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Communication

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Cloning and Characterization of Muscarinic Receptor Genes from the Nile Tilapia (*Oreochromis niloticus*)

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To investigate the regulatory mechanism underlying the contractile response in the intestinal smooth muscle of the nile tilapia (*Orechromis niloticus*), we used pharmacologic and molecular approaches to identify the muscarinic subreceptors and the intracellular signaling pathways involved in this motility. Myography assays revealed that an M1- and M3-subtype selective antagonist, but not a M2-subtype selective antagonist, inhibited carbachol HCl (CCH)-induced intestinal smooth muscle contraction. In addition, a phospholipase C inhibitor, but not an adenylate cyclase inhibitor, blocked the contractile response to CCH. We also cloned five muscarinic genes (*OnM2A*, *OnM2B*, *OnM3*, *OnM5A*, and *OnM5B*) from the nile tilapia. In the phylogenetic analysis and sequence comparison to compare our putative gene products (*OnMs*) with the sequences obtained from the near complete teleost genomes, we unexpectedly found that the teleost fish have respectively two paralogous genes corresponding to each muscarinic subreceptor, and other teleost fish, except zebrafish, do not possess muscarinic subreceptor *M1*. In addition, the expression pattern of the nile tilapia muscarinic subreceptor transcripts during CCH-induced intestinal smooth muscle contraction in the proximal intestinal tissue was analyzed by real-time PCR surveys and it was demonstrated that CCH increased the *OnMs* mRNA expression rapidly and transiently.

INTRODUCTION

Muscarinic acetylcholine receptors (M), like other members of the G protein-coupled receptor superfamily, contain seven putative transmembrane domains and initiate intracellular responses by interacting with heterotrimeric G proteins. Mammals possess five known muscarinic receptor subtypes (M1-M5), each of which is encoded by an intronless gene exhibiting a unique pharmacologic profile (Bonner et al., 1988; Eglen and Nahorski, 2000; Peralta et al., 1987). The muscarinic receptors are divided into two groups according to their biological function. M_{odd} (M1, M3, and M5) preferentially stimulates phospholipase C (PLC) activity, which hydrolyzes phosphatidylinositol 4,5-bisphosphate into the second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), which mediate calcium mobilization and protein kinase C activation, respectively. In contrast, M_{even} (M2 and M4) preferentially inhibits adenylyl cyclase (AC) activity, thereby decreasing cyclic AMP (cAMP) levels (Eglen, 2005; Felder, 1995; Wess, 1996).

In mammals, muscarinic receptors play an essential role in maintaining the contractile state of smooth muscle throughout the body (Ehlert et al., 1997; Kim et al., 2006), but intestinal mobility as a regulatory parameter in aquatic species is a poorly-studied topic. After Burnstock first studied vagal nerve control in the fish intestine (Burnstock, 1958), more reports demonstrated that acetylcholine and serotonin (5-hydroxytryptamine) are the neurotrasmitters mediating contractility of rainbow trout (*Oncorhychus mykiss*) intestinal smooth muscle (Burka et al., 1996; Nilsson and Holmgren, 1993). These observations demonstrated that both serotonergic and muscarinic receptors must be present in fish intestinal smooth muscle.

Recent research regarding motility regulation of the fish intestine has mainly focused on a few trout species (Aronsson and Holmgren, 2000), while other teleosts have not been studied. These previous studies have suggested that the M2 subreceptor is the sole cholinergic receptor in the rainbow trout intestine. In addition, trout intestinal contraction requires the recruitment of extracellular calcium through voltage-gated channels (Aronsson and Holmgren, 2000; Burka et al., 1996).

In this present study, therefore, we investigated how several signaling pathways may be involved in muscarinic receptor-mediated contraction of the nile tilapia intestinal smooth muscle. We also isolated five muscarinic subreceptor genes from the nile tilapia. During the course of cloning, we unexpectedly isolated two unique clones corresponding to each of the muscarinic receptor *M2* and *M5* genes. This finding suggests that the nile tilapia genome might possess more muscarinic receptor genes than in the mammalian genomes described to date.

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Thus, we report herein that the muscarinic receptor gene family has undergone a unique gene evolution in the early vertebrate.

MATERIALS AND METHODS

Fish maintenance and muscle strips

Nile tilapia (120-300 *g*) were obtained from the fish farm attached to Pukyong National University. Fish were maintained in aerated aquaria, equipped with flow-circulator systems and maintained at 25°C, for 10 d before use. Fish were removed from the aquarium with a dip-net and immediately euthanized by anesthetic tricaine methanesulfonate (MS-222). The proximal intestine (~6 cm) was quickly excised and placed in a Petri dish filled with modified Krebs-Henseleit solution (mKHS; NaCl 118 mM; KCl 4.7 mM; $CaCl_2$ 2.2 mM; $MgSO_4$ 1.2 mM; KH_2PO_4 1.2 mM; D-glucose 11.1 mM; HEPES 24.9 mM, adjusted to pH 7.5). The excised muscle was completely separated from other tissues, washed, trimmed, and cut to yield a 20-mm longitudinal segment.

Myography assay

Strips were mounted in a 10-ml organ bath filled with mKHS solution, which was aerated with a mixture of 95% O_2 and 5% CO_2 at 25°C. The strips were subjected to a baseline tension of 1.5 *g* and allowed to equilibrate for 70 min, during which the mKHS solution was exchanged with fresh buffer every 15 min. Changes in tension were measured with a myograph force transducer (Narco F-60; Narco Bio-System Inc., USA) and recorded on linear chart recorders. At the end of each day, the tissues were removed from the organ baths, blotted to remove surface moisture, weighed, and measured (length). To investigate muscarinic subreceptors and signaling pathways, the tissues were allowed to equilibrate for approximately 80 min, with regular changes of the bath fluids. Each strip was exposed to cumulative concentrations of CCH, differing by three logs of 10 (10^{-8}-10^{-5} M CCH), to examine their response in the organ bath. Tissues were exposed to the agonists for 1 min, until the response to the previous concentration had reached a plateau. In the presence of various muscarinic antagonists (M1 antagonist, pirenzepine; M2 antagonist, gallamine HCl; M3 antagonist, 4-DAMP mustard HCl), the strips were activated with the prior concentration before proceeding to the next. All values are the means ± SEM.

Cloning and characterization of muscarinic receptor genes

Total RNA was extracted from various nile tilapia tissues using TRIzol® (Invitrogen) and quantified by ND 1000 (Nanodrop Co., USA). The isolated total RNA (4 ug) was reverse-transcribed using a SuperScript™ First-Strand kit (Invitrogen), according to the manufacturer's instructions. Reverse transcription was carried out at 42°C for 60 min using a thermocycler (Bio-Rad), and the resulting cDNA was used to clone the initial full-length cDNA fragments (as described below) and determine the tissue distribution of each muscarinic receptor gene. Initially, partial cDNA fragments of muscarinic receptor genes were obtained by nested PCR amplification. Primers for amplifying the cDNA fragments were designed to span highly-conserved regions of known *M1-M5* sequences from teleost fish and other vertebrates (Table 1). A set of common primers, MUSR-DegF and MUSR-DegR, designed to clone the *M1-M5* genes, was used in the first round of nested PCR. The resulting PCR product was used as a template for the second round of PCR, using appropriate primer sets corresponding to each muscarinic receptor gene (Table 1; MUSR2&4F and MUSR2&4R for M_{even} genes; MUSR1,3&5F and MUSR1,3&5R for M_{odd} genes). Amplified DNA products were separated by 1% agarose gel electrophoresis, purified, ligated, cloned into the pGEM®-T Easy vector (Promega), and sequenced with an ABI 377 Sequencer (Perkin Elmer). To subsequently identify the full-length coding regions of the muscarinic receptor genes of the nile tilapia, we used rapid amplification of cDNA ends (RACE); specifically, we used a SMART™ RACE cDNA Amplification kit (Clontech laboratories, Inc.), according to the manufacturer's instructions, along with newly-designed, gene-specific primers (Table 1).

Sequence and phylogenetic analysis

Nucleotide and predicted protein sequences were analyzed using DNAsis for Windows version 2.5 (Hitachi Software Engineering), BioEdit Sequence Alignment Editor version 5.0.9. (Hall, 1999) and BLAST programs in non-redundant databases of the National Center for Biotechnology Information (NCBI BLAST, http://www.ncbi.nlm.nih.gov/BLAST/). For phylogenetic analyses, the protein and DNA sequences of the cloned *OnMs* were aligned with muscarinic receptor genes of other species obtained from the Ensembl genome database (http://www.ensembl.org), using CLUSTAL W, version 1.8 (Thompson et al., 1994), as implemented in BioEdit (Hall, 1999). Extensive modeling was performed using BLOSSUM and PAM matrices that employed varying gap-open and gap-extension penalties. Phylogenetic analyses were performed with MrBayes, version 3 (Ronquist and Huelsenbeck, 2003) for Bayesian estimation, and with PAUP*4.0b10 (Swofford, 2000) for maximum parsimony, neighbor-joining and maximum likelihood estimations. Tree reconstruction was visualized with Tree-View (Page, 1996). Muscarinic receptor genes of Caenorhabditis elegans (GenBank accession no. AAD48771.1) and Drosophila melanogaster (GenBank accession no. NP_523844.2) were used as outgroups.

Real-time RT-PCR of muscarinic receptor genes in intestinal tissue exposed to CCH

To investigate the *OnM2-OnM5* gene expression pattern in CCH-treated tissue from the proximal intestine, the tissue was exposed to an EC50 dose (10^{-7} M) of CCH, total RNA was extracted from the tissue, and the RNA was used to synthesize first-strand cDNA using the methods described *vide supra*. Each real-time PCR amplification reaction was performed in a 25 μl volume containing cDNA (200 ng), 0.2 μM each of specific forward and reverse primers (Table 1), and 12.5 μl of iQ™ SYBR Green Supermix (Bio-Rad). Reaction conditions were as follows: 94°C/4 min; 30 cycles of 94°C/30 s, 55°C/30 s, 72°C/30 s; and 72°C/10 min. To confirm that the desired specific products had been amplified, melting curve cycles were conducted with the following parameters: 95°C /1 min, 55°C /1 min, 80 cycles of 55°C /10 s, with a 0.5°C increase per cycle. iQ™ SYBR Green supermix (Bio-Rad) was used to detect specific PCR products. Amplification and detection of SYBR Green were performed with an iQ5 Multicolor Real-Time PCR (Bio-Rad). The nile tilapia *β-actin* gene was used as a housekeeping reference gene to normalize expression levels between the samples and differences in reverse transcriptase efficiency. All of the data were gathered from triplicate experiments and were expressed as fluorescence relative to *β-actin*. Data was collected as the *C*t (the PCR cycle number at which fluorescence is detected above threshold, which decreases linearly with increasing input target quantity) using the iQ5 Multicolor Real-Time PCR system software version 1.0 (Bio-Rad). The *C*t of each sample was used to calculate Δ*C*t values (target gene Δ*C*t subtracted from the *β-actin C*t). Fold changes in gene expression compared to controls were determined by the $2^{-\Delta\Delta Ct}$

Table 1. Oligonucleotide primers used to amplify *OnM1-OnM5* for expression studies

Primer name	5′-3′ sequence	Information
MUSR-DegF	AGYAAYGCNTCNGTNATGAA	Primers used in the first round of nested PCR to obtain
MUSR-DegR	GTRCTRTTGAYRTARCANARCCARTA	initial M1-M5 gene fragments
MUSR1,3&5F	TBATGATHGGYYTVGCNTGG	Primers used in the second round of nested PCR to obtain
MUSR1,3&5R	ACCATGATRTTRTANGGNGTCCA	initial M1, M3, and M5 gene fragments
MUSR2&4F	GCNATYYTSTTCTGGCAGTT	Primers used in the second round of nested PCR to obtain
MUSR2&4R	RTTVATVAGSACCATVACRTTGTA	initial M2 and M4 gene fragments
5′Mus2-R1	CTCTGGAGATCTGCCAGTAGAGCTGAATCA	Specific primer for 5′RACE of M2A
5′Mus2-R2	ACTGTCCGCCCACCAACAATGAACT	
3′Mus2-F1	CACACCATCTAATGCCACTGTTGAGATTGT	Specific primer for 3′RACE of M2A
3′Mus2-F2	ATGTGGCTCGGAAGATTGTGAAGATGACT	
5′Mus4-R1	CCTGAGGTCTTCCTGCTGTCTTTCCTCA	Specific primer for 5′RACE of M2B
5′Mus4-R2	GGAGATGCGCCAGTAAAGGTAGATCATG	
3′Mus4-F1	CACTCAAATGCTAGTCACGAAAACACTGTTTAAGG	Specific primer for 3′RACE of M2B
3′Mus4-F2	AYGAGTGAAAACGCTGTGGCAAAGTG	
5′Mus3-R1	CCTTGTAAATGCGCCAGTAGAGAACACTCA	Specific primer for 5′RACE of M3
5′Mus3-R2	GCACTGTCCTTTCACCCTCAAGAAACTG	
3′Mus3-F1	CAGCTCAAACTCTCAGTGCCATCCTCTT	Specific primer for 3′RACE of M3
3′Mus3-F2	TCTGTGGGCAGTAGGTTACTGGCTATGCTAC	
5′Mus5A-R1	CCTTGTAGATTCTGCAGTACAGGATTGTCATG	Specific primer for 5′RACE of M5A
5′Mus5A-R2	CGGGGACAGTCCTCTTTCCAACAAAGTACT	
3′Mus5A-F1	AACATTCTCCTCGGCCGAGTCCAT	Specific primer for 3′RACE of M5A
3′Mus5A-F2	ATGTCCAAGCCCATAGACACAACGCTG	
5′Mus5B-R1	GTGAGCCCTTGCAGCTCTGCTAGATCT	Specific primer for 5′RACE of M5B
5′Mus5B -R2	AGAGACTGGGATGTAGAACGCAGCAATG	
3′Mus5B -F1	AACGGCAATCCTATGAGCGCAACAT	Specific primer for 3′RACE of M5B
3′Mus5B -F2	GGACACTGCCCTGAAGAACCAGATTACTAAGA	
Mus2-real-F	GGCAGATCTCCAGAGCAAGCAAG	Real-time RT-PCR Primers for expression studies
Mus2-real-R	TTGTTTCTCCTCTGGCCTGGTG	
Mus3-real-F	CTGGCGCATTTACAAGGAGACCC	″
Mus3-real-R	CTACCAGTTCCCGACATGTGCTC	
Mus4-real-F	TTTCTACCTACCTGTGGCCATCATG	″
Mus4-real-R	AGTGCCTGAGGTCTTCCTGCTG	
Mus5A-real-F	CCAGGAGCAACGCGGTCAAATC	″
Mus5A-real-R	TGGAAGCCTCCTGGAGACACTG	
Mus5B-real-F	TCATTTCACCCGAGAGCCAAGAG	″
Mus5B-real-R	CTCTTCTTCTGAGGACGTGTA	
β-actin-F	TGACAATGGATCCGGTATGTGCG	GenBank accession no: EF206796.1
β-actin-R	TGACAATACCGTGCTCAATGGG	

Deg, degenerate primer; GSP, gene-specific primer; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; F, forward; R, reverse; S,C or G; M,A or C; Y,C or T; R,A or G.

method (Giulietti et al., 2001). All data were expressed as the means ± S.D. and analyzed by unpaired Student's *t*-test after normalization. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Effects of muscarinic subtype inhibitor on CCH-induced intestine contraction

To investigate the presence of the M subreceptors within the intestinal smooth muscle of nile tilapia, we exposed each muscle strip to 10^{-8}-10^{-4} M CCH in the absence (■) or presence of increasing concentrations of a selective M1 antagonist (pirenzepine HCl [PZ]; ▲, 10^{-9}; ▽, 3×10^{-9}; ○, 10^{-8}; ◆, 3×10^{-8} M), an M2 antagonist (gallamine HCl [GM]; ▲, 10^{-9}; ▽, 3×10^{-9}; ○, 10^{-8}; ◆, 3×10^{-8} M), or an M3 antagonist (4-DAMP mustard HCl [DP]; ▲, 10^{-9}; ▽, 3×10^{-9}; ○, 10^{-8} M). The cumulative dose-response curve shifted significantly to the right under increasing concentrations of the selective M1 antagonist (PZ; Fig. 1A), and the M3 antagonist (DP; Fig. 1B), but not in the presence of the M2 antagonist (GM; data not shown). In addition, high concentrations of GM (> 10^{-4} M) did not completely

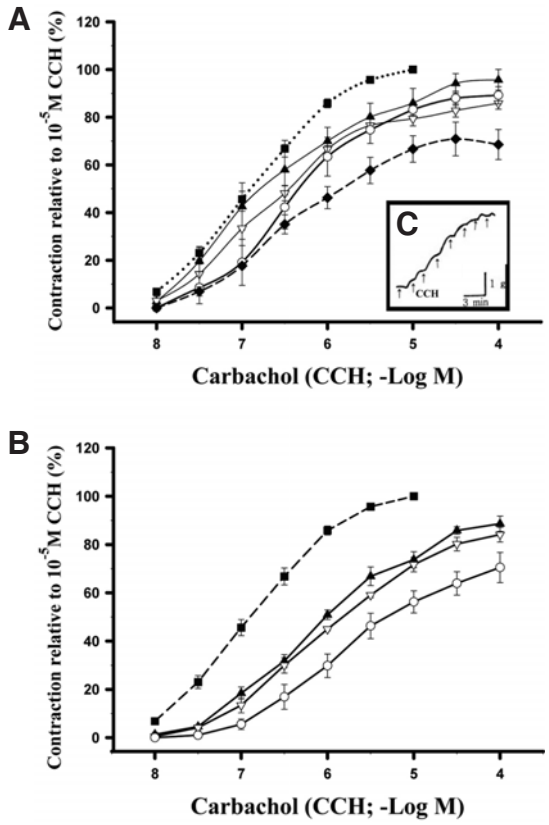

Fig. 1. Cumulative dose-response curve to CCH in nile tilapia intestine. Intestinal strips were exposed to 10^{-8}-10^{-4} M CCH in the absence (■) or presence of increasing concentrations (▲, 10^{-9}; ▽, 3×10^{-9}; ○, 10^{-8}; ◆, 3×10^{-8} M) of pirenzepine HCl (A) or 4-DAMP mustard HCl (B). Values represent percentages of the maximal response to CCH in the absence of antagonist. Data points are the means ± S.E.M (n = 6-40, weight = 205 ± 49.567 g). Traces resulting from CCH (C) treatment of the nile tilapia proximal intestine are shown in the inner panel. Each of the intestinal strips was exposed to 10^{-8}-10^{-4} M CCH.

inhibit CCH-induced contraction, but the presence of 10^{-5} M of PZ or 10^{-5} M of DP completely inhibited contraction (data not shown).

In mammalian gastrointestinal smooth muscle, the most common muscarinic subtypes are M2 and M3, with M2 accounting for about 70-80% and M3 for 20-30% of the muscarinic receptors in this tissue (Ehlert et al., 1997; Zhang et al., 1991). Burka et al. reported that M2 mediated acetylcholine-induced contraction in rainbow trout (Burka et al., 1996), but the present results demonstrated that the M1 and M3 subreceptors more strongly influenced CCH-induced contraction of the nile tilapia intestinal smooth muscle than did the M2 subreceptor.

Effects of various antagonists on CCH-induced intestinal contraction

To examine whether the muscarinic receptors mediated stimulation of PLC activity or AC inhibition, we investigated how an AC inhibitor, MDL 12330A HCl (Lippe and Ardizzone, 1991), or a selective PLC inhibitor, 2-nitro-4-carboxyphenyl-N,N- diphenylcarbamate (NCDC; Baker et al., 1999), affected CCH-induced contraction in the intestinal smooth muscle of the nile tilapia. In the presence of high concentration of MDL 12330A (10^{-5} M), the maximum contractile activity of the intestinal smooth muscle did not significantly decrease. However, when exposed the intestinal smooth muscle strips to the ten-fold dilutions (3×10^{-4} M or 3×10^{-3} M) NCDC for 20 min, the maximum contractile activity decreased significantly to 72.01 ± 4.63 and 37.44 ± 1.31 (data not shown).

Cloning of muscarinic receptor genes from the nile tilapia

To investigate whether the genes corresponding to muscarinic receptors, which were identified by our pharmacology study, really exist in the intestinal smooth muscle of the nile tilapia, we obtained full-length cDNAs for the five muscarinic subreceptors (*OnM2A*, *OnM2B*, *OnM3*, *OnM5A*, and *OnM5B*) from the nile tilapia brain first-strand cDNA, and classified these clones as type A (high similarity to a human muscarinic receptor isoform) or type B (low similarity to a human muscarinic receptor isoform). These full-length muscarinic subreceptor cDNAs were summarized as follows: *OnM2A* was a 2,630-bp gene containing a 1,509-bp open reading frame (ORF) that encoded 502 amino acids (GenBank accession no: EU729324); the full-length cDNA of *OnM2B* yielded a 2,189-bp sequence. The first possible start codon was located at position 114, with an open reading frame of 1,359-bp, encoding a protein of 452 amino acids (GenBank accession no: EU729326); *OnM3* cDNA was 2,323-bp long, 1,938-bp for ORF length, and this translated protein had 645 amino acid residues (GenBank accession no: EU729325); *OnM5A* cDNA was 2,966-bp long. An ORF of 1,578-bp from nucleotide 794 of an initiation codon ATG to nucleotide 2371 of an in-frame stop codon was identified, which codes for a protein of 525 amino acids (GenBank accession no: EU729327); *OnM5B* cDNA consists of 3,213-bp and contains an ORF of 1,533-bp with ATG initiation codon at position 502 and a termination codon at position 2034, encoding a protein of 510 amino acids (GenBank accession no: EU729328).

Sequence comparisons of the nile tilapia muscarinic subreceptors (*OnMs*) demonstrated that the subtypes were 36.4-67.4% (for DNA) and 31.2-56.3% (for amino acids) identical to each other on the DNA and amino acid level (Supplemental Table 1). The amino acid sequence comparison between each of the OnMsA and B paralogous genes revealed high percentages of sequence identity (58% for OnM2 and 68.5% for OnM5) compared to other OnM subtypes. Comparisons of the DNA and amino acid sequences of OnMs with human chrMs (HschrMs) revealed that OnMs showed 36.5-64.2% (for DNA) and 30.3-67.6% (for amino acids) overall identities with all HschrMs (Supplemental Table 1). Each OnMs shared a relatively high identity with the gene corresponding to each HschrM (55.9-64.2% for DNA and 55.9-67.6% for amino acids; Supplemental Table 1). Structural analysis by using the deduced amino acid sequences of the nile tilapia muscarinic receptor family also demonstrated that each nile tilapia muscarinic subreceptor contain the seven transmembrane (TM) segments (I-VII) that typify muscarinic receptors (Supplemental Fig. 1). Altogether, these sequence comparisons and structural features indicate that the genes cloned in this study are clearly members of the muscarinic receptor family.

Muscarinic genes in teleost fish

Phylogenetic analysis of nile tilapia muscrinic receptor genes can help reveal their origins and relationships to other muscarinic receptor genes. Both *CeM* (*Caenorhabditis elegans*) and *DmM* (*Drosophila melanogaster*) served as outgroups to root the tree. In our phylogenetic tree, *OnM2s* (A and B), *OnM3* and *OnM5s* (A and B) belonged to the *HsM2*, *HsM3* and *HsM5* clades, respectively, and formed their respective groups (Fig. 2).

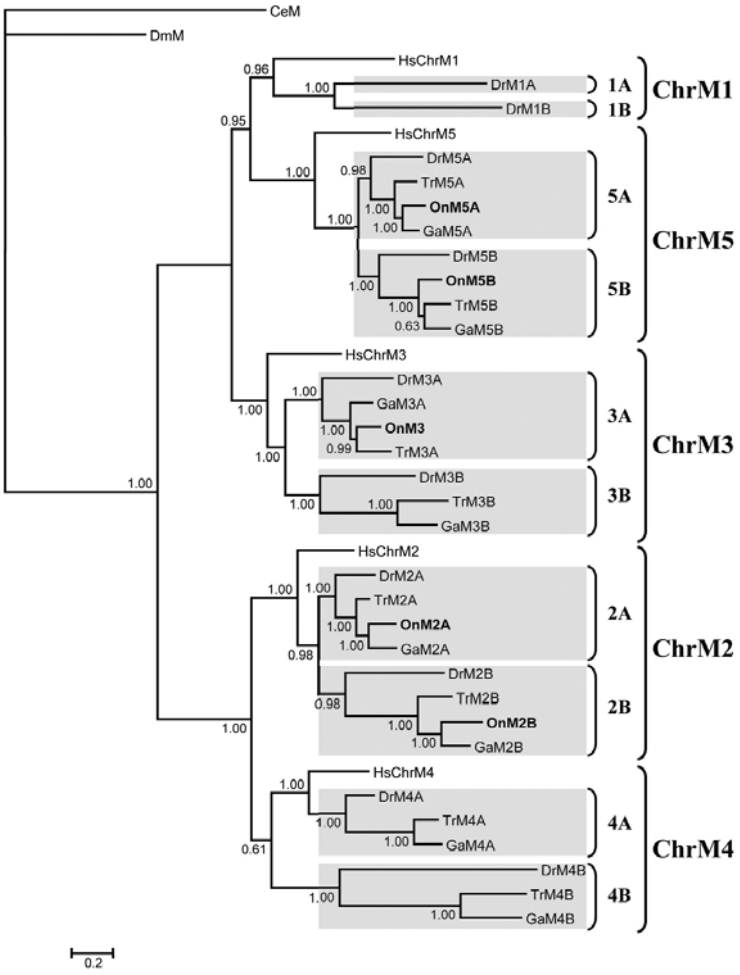

Fig. 2. Phylogenetic reconstruction of muscarinic receptor genes using *C. elegans* (CeM) and *D. melanogaster* (DmM) as outgroups. Bayesian analysis estimated under the GTR + I + G model of substitution with teleost fish and human muscarinic receptor gene sequences (Table 2). For the analysis MrBayes was executed with 2,000,000 generations using Markov Chain Monte Carlo 4 chains. To get a statistically significant tree, the burnin was set to 100. Scale bar refers to estimated number of nucleotide substitution per site. Posterior probability of clade support is indicated above and below branch. The five muscarinic genes from the nile tilapia were named respectively as *OnM2A*, *OnM2B*, *OnM3*, *OnM5A*, and *OnM5B*, and classified these genes as type A (high similarity to a human muscarinic receptor isoform) or type B (low similarity to a human muscarinic receptor isoform). The abbreviations of other sequences are given in Table 2.

This study also revealed that the nile tilapia *OnM2* and *OnM5* subreceptors existed respectively as two paralogous genes (A and B), which exhibited significant sequence homology with the mammalian muscarinic receptor *M2* and *M5* genes at both the nucleotide and amino acid level (Supplemental Table 1 and Supplemental Fig. 1). This finding suggests that the nile tilapia genome might possess more muscarinic receptor genes as well as five genes cloned in this study than the mammalian genomes described to date. The completely sequenced *Takifugu* and zebrafish genomes and the almost completed stickleback genomes provide an opportunity to confirm the total number of muscarinic receptor genes and the gene evolution of muscarinic receptor genes in teleost fish of the early vertebrate. With this in mind, we searched the genome database (Ensembl Genome Browser) containing *Takifugu*, stickleback and zebrafish genes using the nile tilapia muscarinic receptor genes obtained from this study. As reported in Table 2, we found respectively 10 muscarinic subreceptor genes in zebrafish and 8 in other teleost fish, except the zebrafish, compared to 5 of these genes in mammalian genomes. This result indicates that these teleost fish have two paralogous genes corresponding to each muscarinic subreceptor, and that these two paralogous genes originated from fish-specific genome duplication (or gene duplication) prior to the divergence of teleost fish. Several pieces of evidence have emerged that support such an event, most notably the observation that teleost fish have more copies of many genes than species in their sister group, the tetrapods (lobed-finned fish, amphibians, reptiles, birds and mammals; Meyer and Schartl, 1999; Van de Peer et al., 2002; Vandepoele et al., 2004). For instance, seven unlinked *Hox* gene clusters were identified in zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) genomes, compared to four of these gene clusters in mammalian genomes. More recently, many studies have demonstrated that many genes in addition to the *Hox* clusters are duplicated within fish genomes, including genes encoding insulin, the tyrosine kinase receptor, and ATPase (Finn and Kristoffersen, 2007; Irwin, 2004; Meyer and Schartl, 1999; Volff and Schartl, 2003). Like this, through genome duplication, teleost fish are believed to have more genes related to the muscarinic receptor than mammals.

Interestingly, when we searched the Ensemble Genomic Database for genes related to muscarinic receptors from five species of fish (zebrafish, *Takifugu*, and stickleback), we found that, with the exception of zebrafish, a sequence closely related to *M1* had not been identified. We also failed to identify a cDNA sequence corresponding to the nile tilapia *M1* subreceptor using a PCR survey and other methods. Furthermore, there is currently no report of the molecular cloning and biochemical characterization related to the M1 receptor in teleosts. This suggests that the *M1* subreceptor was probably lost in a common ancestor of teleosts after the divergence of zebrafish. However, we showed in this study that pirenzepine, a M1 selec-

Table 2. Sequences used in this study

Sequence for comparison	Species (common name)	Ensembl genomic DNA ID	Ensembl transcript ID	Ensembl peptide ID	Chromosome or genomic location
HsChrM1	Homo sapiens (human)	ENSG00000168539	ENST00000306960	ENSP00000306490	Chr. 11
HsChrM2		ENSG00000181072	ENST00000320658	ENSP00000319984	Chr. 7
HsChrM3		ENSG00000133019	ENST00000255380	ENSP00000255380	Chr. 1
HsChrM4		ENSG00000180720	ENST00000316350	-	Chr. 11
HsChrM5		ENSG00000184984	ENST00000383263	ENSP00000372750	Chr. 15
DrM1A	Danio rerio (zebrafish)	ENSDARG00000037292	ENSDART00000054245	ENSDARP00000054244	Chr. 14
DrM1B		ENSDARG00000009121	ENSDART00000020646	ENSDARP00000024008	Chr. 5
DrM2A		ENSDARG00000055577	ENSDART00000077955	ENSDARP00000072421	Chr. 6
DrM2B		ENSDARG00000029994	ENSDART00000042166	ENSDARP00000042165	Chr. 25
DrM3A		ENSDARG00000071091	ENSDART00000104893	ENSDARP00000095663	Chr. 17
DrM3B		ENSDARG00000071298	ENSDART00000105331	ENSDARP00000096103	Chr. 12
DrM4A		ENSDARG00000069254	ENSDART00000100639	ENSDARP00000091412	Chr. 7
DrM4B		ENSDARG00000017722	ENSDART00000018239	ENSDARP00000019994	Chr. 25
DrM5A		ENSDARG00000004026	ENSDART00000012932	ENSDARP00000006023	Chr. 17
DrM5B		ENSDARG00000069598	ENSDART00000101418	ENSDARP00000092192	Chr. 20
TrM2A	Takifugu rubripes (pufferfish)	ENSTRUG00000015946	ENSTRUT00000040917	ENSTRUP00000040775	Scaffold 21
TrM2B		ENSTRUG00000010086-	ENSTRUT00000025475	ENSTRUP00000025372	Scaffold 105
TrM3A		ENSTRUG00000007974	ENSTRUT00000019938	ENSTRUP00000019857	Scaffold 121
TrM3B		ENSTRUG00000008654	ENSTRUT00000021808	ENSTRUP00000021720	Scaffold 29
TrM4A		ENSTRUG00000004253-	ENSTRUT00000010132	SINFRUP00000170509	Scaffold 151
TrM4B		NEWSINFRUG00000139069.3	SINFRUT00000147533	SINFRUP00000147533	Scaffold 105
TrM5A		ENSTRUG00000014549	ENSTRUT00000037331	-	Scaffold 23
TrM5B		ENSTRUG00000018108	ENSTRUT00000046507	ENSTRUP00000046349	Scaffold 78
GaM2A	Gasterosteus aculeatus (stickleback)	ENSGACG00000019948	ENSGACT00000026406	ENSGACP00000026355	Group IV
GaM2B		ENSGACG00000011914	ENSGACT00000015788	ENSGACP00000015757	Group XIX
GaM3A		ENSGACG00000010124	ENSGACT00000013390	ENSGACP00000013365	Group VI
GaM3B		ENSGACG00000015318	ENSGACT00000020240	ENSGACP00000020201	Scaffold_48
GaM4A		ENSGACG00000015349	ENSGACT00000020282	ENSGACP00000020243	Group I
GaM4B		ENSGACG00000007854	ENSGACT00000010430	ENSGACP00000010408	Group XIX
GaM5A		ENSGACG00000006035	ENSGACT00000008002	ENSGACP00000007983	GroupXV
GaM5B		ENSGACG00000007923	ENSGACT00000010528	ENSGACP00000010506	GroupXVIII

These sequences were used to design the RT-PCR primer sets for cloning of the initial cDNA clones corresponding to each of the nile tilapia muscarinic subreceptors genes, and to construct the phylogenetic tree with the protein and DNA sequences of the cloned *OnMs*.

tive muscarinic antagonist, inhibited CCH-induced intestinal smooth muscle contraction (Fig. 1). This evidence is contradictory to "the absence of the *M1* gene in the teleost fish genome" explained earlier. Therefore, this implies that the mechanism of action of pirenzepine might mediate probably its effect through a non-M1 mechanism. In 2004, Yin et al. reported that the chick does not possess a functional M1 receptor, and that muscarinic antagonist which prevents the progression of myopia in the chick either work through another muscarinic receptor subtype, most likely M2, or through non-specific or non-receptoral mechanisms (Yin et al., 2004). Although pirenzepine was described as a M1-selective antagonist in mammals (Tietje and Nathanson, 1991), Hsieh and Liao have reported that pirenzepine binds the M2 receptor isolated from zebrafish with a submicromolar dissociation constant (Hsieh and Liao, 2002). In addition, Volff described that the common ancestors of the zebrafish and channel catfish diverged about 110-160 MYA (million years ago) from the teleost fish lineage that includes *Takifugu*, *Tetraodon*, stickleback, medaka and nile tilapia (Volff, 2005).

Putting these results together, we estimate that zebrafish diverged earlier from the common ancestor and have candidate genes related to the *M1* receptor, but *Takifugu*, stickleback, and nile tilapia diverged from this common ancestor into the linage of each fish species after the *M1* gene was lost. In addition, it is believed that teleost fish, except zebrafish, have another system that can surrogate the function of the M1 receptor, most likely M2, or a non-M1 mechanism, including products caused by the duplicate genes of *M2-M5*, which might come to exist by fish-specific genome duplication. To determine the possibility of this presumption, muscarinic receptor genes from the evolutionary links between chordates and vertebrates (organisms

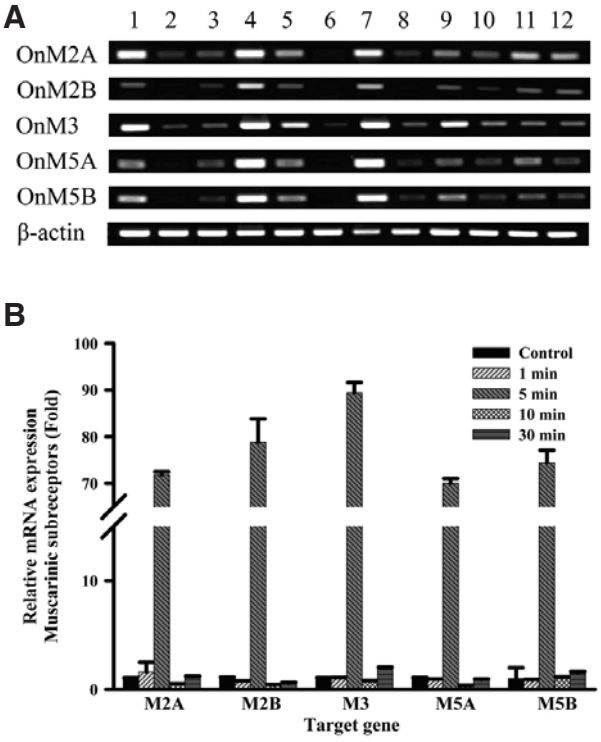

Fig. 3. (A) Nile tilapia muscarinic subreceptor mRNA expression levels in various tissues, as detected by RT-PCR. β-actin served as a positive control. Lane 1, brain; lane 2, body kidney; lane 3, head kidney; lane 4, spleen; lane 5, heart; lane 6, liver; lane 7, gill; lane 8, muscle; lane 9, stomach; lane 10, proximal intestine; lane 11, midgut intestine; 12, distal intestine. (B) Real-time RT-PCR analysis of nile tilapia muscarinic subreceptor mRNA expression in proximal intestinal tissue stimulated with CCH (10^{-7} M) for 1, 5, 10, or 30 min.

such as amphioxus, lamprey, and cartilaginous fish) would need to be identified and characterized completely through further experiments.

Tissue distribution of the nile tilapia muscarinic subreceptor genes

We conducted RT-PCR to elucidate the tissue-specific expression of *OnM2A*, *OnM2B*, *OnM3*, *OnM5A*, and *OnM5B* in the nile tilapia. mRNA expression levels of all the muscarinic subreceptors were high in the spleen, brain, and gill tissues, but generally low in body kidney, head kidney, muscle, and liver tissues (Fig. 3A), and this is the first molecular evidence for the presence of muscarinic receptor subtypes in the spleen and gill tissues of fish. M2, M3 and M5 are known to have a wide distribution in the brain (Bymaster et al., 2003), and this pattern of expression is consistent with our result, and additionally, in the gill and spleen of the nile tilapia all *OnM* genes investigated in this study had a uniformly high level of expression. Phatarpekar et al. studied the expression of the M2 and M5 muscarinic receptors in the retina and retinal pigment epithelium along with brain and heart of the bluegill, and reported that *M2* expression predominated in the heart, while the *M5* gene was highly expressed in the eye and brain, but not in the heart tissue (Phatarpekar et al., 2004). In our experiment, including the pattern of expression of M3, as well as M2s and M5s, the result also demonstrated that in heart tissue, *OnM3* subreceptor mRNA levels were higher than the *OnM2s* and *OnM5s* mRNA levels. However, except for the M3 reported in here, this pattern in the heart showed similarity to that expressed in the bluegill.

mRNA expression of muscarinic subreceptor genes in CCH-treated proximal intestine

Many report demonstrated that cholinergic agent altered mRNA level of the muscarinic receptor gene (Chou et al., 1993; Fraser and Lee, 1995; Ganzinelli et al., 2007). By the previous report, mRNA expression level of *M1* and *M3* receptors was down-regulated between 3 and 9 h when exposed to carbamylcholine, which is musacrinic agonist, in IMR-32 cells (Fraser and Lee, 1995). In addition, CCH rapidly increased mRNA levels of *M1* and *M2* receptors with the up-regulation of mRNA expression level of nitric oxide synthase in rat striatum and cardiac atria (Chou et al., 1993; Ganzinelli et al., 2007).

Therefore, to examine whether CCH (EC50, 10^{-7} M) exposure altered gene expression of the nile tilapia muscrinic subreceptor genes, we carried out real-time RT-PCR on proximal intestinal tissue after determining the gene expression patterns from other tissues (midgut and distal intestine; Fig. 3B).

In result, mRNA expression level of all the *OnM* genes cloned in this study increased greatly at 5 min and decreased sharply after 10 min in the proximal intestine when exposed to CCH. This was consistent with the report that the mRNA level of *M1* genes in the rat cardiac atria only increases at 30 min after exposure to CCH except time difference of gene expression. Furthermore, our results showed, when compared to control, a decrease in the mRNA levels of all the *OnMs* after an hour from exposure to CCH (data not shown). This result is similar with report that mRNA level of *M1* gene is rather down-regulated after several hours from exposure to CCH (Chou et al., 1993; Fraser and Lee, 1995). Most of previous reports showed the expression patterns of muscarinic receptor genes after one or three hours from exposure to CCH. However, in our result, we report that the gene expression patterns of M subreceptors, when exposed to CCH, were monitored in short time interval. Thus, this result is expected to be help understanding the early response mechanism of receptor genes against agents.

In summary, our data suggest that CCH-induced intestinal smooth muscle contraction occurs through a PLC-dependent signaling pathway involving the M3 or non-M1 subreceptors. Furthermore, we discussed the gene duplication of the muscarinic receptor through searching and analyzing genes related to the muscarinic receptor using the nile tilapia muscarinic receptor genes obtained from this study and the genome sequences of other teleost fish. The present report will aid significantly in designing further research on muscarinic receptors in fish. Additionally, the identification of muscarinic receptor homologs in various organisms will contribute to provide various information concerning muscarinic receptor function and their characterization. Our research group will continue to research the function and characteristics of the fish muscarinic receptor duplicates reported herein. In addition, this is the first report that discussed the existence of muscarinic subreceptors in a fish intestine, and the existence of two paralogous genes corresponding to each muscarinic subreceptor by gene duplication or genome duplication in teleost fish.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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REFERENCES

Aronsson, U., and Holmgren, S. (2000). Muscarinic M3-like receptors, cyclic AMP and L-type calcium channels are involved in the contractile response to cholinergic agents in gut smooth muscle of the rainbow trout *Oncorhynchus mykiss*. Fish Physiol. Biochem. *23*, 353-361.

Baker, E.N., Kerkhof, C.J., and Sipkema, P. (1999). Signal transduction in spontaneous myogenic tone in isolated arterioles from rat skeletal muscle. Cardiovasc. Res. *41*, 229-236.

Bonner, T.I., Young, A.C., Brann, M.R., and Buckley, N.J. (1988). Cloning and expression of the human and rat M5 muscarinic acetylcholine receptor genes. Neuron *1*, 403-410.

Burka, J.F., Briand, H.A., Wartman, C.A., Hogan, J.E., and Ireland, W.P. (1996). Effects of modulatory agents on neurally-mediated responses of trout intestinal smooth muscle *in vitro*. Fish Physiol. Biochem. *15*, 95-104.

Burnstock, G. (1958). The effect of drugs on spontaneous motility and on response to stimulation of extrinsic nerves of the gut of a teleostean fish. Br. J. Pharmacol. *13*, 216-226.

Bymaster, F.P., Mckinzie, D.L., Felder, C.C., and Wess, J. (2003). Use of M_1-M_5 muscarinic receptor knockout mice as novel tools to delineate the physiological roles of the muscarinic cholinergic system. Neurochem. Res. *28*, 437-442.

Chou, H., Ogawa, N., Asanuma, M., Hirata, H., Kondo, Y., and Mori, A. (1993). Rapid response of striatal muscarinic M1-receptor mRNA to muscarinic cholinergic agents in rat brain. Mol. Brain Res. *19*, 211-214.

Eglen, R.M. (2005). Muscarinic receptor subtype pharmacology and physiology. Prog. Med. Chem. *43*, 105-136.

Eglen, R.M., and Nahorski, S.R. (2000). The muscarinicM5 receptor: a silent or emerging subtype? Br. J. Pharmacol. *130*, 13-21.

Ehlert, F.J., Ostrom, R.S., and Sawyer, G.W. (1997). Subtypes of the muscarinic receptor in smooth muscle. Life Sci. *61*, 1729-1740.

Felder, C.C. (1995). Muscarinic acetylcholine receptors: signal transduction through multiple effectors. FASEB J. *9*, 619-625.

Finn, R.N., and Kristoffersen, B.A. (2007). Vertebrate vitellogenin gene duplication in relation to the "3R Hypothesis": Correlation to the pelagic egg and the oceanic radiation of teleosts. PLoS One 2, e169.

Fraser, C.M., and Lee, N.H. (1995). Regulation of muscarinic receptor expression by changes in mRNA stability. Life Sci. *56*, 899-906.

Ganzinelli, S., Joensen, L., Borda, E., Bernabeo, G., and Sterin-Borda, L. (2007). Mechanisms involved in the regulation of mRNA for M2 muscarinic acetylcholine receptors and endothelial and neuronal NO synthases in rat atria. Br. J. Pharmacol. *151*, 175-185.

Giulietti, A., Oververgh, L., Valckx, D., Decallonne, B., Bouillon, R., and Mathieu, C. (2001). An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods *25*, 386–401.

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. *41*, 95-98.

Hsieh, D.J., and Liao, C.F. (2002). Zebrafish M_2 muscarinic acetylcholine receptor: cloning, pharmacological characterization expression patterns and roles in embryonic bradycardia. Br. J. Pharmacol. *307*, 782-792.

Irwin, D.M. (2004). A second insulin gene in fish genomes, Gen. Comp. Endocrinol. *135*, 150-158.

Kim, B.J., So, I., and Kim, K.W. (2006). Involvement of the phospholipase Cb1 pathway in desensitization of the carbachol-activated nonselective cationic current in murine gastric myocytes. Mol. Cells *22*, 65-69.

Lippe, C., and Ardizzone, F. (1991). Actions of vasopressin and isoprenaline on the ionic transport across the isolated frog skin in the presence and the absence of adenyl cyclase inhibitors MDL 12330A and SQ22536. Comp. Biochem. Physiol. *99C*, 209-211.

Meyer, A., and Schartl, M. (1999). Gene and genome duplications in vertebrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions. Curr. Opin. Cell Biol. *11*, 699-704.

Nilsson, S., and Holmgren, S. (1993). Autonomic nerve funtions. In The physiology of fishes. D.H., Evans, ed., (Boca Raton, USA: CRC Press), pp. 279-313.

Page, R.D. (1996). TreeView: an application to display phylogenetic trees on personal computer. Comput. Appl. Biosci. *12*, 357-358.

Peralta, E.G., Ashkenazi, A., Windlow, J.W., Smith, D.H., Ramachandran, J., and Capon, D.J. (1987). Distinct primary structures, ligand-binding properties and tissue-specific expression of four human muscarinic acetylcholine receptors. EMBO J. *6*, 3923-3929.

Phatarpekar, P.V., Durdan, S.F., Copeland, C.M., Crittenden, E.L., Neece, J.D., and García, D.M. (2004). Molecular and pharmacological characterization of muscarinic receptors in retinal pigment epithelium: role in light-adaptive pigment movements. J. Neurochem. *95*, 1504-1520.

Ronquist, F., and Huelsenbeck, J.P. (2003). MrBayes: Bayesian phylogenetic inference under mixed models. Bioinformatics *19*, 1572-1574.

Swofford, D.L. (2000). Phylogenetic Analysis Using Parsimony and Other Methods (Software). Sinauer Associates, Sunderland, USA.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. *22*, 4673-4680.

Tietje K.M., and Nathanson N.M. (1991). Embryonic chick heart expresses multiple muscarinic acetylcholine receptor subtypes. Isolation and characterization of a gene encoding a novel m2 muscarinic acetylcholine receptor with high affinity for pirenzepine. J. Biol. Chem. *266*, 17382-17387.

Van de Peer, Y., Taylor, J.S., Joseph, J., and Meyer, A. (2002). Wanda: a database of duplicated fish genes. Nucleic Acids Res. *30*, 109-112.

Vandepoele, K., De Vos, W., Taylor, J.S., Meyer, A., and Van de Peer, Y. (2004). Major events in the genome evolution of vertebrates: paranome age and size differ considerably between ray-finned fishes and land vertebrates. Proc. Natl. Acad. Sci. USA *101*, 1638-1643.

Volff, J.-N. (2005). Genome evolution and biodiversity in teleost fish. Heredity *94*, 280-294.

Volff, J.-N., and Schartl, M. (2003). Evolution of signal transduction by gene and genome duplication in fish. J. Struct. Funct. Genomics *3*, 139-150.

Wess, J. (1996). Molecular biology of muscarinic acetylcholine receptors. Crit. Rev. Neurobiol. *10*, 69-99.

Yin, G.C., Gentle, A., and McBrien, N.A. (2004). Muscarinic antagonist control of myopia: a molecular search for the M1 receptor in chick. Mol. Vis. *10*, 787-793.

Zhang, L., Horowitz, B., and Buxton, L.O. (1991). Muscarinic receptors in canine colonic circular smooth muscle. I. Coexistence of M2 and M3 subtypes. Mol. Pharmacol. *40*, 943-951.