Gene Duplications and Losses within the Cyclooxygenase Family of Teleosts and Other Chordates

Justin C. Havird,† Michael M. Miyamoto,* Keith P. Choe,‡§‖ and David H. Evans*†*

*Department of Zoology, University of Florida; †Mount Desert Island Biological Laboratory, Salisbury Cove, ME; ‡Department of Anesthesiology, Vanderbilt University, Nashville, TN; §Department of Pharmacology, Vanderbilt University, Nashville, TN; and ‖Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN

Cyclooxygenase (COX) produces prostaglandins in animals via the oxidation and reduction of arachidonic acid. Different types and numbers of COX genes have been found in corals, sea squirts, fishes, and tetrapods, but no study has used a comparative phylogenetic approach to investigate the evolutionary history of this complex gene family. Therefore, to examine COX evolution in the teleosts and chordates, 9 novel COX sequences (possessing residues and domains critical to COX function) were acquired from the euryhaline killifish, longhorn sculpin, sea lamprey, Atlantic hagfish, and amphioxus using standard polymerase chain reaction (PCR) and cloning methods. Phylogenetic analyses of these and other COX sequences show a complicated history of COX duplications and losses. There are three main lineages of COX in the chordates corresponding to the three subphyla in the phylum Chordata, with each lineage representing an independent COX duplication. Hagfish and lamprey most likely have traditional COX-1/2 genes, suggesting that these genes originated with the first round of genome duplication in the vertebrates according to the 2R hypothesis and are not exclusively present in the gnathostomes. All teleosts examined have three COX genes due to a teleost-specific genome duplication followed by variable loss of a COX-1 (in the zebrafish and rainbow trout) or COX-2 gene (in the derived teleosts). Future studies should examine the functional ramifications of these differential gene losses.

Introduction

Cyclooxygenase (COX) is the enzyme that catalyzes the oxidation and subsequent reduction of arachidonic acid to form Prostaglandin G_2 and Prostaglandin H_2 (PGH_2). PGH_2 can then undergo additional reactions to produce the primary prostaglandins (PGs), which participate in a variety of physiological functions in the vertebrates, including inducing fever, maintaining pregnancy, and regulating ion transport (Harris et al. 1994; McLaren et al. 1996; Steiner et al. 2005). These functions have been extensively studied in mammals (Vane et al. 1998), but comparatively little functional data exist for other animals despite the sequencing of COX genes in several other vertebrates, particularly the teleosts (Zou et al. 1999; Roberts et al. 2000; Choe et al. 2006). Based on functional studies of COX in the teleosts, it seems that some functions are conserved (Brubacher et al. 2000; Sorbera et al. 2001; Holland et al. 2002; Choe et al. 2006), whereas others may be altered in some species (Goetz et al. 1989) or are novel (Cha et al. 2006).

In mammals there are two paralogues of COX. The first was isolated from sheep seminal vesicles in 1988 and later named COX-1 (DeWitt and Smith 1988; Merlie et al. 1988; Yokoyama et al. 1988). A second was isolated from mouse and chicken fibroblast cell cultures in the early 1990s and named COX-2 (Kujubu et al. 1991; Xie et al. 1991; O'Banion et al. 1992). Originally, COX-1 was considered to be a constitutive form that maintained normal cell functions, and COX-2 was considered to be an inducible form that was upregulated in inflammatory responses (Funk 2001). However, studies have shown that this is an oversimplification and that COX-2 is expressed constitutively in the brain (Breder et al. 1995) and kidneys (Harris and Breyer 2001) of mammals. This (along with associated renal and cardiac problems) has led to the abandonment of COX-2 selective inhibitors (e.g., celecoxib and valdecoxib), which were thought to treat inflammatory pain without the negative side effects associated with nonselective COX inhibition. Although structurally and biochemically similar, COX-1 and COX-2 vary in expression and function. For example, COX-1 but not COX-2 is involved in platelet aggregation in mammals (Vane et al. 1998). Although the amino acid (AA) sequences of COX-1 and COX-2 share about 63% similarity, the presence of valine in COX-2 at position 523 instead of isoleucine is thought to be responsible for their differences in substrate selectivity and sensitivity to specific inhibitors (Otto and Smith 1995).

Analyses of genomic sequences and targeted cloning efforts have demonstrated that, like mammals, other vertebrates have COX-1 and COX-2 forms (Järving et al. 2004). However, some variation exists in this COX-1 and COX-2 paradigm, notably in the more evolutionarily ancestral chordates. Teleosts possess additional copies of COX-1 and COX-2 that are likely the result of a teleost-specific genome duplication event (Järving et al. 2004; Ishikawa and Herschman 2007; Ishikawa et al. 2007). However, not all teleosts possess the same forms of COX. The zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*) have two COX-2 genes (named COX-2a and COX-2b) and one COX-1 gene, whereas the green spotted puffer (*Tetraodon nigroviridis*), fugu (*Takifugu rubripes*), and Japanese medaka (*Oryzias latipes*) have two COX-1 genes (named COX-1a and COX-1b) and one COX-2 gene (Ishikawa et al. 2007). This variation is likely due to differential loss of COX genes after duplication (Ishikawa et al. 2007), and this loss of COX duplicates is consistent with the fate of the vast majority of gene duplicates (Lynch and Conery 2000). Also, an unspecified COX form (named sCOX) has been cloned from the spiny dogfish shark (*Squalus acanthias*) (Yang et al. 2002). Furthermore, sea squirts (subphylum Urochordata, *Ciona* species) possess two forms of COX (named COX-a and COX-b) that do not represent the COX-1 or COX-2 of vertebrates but form another independent lineage of COX evolution in the chordates (Järving

Key words: cyclooxygenase, prostaglandins, 2R hypothesis, teleost genome, gene duplication, gene loss.

E-mail: jhavird@zoology.ufl.edu.

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et al. 2004). Similarly, corals also have COX genes (named COX-A and COX-B) that do not correspond to COX-1/2 of vertebrates or COX-a/b of sea squirts (Valmsen et al. 2001; Järving et al. 2004).

The current view of COX evolution represents an interesting but incomplete account of this gene family, both from a phylogenetic and functional standpoint. Therefore, the first goal of this study was to sequence and perform phylogenetic analyses of all COX forms in the euryhaline killifish (*Fundulus heteroclitus*) and longhorn sculpin (*Myoxocephalus octodecemspinosus*) in an attempt to elucidate the history of COX duplication and loss in the teleosts. A second goal was to investigate the early evolutionary history of COX in the chordates by sequencing COX forms from amphioxus (*Branchiostoma lanceolatum*), the Atlantic hagfish (*Myxine glutinosa*), and the sea lamprey (*Petromyzon marinus*) and subjecting COX forms representative of all animals with known COX-mediated PG production to phylogenetic analyses.

Here, we report the cloning of 9 novel COX sequences from the species mentioned above. Sequence alignments show that these COX forms contain AA residues and motifs critical for COX function, suggesting that all chordates possess functional COX enzymes. Also reported is the first phylogenetic analysis of representatives of all COX forms. These results offer a nomenclature for COX classification based on the predicted evolutionary relationships between the different COX enzymes. They also show a complex history of gene duplication and subsequent loss in several major COX lineages. The identities of the lamprey and hagfish sequences likely correspond to basal COX-1 and COX-2 sequences of vertebrates, implying that all vertebrates possess COX-1 and COX-2 genes. This places the origin of COX-1/2 with the first round of genome duplication in the vertebrates, according to the current timing of the 2R hypothesis (Dehal and Boore 2005; Kasahara 2007; Nakatani et al. 2007; Putnam et al. 2008).

Materials and Methods

Sequence Acquisition

All procedures with live animals were approved prior to beginning the experiments by Institutional Animal Care and Use Committees at the University of Florida and the Mount Desert Island Biological Laboratory (MDIBL). Euryhaline killifish were captured and housed as previously described (Choe et al. 2006). Longhorn sculpin and Atlantic hagfish were purchased from fishermen and were housed similarly to the killifish. Female, nonmigratory lampreys were a generous gift from the USGS Great Lakes Science Center at the Hammond Bay Biological Station in Millersburg, Michigan, and were processed there. Lancelets were purchased from Gulf Marine Specimens (Panacea, FL) and were processed upon arrival.

After initial anaesthetization with MS-222 (~600 mg/l), killifish, sculpin, hagfish, and lampreys were pithed and/or decapitated. The gill arches (first and second arches for lampreys, second and third arches for teleosts) or gill baskets (hagfish) were then removed using sterile, RNAse-free dissecting tools. Lancelets were cut in half with sterile, RNAse-free tools. After removal, tissues were immediately placed in liquid nitrogen and stored at −80 °C before further processing.

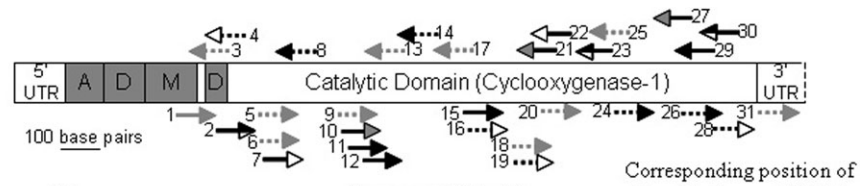
Reverse transcription, polymerase chain reaction (PCR), cloning, and sequencing were performed as described previously (Choe et al. 2006) with slight modifications. Total RNA was isolated from the gills of killifish, lampreys, sculpin, and hagfish as well as the anterior half of lancelets using TRI-reagent (Sigma, St Louis, MO) and reverse transcribed with a Superscript II or Superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol and using oligo-dT as a primer. The resulting cDNA was stored at −20 °C until used for PCR.

Degenerate primers (fig. 1) were first designed against COX sequences from a wide range of chordates to amplify any chordate COX. More specific sets of degenerate primers were then designed to amplify COX-1, COX-1a, and COX-1b sequences in the teleosts. All degenerate primers were designed using the online program Consensus-Degenerate Hybrid Oligonucleotide Primers (Rose et al. 1998). After initial fragments were sequenced, Primer Express software (Applied Biosystems, Foster City, CA) was used to design final primers to extend the original sequences (fig. 1).

Initial PCR was performed on 5% of a reverse transcriptase reaction with a Takara Ex Taq Hot Start DNA Polymerase Kit (Takara Bio Inc., Otsu, Japan) in a PCR Express thermocycler (ThermoHybaid, Franklin, MA) with standard cycling parameters (Hyndman and Evans 2007). PCR products were ligated into PCR4-TOPO vectors and transformed into TOP10 chemically competent cells using a TOPO TA Cloning Kit (Invitrogen) following the manufacturer's protocol. Plasmids were then isolated using a High Pure Plasmid Isolation Kit (Roche, Mannheim, Germany) following the manufacturer's protocol, and plasmid DNA was sequenced in both directions at the Marine DNA Sequencing Center at the MDIBL using ABI 3100 16-capillary sequencers. After initial sequences were extended with specific primers, the 5′ and 3′ ends were sequenced using a Generacer Kit (Invitrogen) following the manufacturer's protocol. All PCR products were visualized by ethidium bromide staining on 1–2% agarose gels to verify that primers amplified fragments of the appropriate size for chordate COX.

Sequence Analysis

Basic local alignment search tool (BLAST; National Center for Biotechnology Information) searches were done with each sequence fragment to confirm that primers amplified COX sequences (Altschul et al. 1990). These sequence fragments were assembled using GeneTools software (BioTools Inc., Edmonton, Alberta), and the resulting sequences were searched for open reading frames. Inferred protein sequences were aligned with other COX proteins using PepTools software (BioTools Inc.) to search for conserved protein domains across COX sequences (Kulmacz et al. 2003; Simmons et al. 2004; Ishikawa et al. 2007) and for phylogenetic analysis. COX sequences from other chordates were obtained from GenBank (release 162, October 2007) and Ensemble (release 47, October 2007) by BLAST



Primers	Sequences (5' to 3')	Corresponding position of 5' end in human COX-1
1) CH COX F1	atggacgactaccagtgygaytgyac	285
2) CH COX1 F2'	gacgtagtacactacatactaacacacttysaytggyt	391
3) Mo COX2 5' RC R3	gaaggagatggagttaatgatgttcc	427
4) Mo COX1a 5' RC R3	tgaccgtcagcactaatctcatg	468
5) hCOX 5' RC M F1	gtttcgagtccacccacatacaa	501
6) hCOX 3' RC F3	agtccacccacatacaatgcc	507
7) CH COX1a F1	ccccaccaacctacaataccaartayggnta	513
8) Fh COX1b 5' RC R2	cagcctctcaaacaacacctgag	653
9) Mo F2	caccagcctgatgtttgcatt	710
10) CH COX F2	gatgtttgcatttttcgctcarcayttyac	720
11) CH COX1 F3	gcacaacacttcacacaccaattyttyaarac	736
12) CH COX1b F1	cggatgggtgtgggcttyacnaargc	775
13) Mo R2	ctccatccaggacctgatatttaag	885
14) Mo COX1b 5' RC R3	aatagccatctgagcctcagga	972
15) CH COX F3	ccactatatggctgcgggarcayaaymg	1037
16) Mo COX1a 3' RC F2	gagggagcataacagactctgtga	1050
17) hCOX 5' RC M R1	cacatcgagtacgtcacacacg	1064
18) Mo COX2 3' RC F3	tcgaggactacgtgcagcac	1165
19) Fh COX1a 3' RC F2	gagtacgtgcagcacctgagc	1171
20) hCOX 3' RC F6	cagtacagcaaccgcatctca	1245
21) CH COX R2	gcatcagcgggtgccartgrta	1284
22) CH COX1a R1	atagggaatgtcgtcnccrtcdat	1321
23) CH COX1 R2'	tgggtgtatgttgtgtcctccnccdatytg	1429
24) Mo COX1b 3' RC F2	ttggcggtggctttaacatc	1433
25) hCOX 5' RC R3	catgtttgatggatgctgttgc	1473
26) Fh COX1b 3' RC F2	agctctacggtgacatcgacact	1610
27) CH COX R1	ccccgaaggtggatggyttccavya	1651
28) Mo COX1a 3' RC F6	gagtatgttggagatgggtgctc	1692
29) CH COX1b R0	ggagaagggagcacccatytcnaccat	1696
30) CH COX1 R1	tcctccgaatgtgcttggyttccarta	1762
31) Mo COX2 3' RC F6	aacatcgtcaacaccgcctc	1810

FIG. 1.—Sequences, orientations, and locations of primers used in PCR amplifications of COX sequences from amphioxus, hagfish, lamprey, killifish, and sculpin. Degenerate primers are denoted with solid lines and subsequent specific primers are denoted with broken lines. Black arrows indicate primers used to amplify teleost COX-1 sequences and lCOX. Gray arrows indicate primers used to amplify teleost COX-2 sequences, hCOX-2, and amphioxus sequences. Arrows outlined in black and shaded gray were used to amplify multiple COX sequences from hagfish, lamprey, and amphioxus. Open and closed arrows indicate primers used to amplify teleost COX-1a and COX-1b sequences, respectively. Half-closed arrows indicate primers used to amplify teleost COX-1a and COX-1b sequences. The primers are aligned against human COX-1 (GenBank accession number NM_000962) and the corresponding position of their 5′ ends are given in the lower right column. The four major COX-functional domains are labeled as follows: A, amino-terminal signal peptide; D, dimerization domains; M, membrane-binding domain and catalytic domain. The 5'- and partial 3'-untranslated regions (UTR) are also indicated (the dashed line indicates that only 136 of 3160 base pairs are shown for the 3' UTR).

searching for sequences with significant similarity to the new COX sequences and human COX-1/2 sequences. The following sequences were included in the study: all novel sequences from the study species (9 sequences), sequences from teleosts with all 3 COX forms represented (19), sequences from nonteleost chordates with two or more COX forms represented and designated as "RefSeqs" in GenBank (22), and sequences from species representing novel evolutionary lineages (11). These criteria resulted in the inclusion of 61 COX sequences for 26 species in the present study.

Phylogenetic Analyses

Phylogenies were generated using the AA alignment of COX sequences. The C- and N-terminal portions of the sequences were not readily alignable and were therefore excluded from phylogenetic analyses (Swofford et al. 1996). Models of evolution to be used in phylogenetic analyses were evaluated to account for different AA replacements, among-site rate variation, and invariable sites. Using likelihood ratio tests (Huelsenbeck and Rannala 1997), a model using the WAG replacement matrix (Whelan and Goldman 2001) and the gamma (Γ) distribution (Yang 1996) for among-site rate variation was chosen as most appropriate for the data set.

A maximum likelihood (ML) phylogeny was generated using the program PhyML (Guindon and Gascuel 2003; Guindon et al. 2005). An optimal phylogeny was generated using the WAG replacement matrix and the gamma distribution with eight substitution rate categories and an

estimated α parameter for site-to-site heterogeneity in rates. Group support was evaluated with 1000 bootstrap replicates. A Bayesian phylogenetic (BP) analysis was also performed using the program MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003), with the same evolutionary model as in the ML analysis and 10 million generations. Trees were sampled every 200 generations, and the first 5,000 trees (10%) were excluded as burn-in when generating the extended majority rule consensus tree.

The relationships of the lamprey sequences were questionable due to their incomplete status (both are only ~50% complete) and tendency to decrease support values for critical groupings (such as the union of COX-1 and COX-2 sequences) when they were included. Therefore, to further investigate early COX evolutionary history in the chordates, lamprey sequences (along with hagfish and shark sequences) were forced to join different lineages at the base of the vertebrate COX-1/2 clade. The resulting increase in log likelihood scores (ln*L*) from these topological changes were examined with PhyML (Guindon and Gascuel 2003; Guindon et al. 2005). Analyses were also repeated excluding these incomplete lamprey COX sequences. In these ways, we were able to test the effects of including these more divergent early vertebrate sequences on the extent and timing of the major COX-1/2 duplication.

Results

Novel Sequences and Key Functional Sites and Motifs

PCR using degenerate and specific primers resulted in the determination of the following novel COX sequences: COX-1a (452 AA for the inferred polypeptide) and COX-1b (598 AA) from the killifish; COX-1a (622 AA), COX-1b (600 AA), and COX-2b (605 AA) from the sculpin (named following Ishikawa et al. 2007); lCOX (286 AA) from the lamprey (named following Yang et al. 2002); hCOX-2 (610 AA) from the hagfish; and COX-c (177 AA) and COX-d (177 AA) from amphioxus (named following Järving et al. 2004). All fragments consistently returned COX sequences from other chordates when subjected to BLAST searches, suggesting that the sequences represent COX forms (GenBank accession numbers for new sequences are given in figs. 2 and 3). Sequences from the teleosts and hagfish were all at least 75% complete (relative to human COX-1). In contrast, the lamprey and amphioxus sequences were 50% and 30% complete, respectively (missing the N and C terminals).

Additional BLAST searches in Ensemble revealed new COX sequences that are analyzed here for the first time. The green anole (*Anolis carolinensis*) genome contains two COX sequences that were confirmed as COX-1 and COX-2 using phylogenetic analyses (see below). The opossum (*Monodelphis domestica*) genome contains four COX sequences that were similarly identified as COX-1 and COX-2. The lamprey genome also contained two sequences with high identity to known COX sequences. These sequences were most similar to the hCOX-2 sequence from the hagfish and had nonoverlapping, but consecutive locations in the multiple sequence alignment (supplementary fig. S1, Supplementary Material online). They were therefore combined into one sequence that was named lCOX-2 based on phylogenetic analyses (see below). During final revisions of this manuscript, a cephalochordate genome (*Branchiostoma floridae*) was published (Putnam et al. 2008) and BLAST searches of this genome returned sequences with high identity to the COX-c and COX-d sequences reported here, indicating that all cephalochordates likely have COX-c and COX-d genes.

A multiple sequence alignment of the novel COX sequences with other COX sequences (supplementary fig. S1, Supplementary Material online) shows that known AAs and motifs critical for COX function are conserved throughout the chordates (Kulmacz et al. 2003; Simmons et al. 2004; Ishikawa et al. 2007). The active sites for COX (tyrosine-385, histidine-388, and serine-530; using ovine COX-1 numbering throughout) and peroxidase activity (glutamine-203 and histidine-207) are strictly conserved throughout the chordates, including the novel COX sequences (table 1). However, only the vertebrate COX-1 and COX-2 sequences (including hCOX-2) contain the first heme-binding domain and second dimerization domain. Additionally, COX-1 sequences from the vertebrates contain a unique N-terminal insertion of 5–12 AAs, whereas COX-2 sequences (including hCOX-2) have a similar C-terminal insertion (Simmons et al. 2004). The novel COX sequences from the teleosts also show these insertions. The presence of valine at position 523 that is the target of COX-2–specific inhibitors is only COX-2 specific in the eutherian mammals (valine-523 is present in all noneutherian COX-1 and COX-2 sequences). Finally, the sculpin COX-1a sequence shows several differences toward the N- and C-terminal ends, including a replacement of phenylalanine for serine-530 and the loss of several N-terminal cysteines involved in dimerization.

Phylogenetic Analysis of Teleost COX Sequences

As predicted, all teleosts examined contained both COX-1 and COX-2 forms that constitute well-supported ($\geq$80% bootstrap scores and $\geq$95% posterior probabilities) monophyletic lineages within the COX-1 and COX-2 clades of vertebrates in both ML and BP analyses (figs. 2 and 3). Within the teleost COX-1 lineage (fig. 3*A*), there are two well-supported clades: one containing the COX-1a sequences from the killifish, sculpin, medaka, stickleback, fugu, and puffer (hereafter, referred to as the derived teleosts) and the other containing the COX-1 sequences (hereafter, referred to as COX-1b sequences) from the zebrafish and trout as its most basal members and the COX-1b sequences from the derived teleosts as its most derived members. Within the teleost COX-2 lineage (fig. 3*B*), there are also two well-supported clades: one containing the COX-2a sequences from the zebrafish and trout and the other containing the COX-2b sequences from the zebrafish and trout as its most basal members and the COX-2 sequences (hereafter, referred to as COX-2b sequences) from the derived teleosts as its most derived members.

Although, the teleost COX phylogenies generally conform to the predicted species relationships within the COX-1 and COX-2 clades, the zebrafish, and trout COX-1b

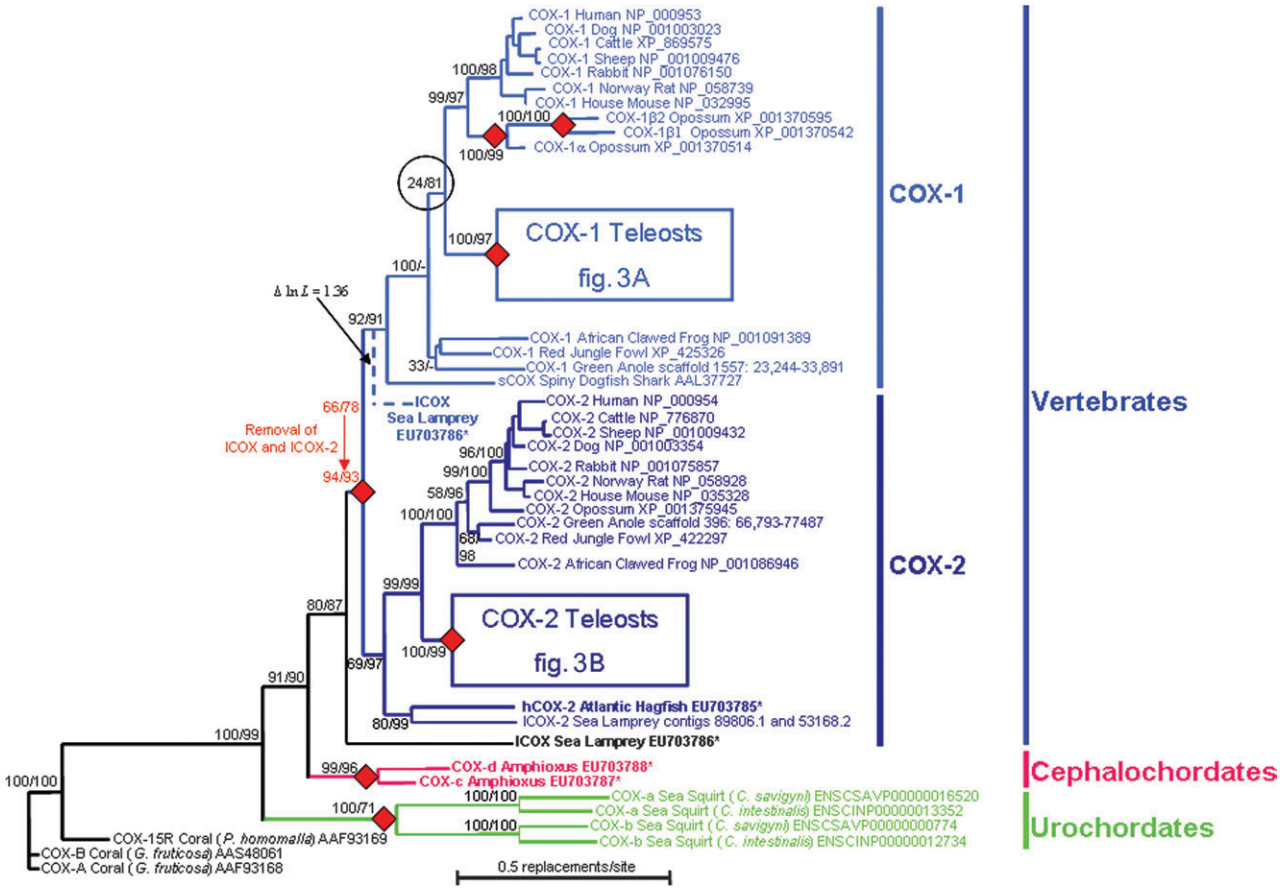


FIG. 2.—Unrooted ML phylogeny of COX proteins. There are three well-supported lineages corresponding to three independent COX duplications found in the chordates: a urochordate lineage (in green) represented by COX-a and COX-b sequences, a cephalochordate lineage (in pink) represented by the novel COX-c and COX-d sequences, and a vertebrate lineage (in blue) represented by COX-1 (in light blue) and COX-2 (in dark blue) sequences. The sequence name is given along with the common name of the animal and the accession number of the sequence in GenBank or Ensemble (scaffold and contig locations are given for anole sequences and lCOX-2). Evolutionary relationships were inferred using ML and BP methods. Numbers at the nodes represent % support values (numbers on the left are based on 1000 bootstrap replicates from the ML analysis and numbers on the right are based on 10 million generations from the BP analysis). Dashes to the right of bootstrap support values indicate relationships that were not supported in the extended majority rule BP consensus tree. The low incidence of these conflicting relationships indicates that the final BP and ML trees were in strong agreement. The lamprey lCOX sequence is shown to produce the smallest change in ln*L* scores when repositioned at the base of the COX-1 lineage (indicated by the dashed line). The support values in red represent the union of the COX-1 and COX-2 sequences from the vertebrates and are shown including and excluding lCOX and lCOX-2 from the analyses, with a drastic increase (by 28% bootstrap support) when these sequences are excluded. This suggests lCOX and lCOX-2 are rogues and do not readily group with a particular lineage. The support values for a biologically irrelevant teleost/mammal union are circled. Novel sequences are bolded and indicated with an asterisk. Diamonds represent proposed gene duplication events. Lineages of interest are indicated on the right. See figure 3 for detailed teleost COX subtrees.

sequences formed a monophyletic group (showing 69% bootstrap and 99% posterior probability support, circled in fig. 3*A*) which is not supported based on other molecular and morphological studies (Ishiguro et al. 2003; Lavoué et al. 2008) or the COX-2 phylogeny. Phylogenetic analyses confirmed that the two teleosts examined here (the euryhaline killifish and the longhorn sculpin) possess two COX-1 genes (COX-1a and COX-1b) and one COX-2b gene that are expressed in the gills.

Phylogenetic Analysis of All COX Sequences

Using ML and BP analyses, several well-supported COX clades are recovered (fig. 2), generally conforming to predicted evolutionary relationships and previous hypotheses of COX evolution (Järving et al. 2004). As predicted, the coral COX sequences are the most basal COX forms and group outside of a well-supported chordate lineage. In all analyses, there are three strongly supported COX lineages within the chordates that correspond to the three subphyla in the phylum Chordata. The COX-a and COX-b sequences from *Ciona intestinalis* and *Ciona savignyi* form a urochordate COX lineage that represents the most basal COX group in the chordates, the COX-c and COX-d sequences from *Branchiostoma* form a novel cephalochordate COX lineage, and the COX-1 and COX-2 sequences of vertebrates form the vertebrate COX lineage. The sCOX sequence from the dogfish (*S. acanthias*) is well supported at the base of the COX-1 lineage, indicating that it is a basal COX-1 sequence (hereafter, referenced as sCOX-1). The new hCOX-2 sequence from the hagfish is consistently placed at the base of the COX-2 lineage, indicating it as a basal COX-2 sequence. Sequences from the anole genome consistently group with

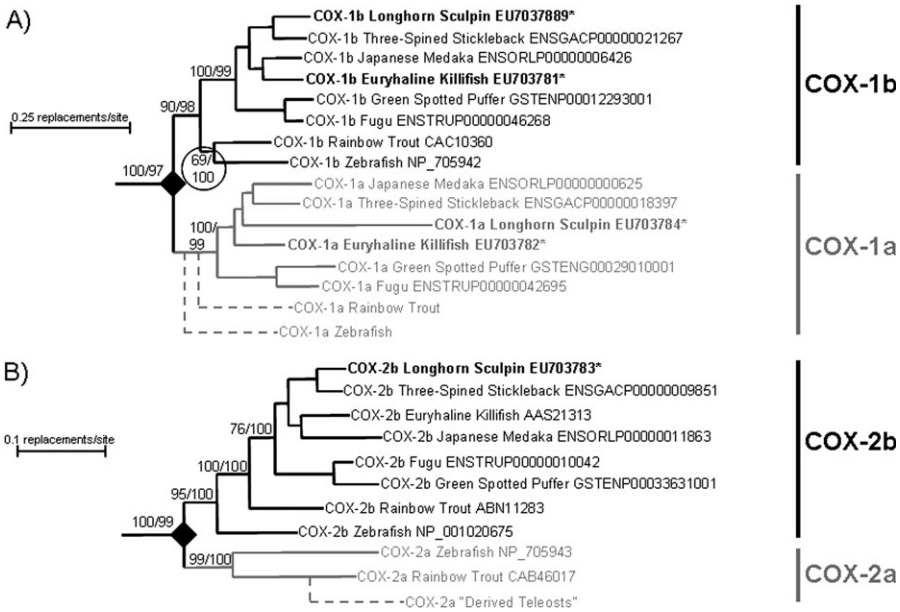


FIG. 3.—Phylogenies of COX in the teleosts as expanded from the overall COX phylogeny (fig. 2). As these subtrees are part of the larger phylogeny, they follow the conventions of the latter in terms of their support values, sequence and group names, accession numbers, and gene duplication events. (*A*) COX-1 in the teleosts, showing well-supported COX-1a (in gray, with independent losses in the zebrafish and rainbow trout indicated by dashed lines) and COX-1b (in black) lineages. The support values for the otherwise refuted union of zebrafish and rainbow trout as sister taxa are circled. (*B*) COX-2 in the teleosts, showing well-supported COX-2a (in gray, with a loss in the derived teleosts indicated by the dashed line) and COX-2b lineages (in black).

known COX-1 and COX-2 sequences within the vertebrate lineages, indicating the anole possesses COX-1 and COX-2 genes. The four COX sequences from the opossum likely represent one COX-2 gene and three COX-1 genes. The three COX-1 genes share AA identities of 79–86%, group together in phylogenetic analyses, and are closely linked on chromosome 1 in the opossum genome (COX-1α: positions 415934521–415959438; COX-1β1: positions 416005263–416019593; and COX-1β2: positions 416118131–416138255 on contigs 41307 and 41309). These results indicate that COX-1 has undergone tandem duplications in the opossum.

When the lamprey COX sequences are included in the analyses, support values for critical and well-known COX groups are reduced. When these sequences are excluded, the support values tend to increase. This is seen most dramatically in the support for the union of vertebrate COX-1 and COX-2 sequences (showing 66% and 94% bootstrap support and 78% and 93% posterior probability support with and without the lamprey sequences, respectively). The lCOX-2 sequence from the lamprey genome consistently groups with hCOX-2 at the base of the COX-2 lineage, identifying it as a basal COX-2. In contrast, the lCOX sequenced from the lamprey is placed in a novel lineage diverging before the vertebrate COX-1/2 lineage in both analyses. Using constrained topologies with altered positions of hCOX-2, lCOX, lCOX-2, and sCOX-1, the smallest change in ln*L* (from −22996.1861 to −22997.5467) was seen when lCOX was moved to the base of the COX-1 lineage (dashed line in fig. 2). Other topologies resulted in changes larger than seven ln*L* units.

Within the COX-1 and COX-2 clades, sequences generally follow the predicted species relationships. However, in the COX-1 lineage the *Gallus*, *Xenopus*, and *Anolis* sequences form a basal clade to a biologically irrelevant teleost and mammal grouping. This grouping is only strongly supported in the Bayesian analysis (with a posterior probability of 81%, circled in fig. 2). This suggests that Bayesian support values may be inflated in this case because the teleosts should be basal to the tetrapods based on overwhelming molecular and morphological data (Delsuc et al. 2006). Furthermore, this clade only shows 24% support in the ML analysis and is not found in the COX-2 lineage.

Discussion

Characteristics of COX Sequences

The presence of known AAs and motifs critical for COX and peroxidase activity in the novel COX sequences confirms the identity of these proteins and suggests that all chordates contain functional COX genes responsible for producing PGs. PGs are also made by insects and aquatic invertebrates (Rowley et al. 2005), and plants produce structural analogues to PGs (Bergey et al. 1996). Interestingly, no COX enzymes have been found in these organisms (only corals and the chordates have known COX enzymes), and it has been hypothesized that these other organisms may possess alternative PG production mechanisms and enzymes (Simmons et al. 2004). Therefore, COX-mediated PG production in the animals may have been lost in invertebrate lineages multiple times and/or independently regained in the chordates.

Only vertebrate COX-1/2 sequences possess the first heme-binding and second dimerization domains, and the complete hagfish sequence also contains these domains, indicating that they originated in the basal vertebrate ancestor.

Table 1
Conservation of Known Functional Domains and Residues among COX Sequences

Domain or Residue	Amino Acid Positions	Presence (+) or Absence (−)							Function and Discussion
		Eutherians		All Vertebrates		Amphioxus	Tunicates	Corals	
		COX-1	COX-2	COX-1	COX-2	COX c/d	COX a/b	COX A/B	
Cyclooxygenase active sites	Y-385	+	+	+	+	?	+	+	Oxidizes arachidonic acid to form PG G_2; All present in all known COX sequences, except Q-388 in opossum COX-1b and F, P-530 in frog COX-1, sculpin COX-1a
	H-388	+	+	+	+	?	+	+	
	S-530	+	+	±	+	?	+	+	
Peroxidase active sites	Q-203	+	+	+	+	?	+	+	Reduces PG G_2 to form PGH_2; Both present in all known COX sequences
	H-207	+	+	+	+	+	+	+	
Substrate-binding site	R-120	+	+	+	+	?	±	+	Binds substrates and some nonsteroidal anti-inflammatory drugs, narrows COX active channel; present in all sequences except COX-a (*Ciona intestinalis*)
Endoplasmic reticulum retention signal	596–599	+	+	±	+	?	±	+	Retains COX proteins inside endoplasmic reticulum; lost in *Ciona savignyi* and some vertebrate COX-1 forms
N-terminal signal peptide	1–24	+	+	+	+	?	+	+	Directs COX into lumen of the endoplasmic reticulum; widespread
Membrane-binding domain	71–115	+	+	+	+	?	+	+	Binds COX to intraluminal surface of microsal membranes; low homology between sequences (sculpin COX-1a has little homology)
N-glycosylation sites	N-68	+	+	+	+	?	+	±	Stabilizes COX via protein folding; N-144 is the only widespread site (Järving et al. 2004; Simmons et al. 2004)
	N-104	+	−	+	−	?	−	−	
	N-144	+	+	+	+	?	+	+	
	N-410	+	+	+	+	?	−	−	
	N-593	−	+	−	+	?	−	−	
Dimerization domains	33–70	+	+	+	+	?	+	+	Holds together COX dimers; second domain is found only in vertebrates (sculpin COX-1a lacks Cys-bridges)
	124–142	+	+	+	+	?	−	−	
Haem-binding domain	312–316	+	+	+	+	−	−	−	Binds iron; first domain is found only in vertebrates
	305–314	+	+	+	+	+	+	+	
N-terminal insertions	23–32	+	−	+	−	?	−	−	Produces more efficient translocation into lumen; only in COX-1 sequences
C-terminal insertions	573–588	−	+	−	+	?	−	−	Function unknown; only in COX-2 sequences
Isoform-specific inhibitor target sites	R-513	−	+	±	+	?	+	−	Allows paralogue-specific inhibition of COX; replacements are only paralogue specific in eutherian mammals
	V-523	−	+	+	+	?	−	−	

NOTE.—Positions of domains and key residues are highlighted in the multiple sequence alignment (supplementary fig. S1, Supplementary Material online) as well as in previously published alignments (Kulmacz et al. 2003; Simmons et al. 2004). Amino acid positions are based on ovine COX-1 numbering (GenBank accession number NP_001009476). The presence (+) or absence (−) of many highlighted domains and residues is unknown for amphioxus due to incomplete (30%) sequencing of its COX-c and COX-d sequences

Vertebrate COX enzymes may therefore possess enhanced iron-binding and dimerization properties when compared with nonvertebrate COX enzymes. The COX-2 characteristic insertions near the C-terminal end in hCOX-2 confirm the phylogenetic classification of this sequence as a basal COX-2. The lack of homologous regions and residues (Asn-68, Val-523, Ser-530, disulfide bond cysteines, and very low homology in the membrane-binding domain) in the sculpin COX-1a sequence suggests that this form may have reduced functionality and that COX-1b or COX-2b may play additional roles in the sculpin as compared with other teleosts. As noted previously (Choe et al. 2006), COX-2–specific inhibitors such as NS-398 which function via a COX-2–specific valine-523 replacement should only be viewed as COX-2–specific inhibitors in the eutherian mammals because in sequences from all other vertebrates this amino acid occurs in COX-1 also.

COX Evolution in the Teleosts

The presence of duplicate COX-1 or COX-2 genes in the teleosts has been proposed previously using model

teleost species such as the zebrafish and green spotted puffer (Järving et al. 2004; Ishikawa et al. 2007; Ishikawa and Herschman 2007). However, this is the first study to examine the identities and relationships of these different forms using a phylogenetic approach. Our results support previous hypotheses and suggest that teleosts possess more COX genes when compared with other vertebrates, which is likely the result of a genome duplication event in the teleost lineage (Taylor et al. 2003) that led to COX-1a, COX-1b, COX-2a, and COX-2b genes. Our phylogenetic analyses strongly indicate that the zebrafish and rainbow trout have retained COX-2a, COX-2b, and COX-1b, while losing COX-1a, and that the killifish, sculpin, medaka, stickleback, fugu, and puffer (the derived teleosts) have retained COX-1a, COX-1b, and COX-2b, while losing COX-2a. The presence of well-supported clades for each of the COX genes in the teleosts offers a novel way of naming and classifying teleost COX genes using a phylogenetic method. For example, it is now clear that zebrafish and trout COX-1 genes are specifically COX-1b genes and should be referenced as such.

The species relationships within the teleost COX-2 phylogeny (fig. 3*B*) generally follow those predicted by other studies (Lavoué et al. 2008) and indicate that the absence of COX-2a in the derived teleosts can be explained by a single loss in their common ancestor. The most exclusive monophyletic group containing these derived teleosts is likely the Acanthopterygii, which also includes the mullets, seahorses, and perches. The loss of COX-2a may have occurred prior to the origin of the acanthopterygiians, but examination of more teleost COX sequences is needed to test this hypothesis.

Although the teleost COX-1 phylogeny (fig. 3*A*) predicts zebrafish and trout as basal sister taxa in a monophyletic group, this relationship has not been supported (or proposed to our knowledge) by any other studies (Ishiguro et al. 2003; Lavoué et al. 2008) and is not supported in the teleost COX-2 phylogeny (fig. 3*B*). Therefore, the absence of COX-1a in the zebrafish and trout cannot be explained by a single loss. Independent losses of COX-1a in the lineages represented by the zebrafish and trout and a single loss of COX-2a in the lineage represented by the derived teleosts after the teleost-specific genome duplication most parsimoniously describes the evolution of COX genes in the teleosts.

The fate of the vast majority of duplicated genes is loss rather than preservation (Lynch and Conery 2000). This has been clearly shown in the genomes of teleosts, which show massive gene loss after their whole-genome duplication (WGD) (Jaillon et al. 2004). After the WGD in the teleosts, the zebrafish and puffer appear to have lost different copies of many duplicate genes, resulting in each species containing the same relative number of duplicate genes but different forms (Woods et al. 2005; Sémon and Wolfe 2007). Our analysis of COX evolution in the teleosts supports this hypothesis, with the zebrafish retaining the two COX-2 duplicates and COX-1b and the puffer retaining the two COX-1 duplicates and COX-2b, giving each species a total of 3 COX genes. This differential retention of duplicated genes may have led to the vast species radiation in the teleosts (Hoegg et al. 2004; Woods et al. 2005; Sémon and Wolfe 2007). Specifically, the variable retention of COX-2 versus COX-1 forms may have caused osmoregulatory (Choe et al. 2006), reproductive (Roberts et al. 2000; Grosser et al. 2002), and immunoregulatory (Brubacher et al. 2000; Holland et al. 2002) differences between species. These hypotheses concerning the functional consequences of differential COX retention and loss between species can be tested by analyses of site-specific evolutionary rate changes between different COX forms (Knudsen and Miyamoto 2001; Wang and Gu 2001).

COX Evolution in the Chordates

Using rigorous phylogenetic analyses of COX sequences, the previously hypothesized relationships for coral, urochordate, and vertebrate COX genes (Järving et al. 2004) are strongly confirmed, and a novel cephalochordate lineage is recovered. The presence of two different duplicate COX genes in each lineage indicates that each underwent an independent COX duplication. There have also been tandem COX-1 duplications in the opossum, which may be present in all metatherians. These genes are named COX-1α, COX-1β1, and COX-1β2 (fig. 2) to reflect their evolutionary origins and avoid nomenclatural confusion with the independent COX-1a/b duplication in the teleosts, which was proposed prior to the publication of the opossum genome (Järving et al. 2004; Mikkelsen et al. 2007).

Interestingly, multiple COX genes appear to have been retained (and are expressed) in each known COX lineage. In contrast, the vast majority of gene duplicates are lost rather than retained (Lynch and Conery 2000). This repetitive retention of two (or more in the teleosts and opossum) COX genes may indicate that COX genes have multiple forms in all animals with COX-mediated PG production due to the many complex regulatory roles of COX (Järving et al. 2004).

Identities and Implications of Hagfish and Lamprey COX Sequences

The "rogue" lamprey sequences appear to decrease support values for critical relationships (e.g., the union of COX-1 and COX-2 sequences) and can group in alternative positions near the base of the vertebrate lineage with relatively small changes in ln*L*. However, the consistent recovery of hCOX-2 and lCOX-2 at the base of the COX-2 lineage and the small change in ln*L* (1.36 ln*L* units) caused from relocating lCOX to the base of the COX-1 lineage suggest that lCOX is a basal COX-1 sequence. Therefore, agnathans most likely have traditional COX-1/2 genes and not basal forms predating their origin. This conclusion predicts that the hagfish and lamprey genomes will each possess copies of COX-1 and COX-2 genes that are orthologous to their COX-1/2 counterparts in the jawed vertebrates. Therefore, it is predicted that the hagfish genome should contain an as of yet undiscovered COX gene, corresponding to a basal COX-1 because all vertebrates have COX-1 and COX-2 genes.

The identification of lamprey and hagfish sequences as basal COX-1 and COX-2 forms indicates that the gene duplication responsible for COX-1 and COX-2 lineages

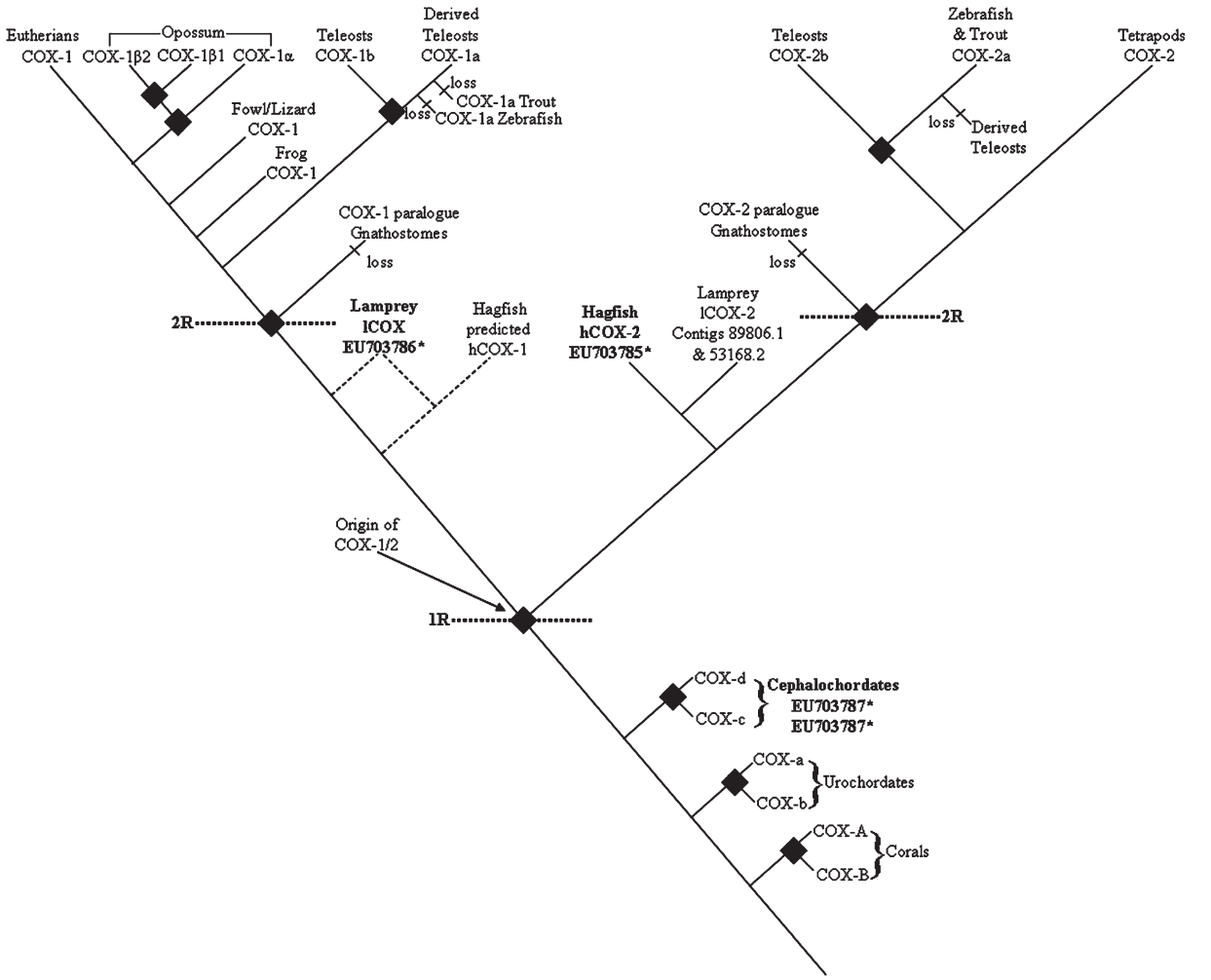


FIG. 4.—Proposed history of COX duplications and losses. This model constitutes our final hypothesis of COX evolution among the corals and chordates along with our corresponding suggested nomenclature for known COX proteins. This hypothesis is based on the inferred COX phylogeny (figs. 2 and 3), current hypotheses about the timing of whole-genome duplication events in the chordates (i.e., based on other gene family phylogenies and linkage analyses of synteny), and the subsequent rapid loss of paralogous genes after duplication events (Lynch and Conery 2000; Dehal and Boore 2005; Kasahara 2007; Nakatani et al. 2007; Putnam et al. 2008). Each of the proposed four major COX lineages (corals, urochordates, cephalochordates, and vertebrates) corresponds to an independent gene duplication event. COX-1 and COX-2 lineages originated with the first round of genome duplication in the vertebrates. The second round of genome duplication resulted in COX-1 and COX-2 paralogues that have subsequently been lost. It is predicted that the hagfish has another COX gene corresponding to a basal member of the COX-1 lineage based on the presence of COX-1 and COX-2 genes in all other vertebrates (unknown relationships indicated by dashed lines). Teleosts underwent another duplication followed by different losses in different groups. COX-1 has undergone tandem duplications in the opossum. Diamonds indicate gene duplication events. Loss events are also indicated. Dotted lines indicate the timing of genome duplications based on the 2R hypothesis (Dehal and Boore 2005; Kasahara 2007; Nakatani et al. 2007; Putnam et al. 2008). Novel sequences are bolded and indicated with an asterisk.

occurred with the origin of the vertebrates and not in the gnathostomes exclusively. This period corresponds to the first round of genome duplication in accordance with the current timing of the 2R hypothesis, which predicts one WGD just before the origin of the agnathans (Dehal and Boore 2005; Kasahara 2007; Nakatani et al. 2007; Putnam et al. 2008). The most current, widely accepted version of the 2R hypothesis then predicts an additional WGD before the origin of the gnathostomes (Lynch 2007). This second, gnathostome-specific WGD is most strongly supported by integrative studies of the phylogenies for different gene families and their patterns of chromosomal linkage and synteny (Dehal and Boore 2005; Nakatani et al. 2007; Putnam et al. 2008).

Thus, in our final hypothesis of COX evolution (fig. 4), the first round of genome duplication led to COX-1 and COX-2 gene lineages which are represented by all vertebrates. The second genome duplication occurring with the origin of the gnathostomes must have then resulted in multiple COX-1 and COX-2 genes in the gnathostome common ancestor. However, only single COX-1 and COX-2 genes are known from gnathostomes (except for teleosts and opossum that underwent later, lineage-specific duplications). Therefore, a single gene loss of each COX isoform in the gnathostome common ancestor most parsimoniously explains the presence of single COX-1/2 forms in extant gnathostomes. This rapid loss of duplicate COX genes is consistent with the fate of the vast majority of other

duplicate genes and their estimated short lifetimes of several million years (Lynch and Conery 2000). These rapid losses of COX duplicates in the gnathostome common ancestor parallel those reported here for COX-1a and COX-2a in the teleost lineages.

The phylogenetic classification of COX sequences reported here provides a reference for further studies investigating COX evolution and function. Although it is now clear that a genome duplication followed by differential loss in the teleosts explains the COX sequences they possess, the timing and type of losses in different teleost lineages as well as their functional consequences remains unknown. For example, do basal teleosts possess all four COX genes? Have any teleosts lost more than one COX duplicate? What functions are conserved or lost in the new COX duplicates? Furthermore, how do invertebrate lineages without COX produce PGs? The presence of multiple COX forms and virtually unknown function of these genes in lineages of teleosts and other chordates (particularly urochordates and cephalochordates) calls for increased taxon sampling, analyses of site-specific evolutionary rate changes between COX forms, and traditional laboratory studies such as expression and localization experiments (Gaucher et al. 2002; Choe et al. 2006).

Supplementary Material

Supplementary figure S1 is available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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