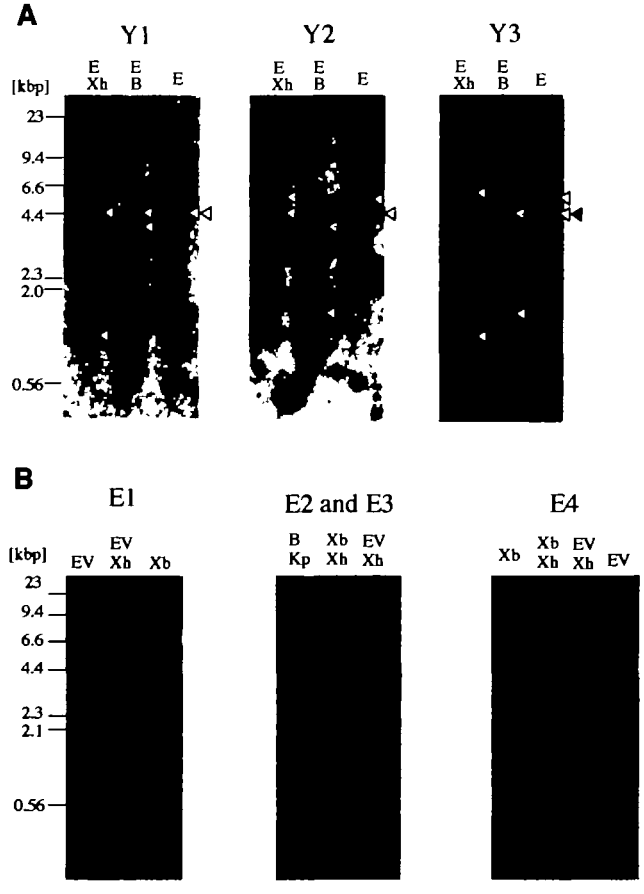

Fig. 2. **Genomic Southern analysis of medaka OR genes.** Ten micrograms of completely digested medaka DNA was blotted and hybridized with probes corresponding to the coding regions of each OR gene, Y1 to Y3 (A) and E1 to E4 (B). Abbreviations for restriction enzymes are B, *Bam*HI; E, *Eco*RI; EV, *Eco*RV; Kp, *Kpn*I; Xb, *Xba*I; Xh, *Xho*I. Strong signals marked by filled arrowheads indicate the detection of restriction fragments containing the coding regions of the OR gene. Signals marked by open arrowheads are the results of cross-hybridizations between subfamily members. Other signals may correspond to additional members of each subfamily that were not isolated in the screening.

to overlap in 10 to 20 kbp regions of the genome (Fig. 1). Further sequencing analysis revealed that these regions contain open reading frames (ORF) encoding ORs designated Y1 to Y3 of subfamily Y and E1 to E4 of subfamily E. As indicated by the open arrows in Fig. 1, the ORFs are closely linked and tandemly reiterated with intergenic distances of about 5 kbp. However, the linkages between the Y1 ORF and Y2 or Y3 ORF have not yet been confirmed, although long distance PCR was tried using several specific primers. Next, we performed genomic Southern analysis using the coding regions of these ORFs as probes (Fig. 2, A and B). Figure 2A shows the results obtained with probes corresponding to each member of subfamily Y. Strong signals were detected in each lane (filled arrowheads), with migration distances in agreement with the molecular weights of restriction fragments containing each of the ORFs of subfamily Y (Fig. 1). Signals marked by open arrowheads are cross-hybridizations between members of our cloned subfamily Y. In addition, three or more weak signals, not corresponding to any of Y1 to Y3, were observed in each lane. It is possible that these signals reflect other subfamily members that have not yet been cloned. Similar results were obtained with subfamily E probes (Fig. 2B). In brief, strong signals marked by the filled arrowheads appear to correspond to restriction fragments containing subfamily E members. In addition, one gene (*Xba*I-*Xba*I 6.6 kbp) closely related to E4 ORF (see "DISCUSSION") was detected in the blot probed with E4 ORF. Taken together, we conclude that both subfamilies Y and E consist of about five members in the medaka genome.

Amino Acid Sequences of Medaka ORs—First, we compared the members of each subfamily with other members of the same subfamily (Fig. 3, A and B). As shown by the alignment in Fig. 3A, the three members of subfamily Y show high similarities to each other, 69% (Y1 to Y2), 72% (Y1 to Y3), and 79% (Y2 to Y3). Also, the four members of subfamily E show high similarities to each other (greater than 71%) (Fig. 3B). Especially, the E2 and E3 ORs, show 91% similarity to each other. In contrast to the high similarities observed among members of the same subfamilies, low similarities were observed between members of subfamily Y and subfamily E. For instance, the similarity between Y1 and E1 was 29%, which is close to the value (30%) observed between Y1 and mammalian ORs (see below).

Next, we compared the amino acid sequences of medaka ORs with other vertebrate ORs. We used Y1 and E1 as representatives of each subfamily and searched protein on-line databases for ORs showing significant similarity to either of them. Several vertebrate ORs, catfish OR 3 and rat OR F6 for Y1 and catfish OR 3, 1, and 32A for E1, are listed at the top with their amino acid sequences aligned with those of Y1 and E1 (Fig. 3C). We also calculated the similarities between medaka and several vertebrate ORs,

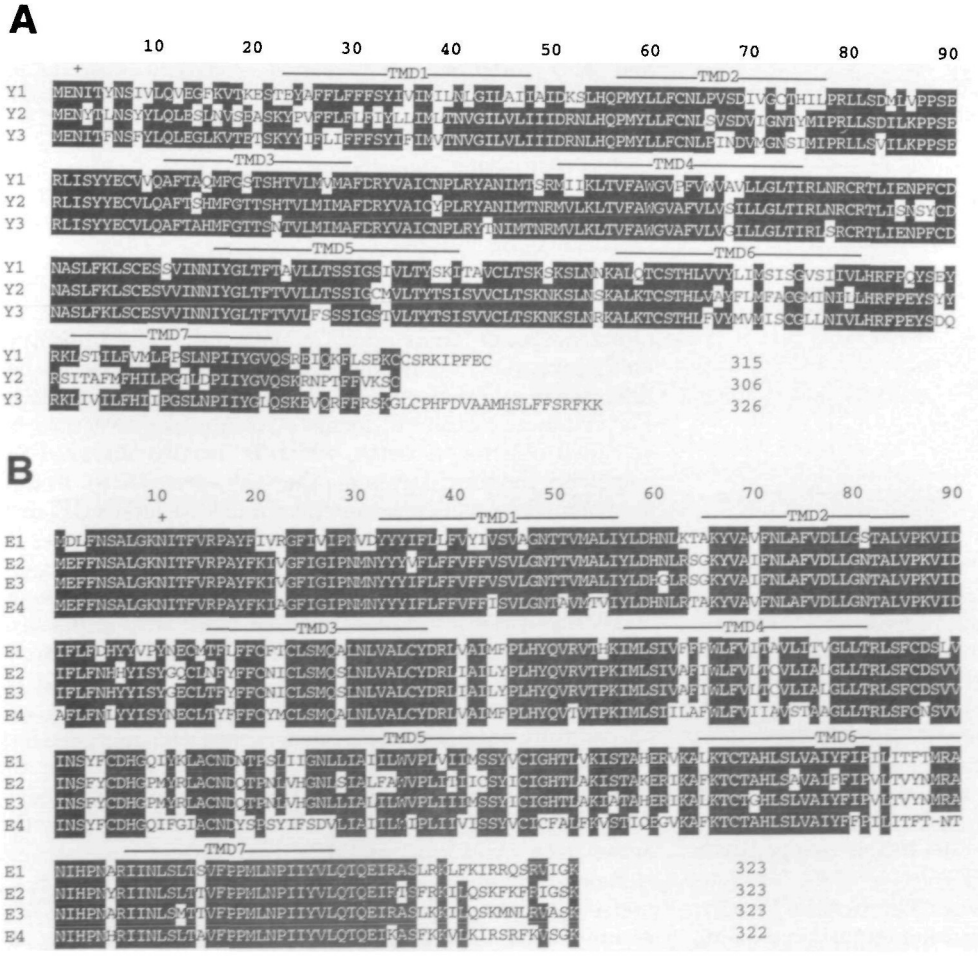

Fig. 3. **Comparison of amino acid sequences among subfamily Y and E ORs and other vertebrate ORs.** Amino acid sequences are compared among members of subfamilies Y (A) and E (B). Amino acids identical in at least two thirds of the ORs are written in reversed letters. A potential asparagine-linked glycosylation site in the amino-terminal extracellular domain conserved among all members is marked by a cross. Seven transmembrane domains (TMDs) are indicated by horizontal bars. (C) Amino acid sequences of medaka OR Y1 and E1 are aligned with those of catfish ORs 3 and 32A and rat OR F6. Numbering of the catfish and rat ORs is the same as described previously (*1, 2*). Amino acids identical among three or more ORs are written in reversed letters. Abbreviations are the same as in (A) and (B). (D) Phylogenic tree showing amino acid differences among medaka ORs and several vertebrate ORs. The tree was drawn using GENETYX software (Software Development) following the neighbor-joining method. The numbers at the nodes are bootstrap values (%) for one hundred trials. As shown by the alignments and the phylogenic tree, type E ORs belong to the pedigree of fish ORs together with catfish ORs 1, 3, and 32A. On the other hand, type Y ORs belong to a new pedigree comprising fish ORs and mammalian ORs.

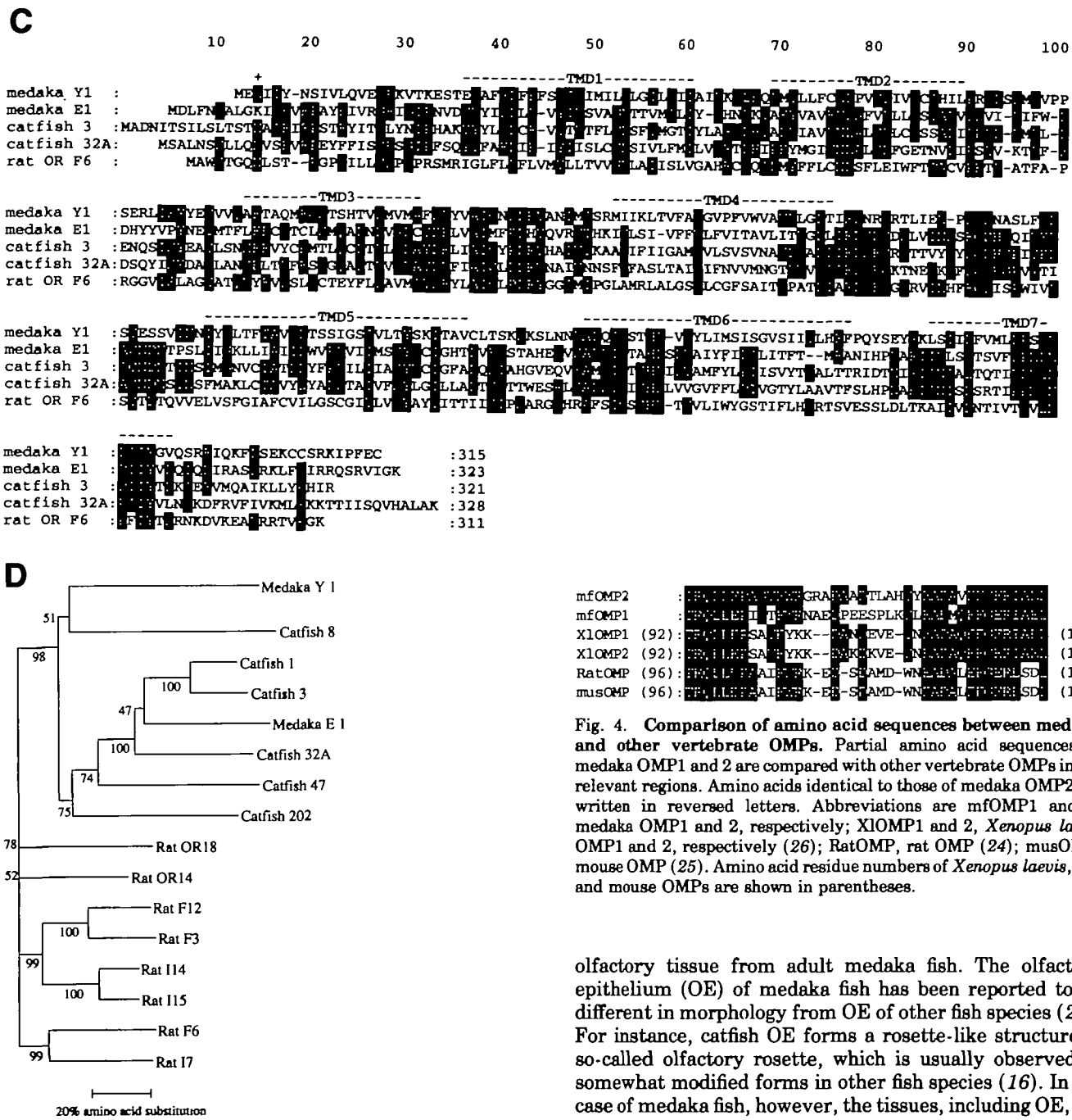

Fig 3, C and D

Fig. 4. **Comparison of amino acid sequences between medaka and other vertebrate OMPs.** Partial amino acid sequences of medaka OMP1 and 2 are compared with other vertebrate OMPs in the relevant regions. Amino acids identical to those of medaka OMP2 are written in reversed letters. Abbreviations are mfOMP1 and 2, medaka OMP1 and 2, respectively; XlOMP1 and 2, *Xenopus laevis* OMP1 and 2, respectively (*26*); RatOMP, rat OMP (*24*); musOMP, mouse OMP (*25*). Amino acid residue numbers of *Xenopus laevis*, rat, and mouse OMPs are shown in parentheses.

including the ORs listed above, and represented them as a phylogenic tree (Fig. 3D). Y1 is most similar to catfish OR 3 (32%), but this value is close to the similarities to rat OR F6 (30%) and other ORs (around 28%). As a result, subfamily Y, like catfish OR 8, constitutes an independent pedigree in the vertebrate OR family (Fig. 3D). On the other hand, E1 shows relatively high similarity to catfish ORs, 49% to catfish OR 3, 46% to catfish OR 1, and 43% to 32A, indicating that subfamily E ORs belong to a pedigree of known fish OR families (Fig. 3D).

Differential Expression of Medaka OR Genes—Finally, we performed an *in situ* hybridization experiment using olfactory tissue from adult medaka fish. The olfactory epithelium (OE) of medaka fish has been reported to be different in morphology from OE of other fish species (*27*). For instance, catfish OE forms a rosette-like structure, a so-called olfactory rosette, which is usually observed in somewhat modified forms in other fish species (*16*). In the case of medaka fish, however, the tissues, including OE, are essentially smooth and contain no grooves, which makes it difficult to discern OE. To clarify the OE in medaka tissue, we attemped to use an medaka olfactory marker protein (OMP) as a tissue marker, because OMP transcripts are known to be specifically expressed in mature olfactory neurons in the main OE and vomeronasal organ, at least in the case of mammals and amphibians (*24-26*). By PCR, we successfully obtained two kinds of genes encoding medaka OMPs (mfOMPs) that show significant similarities to mammalian and amphibian OMPs (Fig. 4), and used them as probes. When the sections were hybridized with antisense RNA probes of mfOMPs, both probes gave strong signals in the midial to apical layer of the tissue (Fig. 5, C and D), which we conclude to be the OE of medaka. We examined the expression of medaka OR genes focusing on

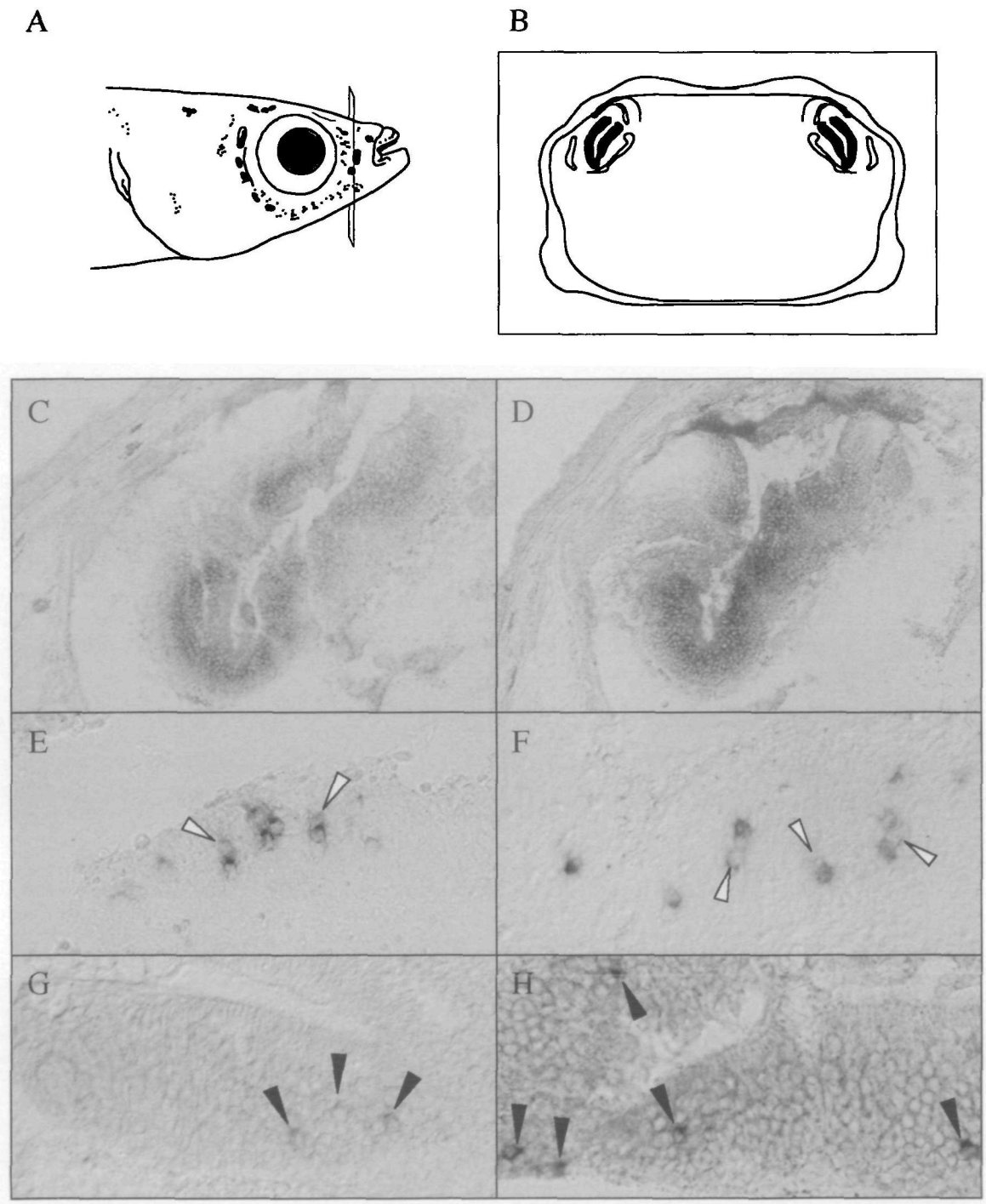

Fig. 5. **Expression of subfamily Y and E genes in the olfactory epithelium of medaka.** (A) Lateral view of an adult fish head. The plane at the middle position of the serial sections is indicated by a parallelogram. (B) Olfactory epithelium (OE) in one coronal section is indicated by the black area. Sections ranging about 200 μm (about 20 sections) along the body axis were hybridized with digoxigenin-labeled antisense RNA probes corresponding to the following molecules: (C) medaka OMP1, (D) medaka OMP2, (E) medaka OR Y2, (F) Y3, (G) E1, and (H) a mixture of E1, E2, and E4. (C), (D) Olfactory neurons positive to medaka OMP probes in the midial to apical layer of OE. (E), (F) Clusters of cells highly positive to Y2 and Y3 probes, which constitute about 2% of the cells in the presumptive mfOMP-positive region. Open arrowheads indicate weak signals adjacent to strong signals supposed to be cross-hybridizations between subfamily Y members. (G) The E1 probe gave signals in about 0.5% of cells (filled arrowheads) in this region. (H) The frequency of positive cells (filled arrowheads) increased to about 1% when the OE was hybridized with a mixture of E1, E2, and E4 probes.

this region.

First we used Y2 and Y3 ORFs as probes. In both cases, strong signals were observed in about 2% cells in the presumptive mfOMP-positive regions (Fig. 5, E and F). In addition, weak signals were often observed in cells near those that gave strong signals (open arrowheads in Fig. 5, E

and F); these are thought to result from cross-hybridizations between the members of subfamily Y. As a whole, the signals tended to appear in clusters scattered throughout the sections. Next we examined the expression of subfamily E ORFs. The results obtained with the E1 probe (Fig. 5G) and a mixture of the E1, E2, and E4 probes (Fig. 5H) are shown. Unlike subfamily Y genes, subfamily E probes gave a few signals, as indicated in Fig. 5G by the filled arrowheads. The frequency of positive cells was estimated as about 0.5% in the presumptive mfOMP-positive regions. This value increased to about 1% when the OE was hybridized with a mixture of the E1, E2, and E4 probes (Fig. 5H). Taken together, subfamily Y and subfamily E ORs are shown to have differential expression patterns in terms of the frequency of positive cells and their distributions in OE.

DISCUSSION

We have cloned and characterized two subfamilies of medaka ORs, subfamily Y and subfamily E, each comprising about five closely related members. We also observed the expression of OR genes in medaka OE, which shows unique anatomical features. Physical mapping analysis showed each member of the two OR gene subfamilies to be tightly linked and reiterated tandemly in the genome with intergenic distances of about 5 kbp. The clustered organization of OR genes has also been found in other vertebrates. In the case of zebrafish, a cluster of OR genes was reported and 8 OR genes were shown to exist within a region of about 80 kbp in the genome (*32*). In this case, the average intergenic distance is 10 kbp. In another fish, pufferfish, we also cloned several OR genes and found five OR genes to be tandemly reiterated in a 40 kbp region of the genome with an average intergenic distance of 8 kbp (Suda, T. and Emori, Y., unpublished data). According to reports on OR gene clusters in the human genome (*33*), the average intergenic distance is calculated to be about 20 kbp in mammals. It is thus concluded that a correlation exists between intergenic distances, medaka and pufferfish, 5 kbp to 8 kbp < zebrafish, 10 kbp < human, 20 kbp, and their genome sizes, 400 Mb to 800 Mb < 1,700 Mb < 3,000 Mb. Recently, pufferfish was reported as a model system for genetic studies in vertebrates (*18*). Because of the compactness of the gene organization as described above, it is concluded that the medaka genome is comparable to pufferfish and may be as useful as the pufferfish genome for genetic studies of olfaction.

Subfamily Y ORs differ from subfamily E ORs in their amino acid similarities to other vertebrate ORs. Subfamily Y ORs are equally similar to fish and mammalian ORs (around 30%), while subfamily E ORs are similar to several catfish ORs (around 45%). Recently, four OR genes (mfOR1-4) were cloned from medaka fish (*34*). Among them, mfOR4 is completely identical in amino acid sequence to Y2 OR. Another OR, mfOR3, shows 98% similarity to E4 OR and is thus supposed to be an additional member of subfamily E detected in the blot probed with E4 ORF (Fig. 2B). However, the other ORs, mfOR1 and 2, show 93% similarity to each other but low (about 30%) similarities to members of subfamily Y, subfamily E, and other fish ORs. These results together with our results lead us to assume that medaka share some ORs (subfamily E ORs) with other fish species, and also have distantly related subfamilies (subfamily Y ORs, mfOR1 and 2) whose spectra of ligand reactivity are unique to medaka fish.

Subfamily Y and subfamily E also differ in their expression patterns in the OE of adult fish. The frequency of cells positive to an individual member of subfamily Y (about 2%) is greater than the frequency of cells positive to an individual member of subfamily E (about 0.5%). This difference in signal frequency is not observed in the OE of other fish species such as zebrafish or catfish where the frequency of each OR subfamily member is around 1% (*2, 32*). Further studies on subfamily Y members, such as identification of their ligands, would help to clarify the biological significance of the relatively high expression frequency of subfamily Y genes.

From the total frequency of subfamily Y and E gene-positive cells in OE, it is expected that other ORs exist in medaka. If the average frequency of cells expressing a certain OR gene is 1% in medaka, and the average number of OR subfamily members is five, the total frequency of cells expressing the OR genes identified so far (subfamilies Y, E, and mfOR1) is estimated to be 15%. This value is low enough to allow for the existence of other unrelated OR genes expressed in the OE of medaka.

At first we found it difficult to identify OE in the nasal cavity of medaka fish because they lack the rosette-like structure that is a good marker for OE in other fish species. Therefore, we used medaka OMPs as molecular markers to identify olfactory neurons in sections and found that the OE of medaka fish is morphologically simpler than those of other fishes (Fig. 5, C and D). This characteristic became obvious when the *in situ* hybridization of OR genes was carried out. Probes corresponding to Y2 and Y3 ORF gave signals as clusters of positive cells distributed randomly in the OE (Fig. 5, E and F). This is in contrast to the case of zebrafish where the expression of each OR gene is segregated into concentric circles in a plane parallel to the olfactory rosette (*35*). We propose that medaka have a simpler olfactory system than other fishes in terms of OR gene expression and tissue morphology. Further analyses of the physiological functions of medaka ORs in an evolutionary context using the characteristics of the olfactory systems described above may provide more general and specific information about vertebrate olfactory systems.

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