EVOLUTION OF CYCLOOXYGENASE IN THE CHORDATES

By

JUSTIN CHASE HAVIRD

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
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By

Justin Chase Havird

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Cyclooxygenase (COX) is the enzyme found in animals responsible for converting arachidonic acid into prostaglandins. These prostaglandins can then perform multiple functions including regulation of inflammation responses, changes in vascular tone, and ion transport/osmoregulation. Before this thesis, only two main forms of cyclooxygenase (COX-1 and COX-2) and one ancestral form (*Ciona* COX) were known from the chordates. I sequenced COX genes from several hitherto uninvestigated chordates in order to determine a more comprehensive scenario for the evolutionary history of cyclooxygenase in the chordates.

After designing primers, partial COX sequences were obtained from the Sea Lamprey (*Petromyzon marinus*) and amphioxus (*Branchiostoma lancelolatum*). Complete COX sequences were obtained from the Atlantic Hagfish (*Myxine glutinosa*), euryhaline killifish (*Fundulus heteroclitus*), and longhorn sculpin (*Myoxocephalus octodecemspinosus*). These novel sequences along with other known COX sequences were then subjected to a variety of phylogenetic analyses using standard techniques. The results of these analyses suggest a complex history for the evolution of cyclooxygenase in the chordates.

There appears to have been at least 3 evolutionary independent origins of COX in the chordates. The forms from the sea squirt (*Ciona intestinalis*) (COXa and COXb) represent the

most ancestral forms, the forms from amphioxus represent another independent origin (COXc and COXd), and the vertebrate forms (COX-1 and COX-2) represent yet another origin, with the hagfish and lamprey as likely basal members. Furthermore, the teleosts appear to have duplicate COX-1 (COX-1a and COX-1b) or COX-2 (COX-2a and COX-2b) forms. These results agree with previous studies and reveal novel information about COX forms in the chordates. More intense sampling of other chordates could reveal other novel origins of COX or contribute to a more robust understanding of COX evolution. Finally, the function of most non-mammalian COX forms remains to be investigated.

CHAPTER 1
GENERAL INTRODUCTION

Introduction

Cyclooxygenase (COX) is the enzyme responsible for the rate limiting step that converts arachidonic acid to Prostaglandin G_2 and Prostaglandin H_2. Arachidonic acid is an essential fatty acid required by most mammals and can be obtained by the conversion of linoleic acid found in plants (occurring in herbivores). Some animals cannot convert linoleic acid into arachidonic acid and therefore must obtain it from other animals (obligate carnivores). COX oxidizes arachidonic acid to make the hydroperoxy endoperoxide Prostaglandin G_2, which is then reduced to form the hydroxy endoperoxide Prostaglandin H_2. Prostaglandin H_2 can then be converted to the primary prostanoids (Prostaglandin I_2, Prostaglandin D_2, Prostaglandin E_2, Prostaglandin $F_{2\alpha}$, and Thromboxane A_2) by a variety of enzymatic and non-enzymatic pathways. Therefore, the expression of COX can directly influence the amount of prostaglandins that are synthesized due to the arachidonic acid cascade (Vane *et al*., 1998, Fig. 1-1).

Prostaglandins are found in vertebrates, some invertebrates, and possibly even in plants (Rowley *et al*., 2005; Ryan, 2000). In mammals, they are expressed in virtually all tissues and have a myriad of physiological functions, but they mainly regulate vascular tone in smooth muscle by acting as vasodilators and vasoconstrictors. In addition, prostaglandins are key regulators of such diverse processes as salt and water homeostasis in the mammalian kidney (Harris and Breyer, 2001), protection of stomach lining (Kargman *et al*., 1996), platelet aggregation in blood (Főrster and Parratt, 1996), birth (Challis *et al*., 2002), and central nervous system function (Hopkins, 2007). They also may play prominent roles in pathological states such as fever, inflammation, and cancer (Vane *et al*., 1998).

Prostaglandins were first extracted from semen, prostate (hence the name prostaglandin), and seminal vesicles by Goldblatt and von Euler in the 1930s (von Euler, 1936) and were shown to affect blood pressure. The reaction that catalyzes Prostaglandin G_2 synthesis was first characterized in the 1970s (Hamberg and Samuelsson, 1973; Hamberg *et al*., 1974), but it wasn't until 1988 that cyclooxygenase (COX) was first described as the enzyme responsible for endoperoxide synthesis by three separate groups (DeWitt and Smith, 1988; Merlie *et al*., 1988; Yokoyama *et al*., 1988), all of which described a COX from sheep seminal vesicles which was later named COX-1. However, before COX-1 was isolated there was early evidence that multiple COX forms existed, based on prostaglandin activity in the rat brain and spleen (Flower and Vane, 1972). In the early 1990s, a second COX form was cloned from mouse and chicken fibroblast cell cultures and was named COX-2 (Kujubu *et al*., 1991; Xie *et al*., 1991; O'Banion *et al*., 1992). This discovery would later prove to be of major importance in the field of prostaglandin biology due to the development of drugs that could selectively inhibit the different forms of cyclooxygenase.

Both forms catalyze the reactions that form prostaglandins from arachidonic acid, but early studies suggested that COX-1 was constitutively expressed in many tissues, while COX-2 was an inducible isoform that was expressed in response to physiological stimuli (Funk, 2001). Because basal expression of COX-2 was low in some cell types and COX-2 was found to be expressed much more so than COX-1 in response to mitogens and cytokines, it was assumed that COX-1 was constitutively expressed at basal levels and served a housekeeping function and that COX-2 was inducibly expressed to serve in inflammatory functions (Kujubu *et al*., 1991; Xie *et al*., 1991; O'Banion *et al*., 1992). However, this was later shown to be an oversimplification (Funk, 2001). Notably, it was shown that COX-2 is constitutively expressed in the mammalian

kidney, where it regulates blood flow and ion transport in the Loop of Henle (Harris and Breyer, 2001) and plays a role in cell survival in medullary interstitial cells during dehydration (Yang *et al*., 2002; Yang, 2003).

Despite their potential functional differences in different tissues, COX-1 and COX-2 are biochemically very similar. Both are inhibited by nonsteroid anti-inflammatory drugs (NSAIDs), such as aspirin, due to competition with arachidonic acid for the active sites of the enzymes or irreversible acetylation (Vane, 1971). Both proteins in humans are ~600 amino acids and share about 63% sequence similarity with each other. However, the proteins originate from different genes on different chromosomes and encode different mRNA transcripts. COX-1 comes from a much larger gene (22 kilobases) than COX-2 (8 kilobases). The amino acid residues that form the binding and catalytic sites (the functionally important sites) as well as the adjacent amino acids are all identical in human COX-1 and COX-2, except for two substitutions. At positions 434 and 523, valine is substituted for isoleucine in COX-2 when compared to COX-1. These substitutions have been implicated as the most likely reason behind the biochemical differences between COX-1 and COX-2. COX-1 appears to be more selective in terms of the fatty acids it will accept as substrates than COX-2 (Otto and Smith, 1995). COX-2 selective inhibitors have offered an insight into the biochemistry behind the different forms. These inhibitors can differentiate between COX-1 and COX-2 with over 1000 fold specificity (Griswold and Adams, 1996). The rationale behind these selective inhibitors is that the smaller size of valine on position 523 in COX-2 allows the inhibitor to bind to a side pocket in COX-2, but the larger isoleucine on position 523 of COX-1 appears to prevent this binding. When this position is altered in both forms to the alternate amino acid, the selectivity for inhibitors is reversed (Wong *et al*., 1997; Gierse *et al*., 1996; Guo *et al*., 1996), suggesting that this residue

plays a critical role in differing biochemical activity. COX-2 selective inhibitors were thought to be a major medical breakthrough due to the ability to stop disease induced inflammation without affecting normal prostaglandin levels produced by COX-1 and therefore causing minimal side effects. However, due to the constitutive expression of COX-2 in certain tissues (especially the kidney), COX-2 selective inhibitors may not be as affective as once thought. It is important to remember that COX-1 and COX-2 are very structurally similar and that their main difference is due to expression.

As outlined above, COX form and function have been extensively studied extensively in mammals, especially humans. However, non-mammalian COX forms and their functions have not been examined in great detail in other chordates. The goal of this research was to characterize COX evolution in the chordates, with specific attention on the more evolutionarily ancestral chordates. Describing ancestral COX forms is the first step in elucidating the evolution of COX function, which may lead to a better understanding of overall COX function, and even the development of new drugs. In this chapter, I give a brief overview of COX evolution and function in the chordates, concluding with a brief summary of the research described in the following chapters.

Cyclooxygenase Evolution

Like mammals, most vertebrates appear to have two homologous copies of cyclooxygenase named COX-1 and COX-2. However, other COX forms exist throughout the chordates, including variants of COX-1 and COX-2 as well as forms representing independent origins of COX that are not closely related to COX-1 or COX-2. There are also COX forms in corals and other invertebrates (Jarving *et al*., 2004), but the focus of this section is to review the forms found in the chordates.

Mammals

COX-1 and COX-2 sequences have been either cloned or derived from genomic sequences for most of the classic mammalian model species, including mouse (Accession# NP_032995 and NP_035328), rat (Accession# NP_058739 and NP_058928), dog (Accession# NP_001003023 and NP_001003354), cow (Accession# XP_869575 and NP_776870), sheep (Accession# NP_001009476 and NP_001009432), and rabbit (Accession# NP_001076150 and NP_001075857). COX sequences also exist for rhesus monkey, guinea pig, pig, horse, and mink, but these are either partial sequences or are not available for both forms.

A third COX variant has been proposed to occur in mammals (Flower and Vane, 1972) based on inhibitory studies (see below). This form is known as COX-3 or COX-1b because of its close similarity to COX-1. Recently, a potential COX-3 (Accession# AY547265) was cloned from mouse (Kis *et al*., 2006) and rat (Accession# AY523672, Snipes *et al*., 2005). However, these forms likely do not participate in prostaglandin synthesis (Kis *et al*., 2006) and are not a separate COX form. Furthermore, a similar study using an artificially created human COX-1b found no inhibitory similarity to the proposed COX-3 enzyme (Censarek *et al*., 2006). Therefore, while a third, genuinely different COX variant may exist in mammals, its sequence remains elusive.

Analysis of the recently completed gray short-tailed opossum genome yielded the predicted COX-1 and COX-2 forms (Accession# XP_001370514 and XP_001375945), as well as two additional COX-1 forms, named COX-1b and COX-1b2 (Accession# XP_001370542 and XP_001370595). These forms are complete and differ in amino acid composition and length. These are the first COX-1 variants in a mammal that do not represent modified versions of normal COX-1 and although is has not been suggested, it is possible that one of these forms

represents the elusive COX-3 variant. Functional studies to determine how these forms differ from normal COX have not been published to date.

Fishes

Cyclooxygenase has been sequenced in several fishes, including the rainbow trout (*Oncorhynchus mykiss*) (Zou *et al*., 1999), brook trout (*Salvelinus fontinalis*) (Roberts *et al*., 2000), Atlantic croaker (*Micropogonias undulates*), and zebrafish (*Danio rerio*) (Grosser *et al*., 2002). These fishes, along with genomic sequence data from the stickleback (*Gasterosteus aculeatus*), green spotted puffer (*Tetraodon nigroviridis*), pufferfish (*Takifugu rubripes*), and Japanese medaka (*Oryzias latipes*) suggest that fishes posses both COX-1 and COX-2 forms (Jarving *et al*., 2004). Furthermore, COX-2 has recently been sequenced from European sea bass (*Dicentrarchus labrax*) (Buonocore *et al*., 2005) and euryhaline killifish (*Fundulus heteroclitus*) (Choe *et al*., 2006), supporting this prediction.

A cyclooxygenase has also been sequenced from the spiny dogfish shark (*Squalus acanthias*) (Yang *et al*., 2002). However, this form was not designated as COX-1 or COX-2 because of its near equal identity to both forms (it is only slightly more similar to COX-1). This form was named sCOX by the authors and may represent an evolutionarily distinct form of cyclooxygenase found in the elasmobranchs.

Ishikawa and colleagues have recently further characterized COX evolution in the teleosts by demonstrating that both the zebrafish (Ishikawa *et al*., 2007) and rainbow trout (Ishikawa and Herschman, 2007) have two functional COX-2 genes (named COX-2a and COX-2b). In addition, by analyzing sequence databases, they concluded that not all teleosts have two COX-2 forms, but that the stickleback, green spotted puffer, pufferfish, and Japanese medaka have two COX-1 forms instead (named COX-1a and COX-1b) (Ishikawa *et al*., 2007). They concluded that a genome duplication before the teleosts, and a subsequent loss of either one

COX-1 or COX-2 form characterizes the species mentioned (Ishikawa and Herschman 2007) (Fig. 1-2). This is supported by the apparent genome duplication events in teleosts (Crollius and Wessenbach, 2005).

Other Vertebrates

Although functional studies on prostaglandin roles and cyclooxygenase are not uncommon in other vertebrates, cyclooxygenase sequences from non-mammalian and non-teleost vertebrates are lacking. Cyclooxygenase forms have been predicted from the chicken genome (*Gallus gallus*), suggesting that birds have both COX-1 and COX-2 forms (Urick and Johnson, 2006). Both forms have also been found in the African clawed frog (*Xenopus laevis*) and COX-2 has been found in the western clawed frog (*Xenopus tropicalis*), suggesting that both forms occur in amphibians (Klein *et al*., 2002). Functional studies suggest COX exists in reptiles as well (Seebacher and Franklin, 2003) and the recently completed *Anolis carolinensis* genome contains both COX forms.

Taken together, the presence of COX-1 and COX-2 forms in the mammals, teleosts, birds, and amphibians suggest that both forms originated in the early vertebrates, possibly from the duplication of an ancestral COX gene. The presence of sCOX in the dogfish indicates that COX forms in the early vertebrates may not have diverged (in sequence or possibly in function) to the extent that allows them to be labeled as COX-1 or COX-2, although it is predicted that two COX forms exist in all vertebrates, since sCOX groups with COX-1 and is not ancestral to the COX-1/COX-2 split (Fig. 1-2, unpublished phylogeny generated using the online program PHYML) (Guindon and Gascuel, 2003; Guindon *et al*., 2005).

Other Chordates

Although the subphylum Vertebrata includes the majority of species in the phylum Chordata, two other subphyla are included in the chordates: the Urochordata (sea squirts or

tunicates) and the Cephalochordata (amphioxus or lancelets). These other subphyla are basal to the vertebrates and represent evolutionarily distinct monophyletic groups (Fig. 1-3). There is some debate as to whether the urochordates or cephalochordates represent the base of the chordate phylogeny, although urochordates have been generally been considered basal (Pough *et al*., 2005).

Interestingly, the genomes of sea squirts (*Ciona intestinalis* and *Ciona savignyi*) predict COX genes that are not homologous to COX-1 and COX-2. These forms (named COXa and COXb) are basal to vertebrate COX forms and indicate an evolutionarily distinct branch in COX evolution (Jarving *et al*. 2004) (Fig. 1-4). This separate branch is situated between the COX of corals and the vertebrate COX forms (Fig. 1-4). This suggests that the COX-1 and COX-2 forms originated sometime after the origin of the sea squirts but before the origin of the elasmobranchs (around 550-600 mya).

Cyclooxygenase Function in Mammals

Cyclooxygenase was first described from mammals, and due to its medical importance, its function has been intensely examined in mammals, specifically humans. Since COX stimulates the production of prostaglandins, COX function is intrinsically linked to the roles that prostaglandins play in everything from water balance to reproduction. Here, I review the major functions of COX-1 and COX-2 in various mammalian physiological systems (modeled after Vane *et al*., 1998), but it should be noted that mammalian COX studies are ongoing and that COX likely plays many roles not described here.

Gastrointestinal Tract

In the stomach, prostaglandins play a cytoprotective role, guarding the stomach lining against digestive acids and enzymes. The mechanism behind this protection is an increased mucosal blood flow caused by the vasodilating properties of prostaglandins (specifically

Prostaglandin I_2). It has generally been accepted that these prostaglandins are synthesized by COX-1, although COX-2 is present in small amounts (Kargman *et al*., 1996). This is supported by studies showing that irradiated mice have further reduced numbers of crypt cells (cytoprotective mucosal cells) when indomethacin (a non-specific COX inhibitor) was administered, but not when a COX-2 selective inhibitor was administered (Cohn *et al*., 1997). COX-2 expression in the esophagus of patients with gastroesophageal reflux disease was upregulated in response to acid exposure, suggesting a protective function in the distal esophagus (Lurje *et al*., 2007). This may indicate that throughout the gastrointestinal (GI) track, COX-2 is inducibly expressed in response to inflammation responses.

Furthermore, throughout the GI track, inflammatory mediators such as COX may also serve to repair damaged mucosa (Martin and Wallace, 2006). While it seems that COX-1 is responsible for baseline prostaglandin expression in the GI tract, COX-2 and not COX-1 seems to be more involved in response to GI tract cancers (Ristimäki *et al*., 1997). However, recent studies on patients with gastric cancer from Dalian, China showed that COX-2 expression was infrequent in gastric cancers due to hypermethylation and that a COX-2 focused strategy for treatment would likely be ineffective (Huang *et al*., 2006).

The Kidney

Prostaglandins can modulate blood flow in the kidney, as in other organs (Vane and Botting, 1994). In addition, COX-2 has been shown to play a major role in ion regulation and water balance in the mammalian kidney (Harris *et al*., 1994). It has also been shown that the COX-2 selective inhibitor SC-58236 and several non-selective COX-inhibiting NSAIDs (including sulindac, ibuprofen, and indomethacin) cause medullary interstitial cell (MIC) death in the kidney, but the COX-1 selective inhibitor SC-58560 was 100-fold less potent for inducing MIC death (Hao *et al*., 1999). COX-2 knockout mice also develop terminal nephropathy

(abnormal kidney function) which results in death by the third month (Morham *et al*., 1995). These results suggest that COX-2, and not COX-1, plays a critical role in kidney function *via* MIC cell survival. This is contrary to the original description of COX-1 as the constitutively expressed form responsible for maintaining homeostasis and COX-2 as the inducible form responsible for inflammatory responses.

In the kidney, COX-2 is present in a subset of tubular epithelial cells located in the cortex and outer medulla (Vio *et al*., 1997). These cells are part of the medullary thick ascending limb (MTAL), the part of the nephron that regulates extracellular fluid volume by establishing the osmotic gradient in the medulla that concentrates urine (Hebert *et al*., 1981; Hebert and Andrioli, 1984). *In vitro* MTAL preparations have demonstrated high levels of Prostaglandin E_2 (PGE_2) (Ferreri *et al*., 1984; Hirano *et al*., 1986; Lear *et al*., 1990) which has been shown to inhibit basolateral Na^+/K^+-ATPase (NKA) and apical Na^+/K^+-$2Cl^-$ cotransporter (NKCC) in the MTAL (Kaji *et al*., 1996; Stokes and Kokko, 1976; Wald *et al*., 1990). These proteins are critical for NaCl reabsorption in the MTAL. Furthermore, the COX-2 selective inhibitor NS-398 has been shown to prevent tumor necrosis factor (TNF) mediated PGE_2 production in cultured MTAL cells (Ferreri *et al*., 1999). TNF is a cytokine expressed in the MTAL that may be an important mediator of ion transport by inhibiting ion uptake *via* a prostanoid dependant mechanism (Escalante *et al*., 1994; van-Lanschot *et al*., 1991). Taken as a whole these results show that COX-2 may influence ion uptake *via* TNF mediated prostaglandin production in the MTAL. Therefore, COX-2 is involved in cell survival and ion transport in the mammalian kidney.

This finding is critical for treatment protocols using COX inhibitors. NSAIDs are the most common drugs used to treat pain and inflammation with over 30 million uses per day worldwide (Singh and Triadafilopoulos, 1999). Although the negative side effects

(hypertension, renal ischemia, and GI toxicity) associated with chronic NSAID use have been known for some time, the discovery of COX-2 selective inhibitors suggested a treatment for inflammation without causing disruption of normal COX function (thought to be mediated by COX-1). The role of COX-2 in the kidney has reduced the supposed power of these new drugs and negative side effects associated with COX-2 selective inhibitors are now well documented (see Harris and Breyer, 2006 for a review). Furthermore, in 2005 the Food and Drug Administration (FDA) required additional warning labeling of celecoxib to highlight side effects and required Pfizer to remove valdecoxib from the market, both of which are selective COX-2 inhibitors.

Platelets

Interestingly, COX-1 seems to be the only form expressed in mammalian platelets. COX-2 was not detected in human platelets using western blotting techniques (Reiter *et al*., 2001) or in canine platelets using northern blotting techniques (Kay-Mugford *et al*., 2000). In the platelet, COX-1 is involved in platelet aggregation (clotting) through the production of Thromboxane A_2 (TXA_2). When inhibited with aspirin, COX-1 mediated production of TXA_2 is irreversibly inhibited, causing reduced platelet aggregation (blood thinning). This effect occurs about an hour after oral ingestion of even low doses of aspirin and can inhibit platelet function for several days after a dose (Vane *et al*., 1998; Vane, 1971). Inhibition of COX-1 by resveratrol and other components of red wine is responsible for the anti-platelet activity associated with the cardiovascular benefits of red wine consumption (Szewczuk and Penning, 2004).

Reproduction

Cyclooxygenase and the prostaglandins it produces play critical roles in almost every aspect of reproduction in mammals, although its role in pregnancy has been studied the most

intensely. Both isoforms are likely involved in reproductive processes, with COX-1 expression constitutive and COX-2 expression inducible (Chakraborty *et al*., 1996).

In a recent study, COX-2 was shown to contribute to fertility in male mice (Balaji *et al*., 2007). COX-2 expression was intense in the epithelial cells of mice vas deferens and the COX-2 specific inhibitor nimesulide was shown to decrease sperm motility six hours after administration (Balaji *et al*., 2007). Interestingly, although prostaglandin levels initially dropped after nimesulide administration, they tended to rise after sustained COX-2 inhibition (Balaji *et al*., 2007). This suggests that COX-1 expression may recover COX function or at least contributes heavily to prostaglandin production in the vas deferens. A similar situation exists with respect to human female fertility, in which COX-1 expression can recover function in cases where COX-2 is lost or inhibited (Wang *et al*., 2004). It also seems that COX-2 is critical for follicle development and ovulation in women and that COX inhibitors can have severe effects on female fertility (Sirois *et al*., 2004).

Prostaglandins are also important during pregnancy and labor. Generally, COX-1 is expressed more than COX-2 during normal stages of pregnancy, and COX-1 may play an active role in maintaining a healthy pregnancy (Trautman *et al*., 1996). COX-1 is expressed at high levels is the fetal brain, kidneys, heart, and lungs (Gibb and Sun, 1996). However, both COX-1 and COX-2 are expressed in the uterine epithelium during early pregnancy and may play roles in implantation and placental formation (Chakraborty *et al*., 1996). This was recently confirmed for COX-2 in rats (Diao *et al*., 2007). COX-2 may play a dominant role in inducing labor. During labor, contractions are stimulated by prostaglandins and therefore any effect causing increased COX activity will induce labor. Up-regulation of COX-2 during intrauterine infection may increase prostaglandins to the point of causing premature labor (Spaziani *et al*., 1996).

Furthermore, a similar up-regulation in sheep may reduce progesterone levels to the point that pregnancy cannot be maintained (McLaren *et al*., 1996). However, it was recently shown that during the course of pregnancy there is a gradual change in COX expression, with COX-1 dominating during early pregnancy and COX-2 dominating in later stages (Johnson *et al*., 2006). It was concluded that while COX-2 is expressed at high levels in late stages of pregnancy, it is not up-regulated suddenly to induce labor (Johnson *et al*., 2006). COX inhibitors can halt premature labor by inhibiting prostaglandin production (Sawdy *et al*., 1997). However, as in treatment strategies that use COX inhibitors for other problems, there are negative side effects associated with the kidneys: renal clearance and glomerular filtration were reduced during the first weeks of life in babies that were exposed to COX inhibitors to stop premature birth (Allegaert *et al*., 2006). As in other cases, COX-2 selective inhibitors were thought to eliminate these negative side effects but have not proven more effective than non-specific NSAIDs (Olson 2005).

Nervous System

COX-1 and COX-2 are both expressed in the mammalian nervous system and may participate in nerve transmission, sensory processing, thermoregulation, and pain effects. As in other systems, the use of COX inhibitors to treat neural diseases (such as Alzheimer's) is prevalent, but is accompanied by undesired side effects that are not prevented by using COX-2 selective inhibitors.

The brain is one of the few organs that constitutively expresses COX-2 and contains high amounts of COX-2 in the cortex, hippocampus, hypothalamus, and spinal cord (Breder *et al*., 1995), whereas COX-1 is abundantly expressed in the forebrain, where it may be involved in sensory information processing (Yamagata *et al*., 1993). COX-2 is expressed in both neuronal

and non-neuronal cells in the brain and is up-regulated in response to abnormal nerve activity, suggesting a role in nerve transmission (Breder *et al*., 1995; Breder and Saper, 1996).

COX-2 in the spinal cord has been shown to modulate pain responses (nociception) in both humans and rats due to peripheral pain stimulation (Martin *et al*., 2007; Gardiner *et al*., 1997; Willingale *et al*., 1997). These results are expected since prostaglandins are generated at the ends of sensory neurons during inflammatory pain (Ferreira 1972; Woolf *et al*., 1997). However, the up-regulation of COX-2 over COX-1 in the spinal cord during inflammatory pain suggests a role during pain processing in the central as well as the peripheral nervous system. This up-regulation has been detected *via* increases in COX-2 mRNA (Gardiner *et al*., 1997; Beiche *et al*., 1996) in the spinal cord of rats during inflammatory stimuli. Furthermore, COX-2 selective inhibition also affected the withdrawal reflex of rats when stimulated using a non-inflammatory, electrical stimulus (Willingale *et al*., 1997). This supports the general role of COX-2 in nociception in the spinal cord.

COX-2 also plays a thermoregulatory role in the brain by increasing prostaglandin production in the hypothalamus during periods of fever. Injection of bacterial lippopolysaccarides (LPS) into mammals causes a monophasic fever characterized by a single rise in core body temperature. It has long been known that PGE_2 levels increase in the hypothalamus during fever and likely mediate the response (Ivanov and Romanovsky, 2004). Early studies suggested that an up-regulation of COX-2 was the cause of increased PGE_2 levels during fever (Cao *et al*., 1997). This initial hypothesis has been confirmed, recently in COX-2 deficient mice which do not respond to LPS injection, in contrast to COX-1 deficient mice or control mice (Steiner *et al*., 2005).

Cyclooxygenase Function in Non-Mammals

Although COX has been extensively studied in mammals due to its role in inflammation and the correlating drug market surrounding COX inhibition, studies in non-mammals are lacking. Unfortunately, it seems that although non-mammals posses the mammalian COX-1 and COX-2 forms, these forms may be substantially different (both in structure and function) from their mammalian counterparts (Grosser *et al*., 2002) and may also represent independent evolutionary events, such as duplications of an isoform (teleosts) or independent origins (sea squirts). In this section, I review the major studies investigating COX function in non-mammalian chordates.

Fishes

Cyclooxygenase has likely been studied in the fishes more than other non-mammals due to their status as model organisms. As noted above, COX forms have been found in several fishes and along with genomic sequence data there is a general consensus that fishes possess both COX-1 and COX-2 forms that are homologous to those found in mammals. However, there is recent evidence that teleosts also possess an additional form of COX-1 or COX-2 (Ishikawa *et al*., 2007; Ishikawa and Herschman, 2007) and some functional data also exist for these forms.

Osmoregulation

In fishes, the gills are the dominant site of acid/base regulation, nitrogenous waste secretion, gas exchange, and ion transport (Evans *et al*., 2005). Several studies have investigated the osmoregulatory role of COX in the gills of the euryhaline killifish, *Fundulus heteroclitus*. This teleost can withstand instant salinity transfer between full strength seawater and fresh water without any apparent physiological stress (Wood and Laurent, 2003). One study showed that short circuit current across the opercular epithelium (a tissue with known ion transport capabilities) was reduced by using a non-specific COX inhibitor (Evans *et al*., 2004). This

suggests that COX-2 plays a role in ion transport in fishes that may be similar to its function in the kidneys of mammals. Further studies supported this initial hypothesis by showing that COX-2 is expressed most abundantly in the gill, opercular epithelium, and kidney of the killifish (Choe *et al*., 2006), tissues that are known to be involved in ion transport in fishes. This is supported in the zebrafish, which shows the highest levels of COX-2 in the gills (Grosser *et al*., 2002). Furthermore, COX-2 was localized in mitochondrion-rich cells in the gills of killifish, the main sites of ion uptake and secretion in the gills (Choe *et al*., 2006). Finally, COX-2 expression was shown to significantly increase following either hypotonic or hypertonic salinity transfers, suggesting COX-2 may play a role in maintaining cell homeostasis or promoting cell survival during periods of osmotic shock (Choe *et al*., 2006). Taken together, these results strongly suggest that COX-2 in the gills of teleosts acts in the same way as COX-2 in the kidneys of mammals, including regulating ion transport and promoting cell survival.

Cyclooxygenase has also been shown to play an osmoregulatory role in the rectal gland of the dogfish shark, *Squalus acanthias*. Sharks rely on the rectal gland rather than the gills for regulation of salt secretion and a COX form (sCOX) has been cloned from the rectal gland (Yang *et al*., 2002). This form was expressed most abundantly in the rectal gland of the shark, where PGE_2 production was also high (Yang *et al*., 2002). Finally, using a COX-2 specific inhibitor, vasoactive intestinal peptide mediated chloride secretion decreased in the rectal gland, but then recovered following removal of the inhibitor (Yang *et al*., 2002). Even though sCOX is slightly more similar to COX-1 of mammals than COX-2, this result can be explained by the presence of valine at position 523 instead of isoleucine (and thus conferring COX-2 inhibitory properties). This result suggests that sCOX plays a role in ion transport in the rectal glands of sharks that may be similar to the gills of teleosts or the kidneys of mammals. The osmoregulatory role of

COX in fishes needs to be examined in greater detail using other species and diverse techniques. This may be highly feasible because COX sequences exist for a wide range of teleosts.

Reproduction

As in mammals, COX has been shown to play a role in reproduction in fishes. By using the non-specific COX inhibitor indomethacin, it was shown in the Atlantic croaker (*Micropogonias undulatus*) that COX pathways may play a role in the maturation of ovarian follicles and ovulation through prostaglandin formation, although other proteins may play a more dominant role (Patiño *et al*., 2003). Results from the European sea bass also indicate a similar role for prostaglandins in ovulation, with indomethacin inhibiting follicle maturation (Sorbera *et al*., 2001). However, it has been shown in the brook trout that indomethacin does not block ovulation (Goetz *et al*., 1989), although it does in other fish species (Goetz *et al*., 1991). This apparent loss of function in the brook trout may be explained by changes in the levels of COX-1 and COX-2 during ovulation. It was shown that COX-1 levels remained constant and high during ovulation but that COX-2 levels did not increase prior to ovulation as they do in mammals (Roberts *et al*., 2000). This is supported by data from the zebrafish which show high levels of COX-1 but not COX-2 in the ovaries (Grosser *et al*., 2002). This suggests that COX-2 function in the reproduction of the brook trout (and perhaps other fishes) is different than in other vertebrates. In the Japanese medaka, it was shown that low chronic levels (perhaps comparable to those found in waste water) (Metcalfe *et al*., 2003) of the non-specific COX inhibitor ibuprofen causes altered reproduction by decreasing the number of spawning events but increasing the number of eggs per spawning event (Flippin *et al*., 2007). This conforms to delayed pregnancies associated with NSAID use in mammals. These results indicate that COX likely plays a role in fish reproduction, although it may vary among species.

Differential functions of novel forms

Recently, the discovery of novel COX-2 forms in the zebrafish (Ishikawa *et al*., 2007) and rainbow trout (Ishikawa and Herschman, 2007) and analyses of genomic sequence data indicates that all teleosts may posses at least three COX forms: either COX-1a, COX-1b, and COX-2 or COX-1, COX-2a, and COX-2b. Preliminary data on the described COX-2 forms suggests that they are functionally different from each other. In the zebrafish, COX-2a and COX-2b are expressed at different levels in the gill, kidney, and other tissues and respond differently to 12-O-tetradecanoylphorbol-13-acetate (TPA, which is known to induce COX-2 expression in mouse) (Ishikawa *et al*., 2007). A similar result was found in the rainbow trout, with differential expression of the two forms due to TPA (Ishikawa and Herschman, 2007). Interestingly, COX-2a and not COX-2b was up-regulated in response to lippopolysaccarides (Ishikawa and Herschman, 2007). Clearly, the discovery of these new forms indicates a new chapter in studying COX function and further studies are needed to determine the functional differences among the teleosts' forms of COX.

Other functions

Cyclooxygenase has also been implicated in determining body plan development during embryonic stages in the zebrafish (Grosser *et al*., 2002; Cha *et al*., 2005; Cha *et al.,* 2006a; Cha *et al*., 2006b; Yeh and Wang, 2006). Briefly, COX may function to signal cell motility during gastrulation (Cha *et al*., 2006b), and COX-1 function has been shown to participate in vascular tube formation during development (Cha *et al*., 2005). COX-2 expression has also been shown to increase in response to lippopolysaccarides in the rainbow trout (Brubacher *et al*., 2000; Holland *et al*., 2002), suggesting an immunoregulatory role for COX-2 in fish as in mammals (*e.g*., fever induction). Clearly, the function of COX in fishes is just beginning to be explored,

and further studies will likely indicate more similarities and differences with COX functions in mammals.

Amphibians

As in other vertebrates, there is evidence that COX is involved in ovulation and reproduction in amphibians. Indomethacin was shown to inhibit oocyte ovulation in the frog (*Rana temporaria*), while Prostaglandin $F_{2\alpha}$ was shown to promote ovulation (Skoblina *et al*., 1997). This suggests another mediator may be influencing $PGF_{2\alpha}$ production in the frog, but this contrast between seemingly related proteins has not been confirmed in other species. COX may also function in testosterone synthesis via PGE_2 in the crested newt, *Triturus carnifex* (Gobbetti and Zerani, 2002). Ion and water transport have also been studied and non-specific COX inhibitors reduce short circuit current and voltage potential across the frog skin, bladder, and corneal epithelium (Shakhmatova *et al*., 1997; Carrasquer and Li, 2002). As in reptiles, fever and thermoregulatory changes are behaviorally induced in amphibians. Injection of lippopolysaccarides (LPS) normally causes a behaviorally induced fever in toads. However, when indomethacin is administered, the fever induced by LPS is completely blocked in the toad (*Bufo paracnemis*) (Bicego *et al*., 2002). This indicates that the COX pathway plays a role in the behaviorally induced fever caused by LPS and that this response has an ancient origin that may be conserved in all tetrapods.

Reptiles

Cyclooxygenase has not been studied extensively in the lizards, crocodilians, snakes, and turtles although some data suggest prominent roles in thermoregulation. Reptiles undergo behavioral thermoregulation to adjust their body temperatures, and this process is amplified by changes in heart rate. Specifically, an increase in heart rate (heart-rate hysteresis) accompanies an increase in temperature. Prostaglandins and cyclooxygenase were shown to play a role in this

process in the lizard *Pogona vitticeps*. Inhibition of COX-1 and COX-2 resulted in no increased heart rate during heating (Seebacher and Franklin, 2003). Furthermore, administration of Prostaglandin $F_{2\alpha}$ and Prostaglandin I_2, but not Thromboxane B_2, caused an increased heart rate (Seebacher and Franklin, 2003). However, this effect was not seen in crocodiles (*Crocodylus porosus*) (Seebacher and Franklin, 2004) or the lizard *Phrynocephalus przewalskii* (Liu *et al*., 2006), suggesting that thermoregulatory functions of COX *via* changes in heart rates may vary between closely related species.

Interestingly, COX was also implicated in tail regeneration in the house lizard. Twenty days after tail removal (the time period associated with tissue differentiation), an increase in cyclooxygenase activity occurred along with the appearance of endogenous Prostaglandin E_2, which may signal a cascade resulting in tissue differentiation (Jayadeep *et al*., 1995). Finally, indomethacin was used in the turtle bladder to show that prostaglandins likely do not play a role in the inhibition of Na^+ or H^+ transport due to high intracellular calcium (Arruda, 1982). COX function in reptiles obviously needs further study as many functions associated with COX in mammals have yet to be investigated in reptiles and there have been no COX targeted sequencing efforts.

Birds

Cyclooxygenase and prostaglandin function have been studied somewhat in model avian species, specifically chickens, where there is support that COX is involved in similar processes as in mammals, (*e.g.*, reproduction). COX-2 (but not COX-1) and prostaglandin activity was shown to increase with administration of transforming growth factor in granulosa cells of white leghorn hen follicles, suggesting that COX-2 and not COX-1 plays a role in granulosa cell proliferation during follicular development (Li *et al*., 1996). It was also shown that indomethacin reduced the proliferation of granulosa cells (Jin *et al*., 2006). When using COX-1

and COX-2 specific inhibitors, it was found that both types reduced granulosa cell proliferation, but that COX-2 specific inhibitor showed a stronger effect, supporting original studies that COX-2 is the dominant form in granulosa cell proliferation (Jin *et al*., 2007). However, COX-1 was found to be expressed highly in the brain and seminal vesicle of the chicken (Reed *et al*., 1996). COX-1 (but not COX-2) is also up-regulated in ovarian cancers occurring in hens, suggesting a target for treatment (Urick and Johnson, 2006). COX inhibitors, both specific and non-specific, were shown to decrease sperm motility in the domestic turkey, demonstrating a further role for COX in bird reproduction (Kennedy *et al*., 2003).

As in mammals, COX-2 has been implicated with the detection and persistence of peripheral inflammatory pain in chickens. The number of COX-2 containing neurons increased significantly in laminae under inflammatory conditions, but not under control conditions, 12-24 hours after injection of Freund's adjunvant (Yamada *et al*., 2006). This suggests that the numbers of COX-2 containing neurons are related to inflammatory pain as in mammals. It appears that COX may not play a thermoregulatory role in birds as it does in mammals because indomethacin did not cause any change in rectal temperature during heat stress in chickens (Furlan *et al*., 1998). However, COX and its prostaglandins may play a role in memory retention in birds, because inhibitors produce amnesic effects (Hölscher, 1995). Finally, COX-2 is expressed in the kidneys during development in chickens (Mathonnet *et al*., 2001) and at high levels in the kidneys of adults (Reed *et al*., 1996).

Study Overview

As outlined above, the family of cyclooxygenase genes has an interesting and functionally important history in the vertebrates. Drugs derived from cyclooxygenase function are among the most important and widely used treatment methods in medicine. However, COX function and evolution is not fully understood in the ancestral chordates, specifically the teleosts,

sharks, hagfish, lampreys, cephalochordates, and urochordates. Indeed, the origin of the mammalian forms of COX is unclear since the sea squirt possesses evolutionarily distinct COX forms. Resolving the evolution of COX in the chordates could prove invaluable in determining new targets for drug development (Searls, 2003).

In Chapter 2, I describe the cloning and characterization of 9 new COX genes in the chordates. These include COX-1 and COX-2 forms from teleosts as well as novel forms from the hagfish, lamprey, and amphioxus. In phylogenetic analyses, these forms indicate that several origins of COX occurred during chordate evolution, with the mammalian COX-1 and COX-2 forms likely originating with the hagfish (craniates). Data from the teleosts supports the findings of Ishikawa and colleges that teleosts in the Acanthopterygii posses two COX-1 forms and one COX-2 form, whereas earlier teleosts such as the zebrafish and rainbow trout posses two COX-2 forms and one COX-1 form. Furthermore, analyses of protein alignments from novel sequences indicate conserved and derived functional residues and areas between novel COX forms and the COX-1 and COX-2 forms of mammals.

In Chapter 3, I summarize the findings of this research and suggest future avenues of research based on the results presented here. I also present preliminary data investigating the possible function of cyclooxygenase in these ancestral chordates.

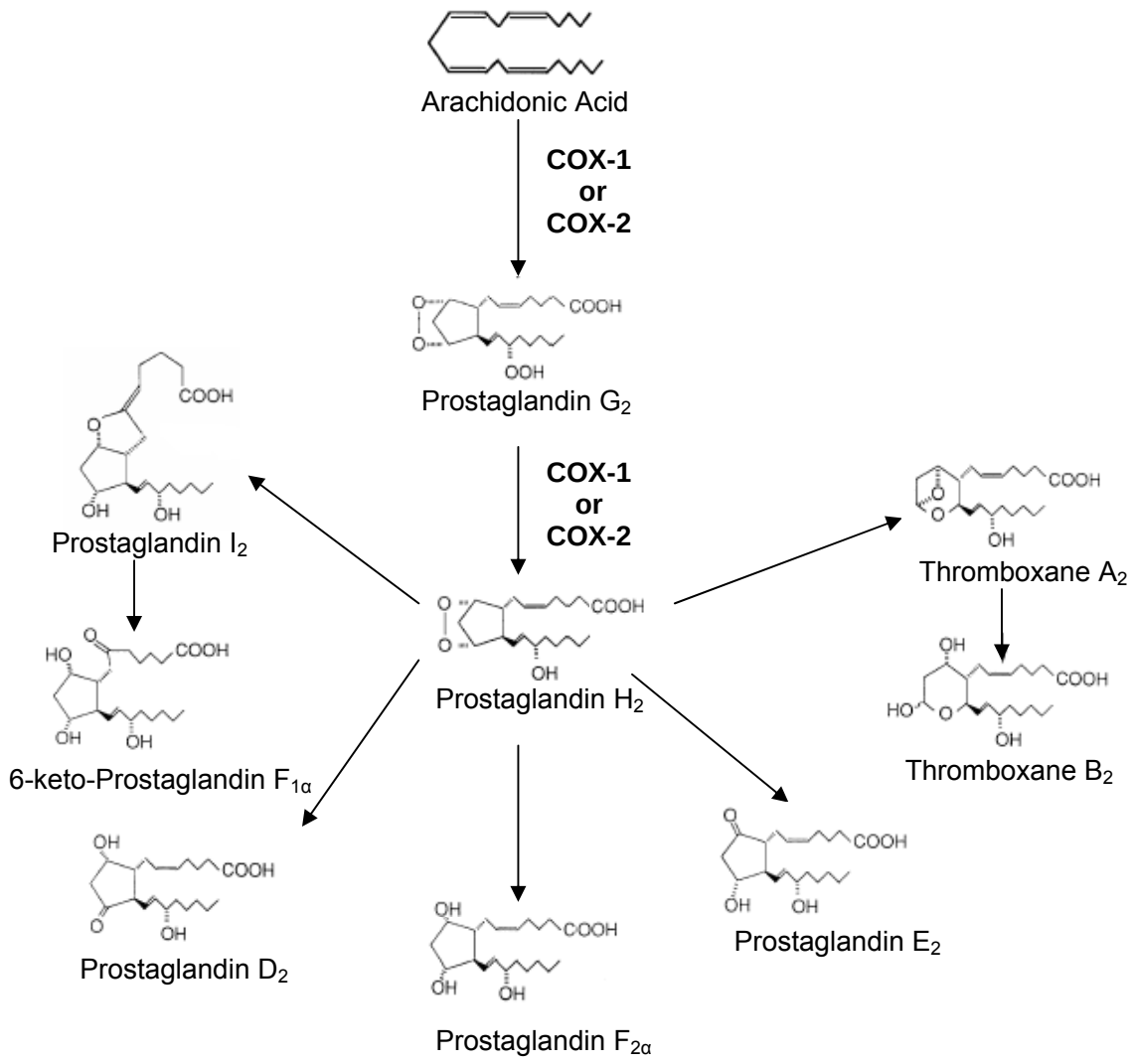

Figure 1-1. Cascade of reactions depicting the conversion of arachidonic acid to the primary prostaglandins *via* catalyzation with either cyclooxygenase (COX) form. Arachidonic acid is obtained from the conversion of linoletic acid found in plants or directly from other animals. It is then oxidized by COX to produce Prostaglandin G_2 which is subsequently reduced by COX to form Prostaglandin H_2. Prostaglandin H_2 can then be converted into the other prostaglandins via other enzymes (Vane et al., 1998).

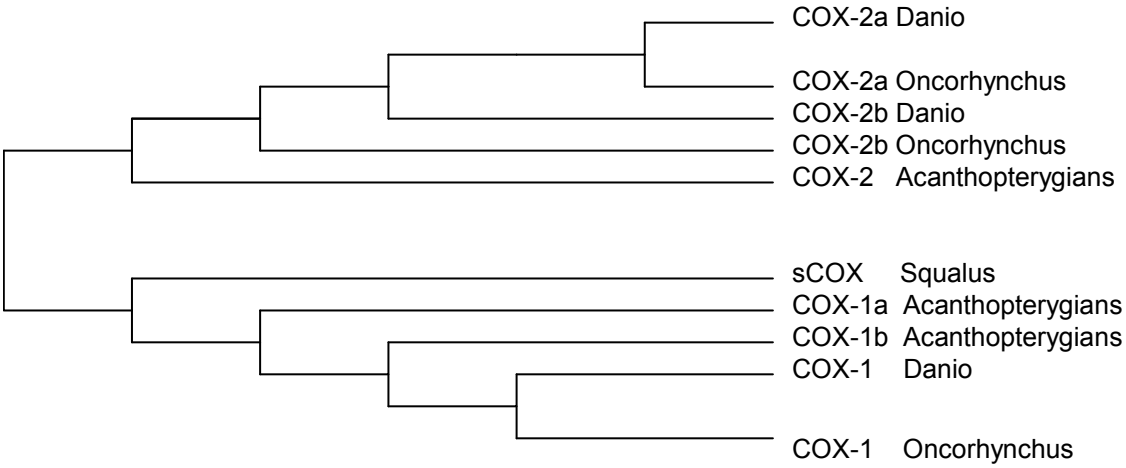

Figure 1-2 General phylogeny representing the different COX forms in the fishes. Based on recent studies, it appears that teleosts have multiple forms of COX-1 or COX-2 (Ishikawa *et al*., 2007; Ishikawa and Herschman, 2007). It is hypothesized that with the origin of the teleosts, COX-1 and COX-2 underwent duplication, but that in one lineage (represented by the zebrafish and rainbow trout) one COX-1 form was lost and in another lineage (represented by the Acanthopterygians) one COX-2 form was lost. Also included is the form cloned from the spiny dogfish (Yang *et al*., 2002) which loosely groups with COX-1 forms, although it shares near equal identity with COX-1 and COX-2. This phylogeny was simplified from an unpublished phylogeny generated using the online program PHYML (Guindon and Gascuel, 2003; Guindon *et al*., 2005). Abbreviations: *Danio* = *Danio rerio* (zebrafish), *Oncorhynchus* = *Oncorhynchus mykiss* (rainbow trout), *Squalus* = *Squalus acanthias* (spiny dogfish). The Acanthopterygians clade is represented by the stickleback (*Gasterosteus aculeatus*), green spotted puffer (*Tetraodon nigroviridis*), pufferfish (*Takifugu rubripes*), and Japanese medaka (*Oryzias latipes*).

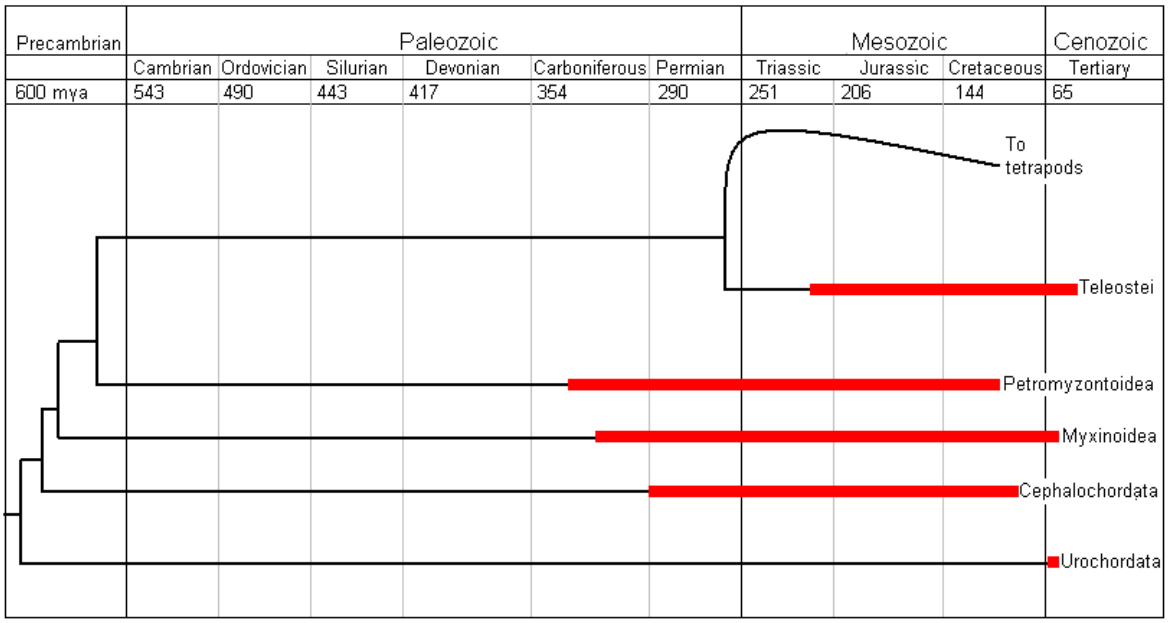

Figure 1-3 Accepted evolution of ancestral chordates (Pough, *et. al*, 2005). Black lines show relationships only; they do not indicate times of divergence nor the unrecorded presence of taxa in the fossil record. Bars shaded red indicate ranges of time when the taxon is known to be present. The subphylum Vertebrata includes the Myxinoidea (hagfishes), Petromyzontoidea (lampreys), Teleostei (teleosts), and tetrapods. There is some controversy over whether the Urochordates (sea squirts) or the Cephalochordates (lancelets) represent the most basal chordate lineage. Also, it has historically been suggested that the hagfish and lamprey constitute a monophyletic group (the Agnathans or Cyclostomes).

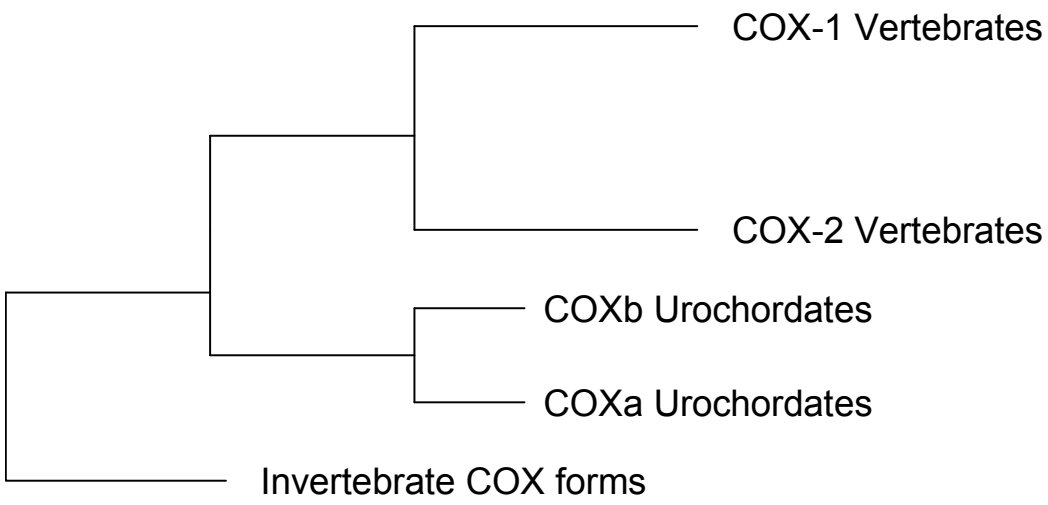

Figure 1-4 Evolution of COX forms in the chordates, based on Jarving *et al*. (2004). The Urochordates (represented by *Ciona intestinalis* and *Ciona savignyi*) have COX forms that represent an evolutionary distinct branch ancestral to the COX-1 and COX-2 forms of the vertebrates. In this tree, the vertebrates are represented by the teleosts, mammals, *Xenopus*, and *Gallus*

CHAPTER 2
CYCLOOXYGENASES IN THE ANCESTRAL CHORDATES: SEQUENCE AND PHYLOGENETIC ANALYSES

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, which act as vascular tone regulators in the vertebrates. Prostaglandins and COX participate in a variety of physiological functions in the vertebrates, including inducing fever, maintaining pregnancy, and regulating ion transport in the kidneys (Steiner *et al*., 2005; McLaren *et al*., 1996; Harris *et al*., 1994). These functions have been extensively studied in mammals (Vane *et al*., 1998), but comparatively little functional data exist for other animals. However, COX has been sequenced in several evolutionarily more ancestral chordates, 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 (Choe *et al*., 2006; Sorbera *et al*., 2001; Brubacher *et al*., 2000; Holland *et al*., 2003) while others may be altered in some species (Goetz *et al*., 1989) or novel (Cha *et al*., 2006b).

In mammals there are two main forms of cyclooxygenase. The first form 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 form 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 up-regulated in inflammatory responses (Funk, 2001). However, studies have shown that this is an oversimplification and COX-2 is expressed constitutively in the brain (Breder *et al*., 1995)

and kidneys (Harris and Breyer, 2001) of mammals. This 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 non-selective COX inhibition. Although structurally and biochemically similar, COX-1 and COX-2 vary in expression in the vertebrates and participate in different functions. For example, COX-2 but not COX-1 plays a role in granulosa cell proliferation in chicken follicles (Li *et al*., 1996; Jin *et al*., 2007). Although the amino acid 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). Inhibitory studies in mammals have suggested a COX-3 form also exists, but it has not been characterized (Kis *et al*., 2006; Censarek *et al*., 2006).

Analyses of genomic sequence and targeted cloning efforts have demonstrated that, like mammals, other vertebrates have COX-1 and COX-2 forms (Jarving *et al*., 2004). However, some variation exists in the COX-1 and COX-2 dichotomy, notably in the more evolutionarily ancestral chordates. Ishikawa and colleagues (Ishikawa *et al*., 2007; Ishikawa and Herschman, 2007) have recently shown that teleosts posses three forms of COX. The zebrafish (*Danio rerio*) and the rainbow trout (*Onchorhynchus mykiss*) both posses two COX-2 forms (named COX-2a and COX-2b) and one COX-1 form, whereas the stickleback (*Gasterosteus aculeatus*), green spotted puffer (*Tetraodon nigroviridis*), pufferfish (*Takifugu rubripes*), and Japanese medaka (*Oryzias latipes*) posses two COX-1 forms (named COX-1a and COX-1b) and one COX-2 form. They also showed that COX-2a and COX-2b are functionally different in the zebrafish and rainbow trout. Also, a COX form (named sCOX) has been cloned from the spiny dogfish (*Squalus acanthias*) and groups with COX-1 in phylogenetic analyses, but shares strong

sequence identity to COX-2 as well (Yang *et al*., 2002). Furthermore, sea squirts (subphylum Urochordata) possess two forms of COX (named COXa and COXb) that do not correspond to the COX-1 or COX-2 of vertebrates and represent an ancestral origin of COX in the chordates (Jarving *et al*., 2004).

The current view of the evolution of cyclooxygenase in the chordates 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 pinpoint the origin of COX-1 and COX-2 in the chordates by searching for COX forms in the sea lamprey (*Petromyzon marinus*), Atlantic hagfish (*Myxine glutinosa*), and amphioxus (*Branchiostoma lancelolatum*). A second goal was to confirm the findings of Ishikawa and colleagues by searching for the 3 forms of COX in the euryhaline killifish (*Fundulus heteroclitus*) and longhorn sculpin (*Myoxocephalus octodecemspinosus*). It was hypothesized that both teleosts would posses COX-1a, COX-1b, and COX-2 since both are in the Acanthopterygii clade of Teleostei and all other acanthopterygiians posses COX-1a, COX-1b, and COX-2. Here, I report the cloning of 9 novel COX sequences from the species mentioned above. Sequence and phylogenetic analyses suggest that ancestral COX forms may have had similar functions as COX-1 and COX-2 in mammals, which likely originated with the origin of the vertebrates, although the hagfish and lamprey may have COX-1 and COX-2 forms that represent novel lineages.

Materials and Methods

Animals and Holding Conditions

All procedures were approved prior to beginning the experiment by the University of Florida Institutional Animal Care and Use Committee (IACUC). Euryhaline killifish (*Fundulus heteroclitus*) were captured from Northeast Creek near the Mount Desert Island Biological Laboratory (MDIBL), Salisbury Cove, Maine using minnow traps. They were transported to the

MDIBL where they were kept in fiberglass tanks containing 100%, flowing seawater from the Gulf of Maine. The tanks were exposed to natural conditions. Longhorn sculpin (*Myoxocephalus octodecemspinosus*) were purchased from fishermen and were housed in a similar way at the MDIBL. Atlantic hagfish (*Myxine glutinosa*) were also purchased from fishermen and were housed at the MDIBL in 100% seawater and maintained on a 12 h light/dark cycle. Female, non-migratory lampreys (*Petromyzon marinus*) were a generous gift from the USGS Great Lakes Science Center at the Hammond Bay Biological Station in Millersburg, Michigan and were dissected there. Lancelets (*Branchiostoma lancelolatum*) were purchased from Gulf Marine Specimens (Panacea, FL) and were kept in the bags they were shipped in for a few days before they were sacrificed. All housed animals were fed to satiation regularly.

Some killifish were shipped to the University of Florida (Gainesville, FL) for further experiments. These killifish were housed as described previously (Choe *et al*., 2006). Some hagfish were also transported to the University of Florida (Gainesville, FL) for further experiments. These hagfish were housed for a few days in a large Rubbermaid tank in 100% seawater at 4 °C before they were killed.

Tissue Collection

After initial anaesthetization with MS-222 (~600 mg L^{-1}), killifish, sculpin, and lampreys were pithed and/or decapitated. The gill arches (1st and 2nd arches for lampreys, 2nd and 3rd arches for teleosts) were then removed using sterile, RNAse free dissecting tools. Hagfish were decapitated and all gill baskets were then removed using sterile, RNAse free 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 for 2 weeks to 4 months before further processing.

Reverse Transcription, Primer Design, PCR, Cloning, and Sequencing

Reverse transcription, PCR, cloning, and sequencing were performed as described previously (Choe *et al*., 2006) with 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 (those labeled as CH COX, Table 2-1) were first designed to amplify non-specific cyclooxygenases in the hagfish, lamprey, and lancelet based on conserved amino acid sequences between COX-1 and COX-2 of *Mus musculus*, COX-2 of *Fundulus heteroclitus*, sCOX of *Squalus acanthias* (GenBank accession numbers: NP_032995, NP_035328, and AAS21313), and predicted COXa and COXb of *Ciona intestinalis* based on genomic data. These primers were also later used to amplify COX-2 in sculpin. Another set of degenerate primers (those labeled as CH COX-1 in Table 2-1) were designed to amplify COX-1 forms specifically over COX-2 forms in teleosts based on conserved amino acid sequences between COX-1 in *Oncorhynchus mykiss*, *Danio rerio*, *Salvelinus fontanalis* (GenBank accession numbers: CAC10360, NP_705942, and AAF14529), and a predicted COX-1 of *Tetraodon nigroviridis* based on genomic data. These primers first amplified a COX-1a form in sculpin and a COX-1b form in killifish (see below). A further set of degenerate primers (those labeled as CH COX-1a in Table 2-1) were designed to amplify COX-1a forms specifically over COX-1b in teleosts based on conserved amino acid sequences between predicted COX-1a forms in *Gasterosteus aculeatus*, *Oryzias latipes*, and *Tetraodon nigroviridis* based on genomic data and a COX-1a found in sculpin earlier in this study (see below). A final set of degenerate primers (those labeled as CH COX-1b in Table 2-1) were designed to amplify COX-1b forms specifically

over COX-1b in teleosts based on conserved amino acid sequences between predicted COX-1b forms in *Gasterosteus aculeatus*, *Oryzias latipes*, and *Tetraodon nigroviridis* based on genomic data and a COX-1b found in killifish earlier in this study (see below). All degenerate primers were designed using the online program Consensus-Degenerate Hybrid Oligonucleotide Primers (CODEHOP) (Rose *et al*., 1998).

Initial PCR reactions were performed on 1/20th of a reverse transcriptase reaction with a TaKaRa Ex Taq™ Hot Start DNA Polymerase Kit (Takara Bio Inc., Japan) in a PCR Express thermocycler (ThermoHybaid, Franklin, MA) with the following parameters: initial denaturing at 95 °C for 5 minutes, then 35 cycles of 30 seconds at 95 °C, 30 seconds at 60 °C, and 1.5 minutes at 72 °C. A final elongation step of 7 minutes at 72 °C was performed for each PCR and then the reaction was held at 4 °C. Initial PCR products were visualized by ethidium bromide staining on 1-2% agarose gels and then ligated into PCR®4-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, Germany) following the manufacturer's protocol and plasmid DNA was sequenced in both directions at the Marine DNA Sequencing Center at the MDIBL.

After initial fragments were sequenced, Primer Express software (Applied Biosystems, Foster City, CA) was used to design specific primers based on the initial fragments (Tables 2-2, 2-3, 2-4, 2-5, and 2-6). These specific primers, along with the original degenerate primers, were used to extend COX sequences. PCR was completed as above with slight modifications to annealing temperature and elongation time for each reaction, as well as nested PCR reactions in some cases to increase specificity. Products were visualized, cloned, and sequenced as above.

5' and 3' Rapid Amplification of cDNA Ends (RACE)

After initial sequences were extended with specific primers, the 5' and 3' ends were sequenced using 5' and 3' RACE. Following the manufacturer's protocols, a Generacer™ Kit (Invitrogen) was used to make 5' and 3' cDNA for hagfish, killifish, and sculpin. Initial sequence data was used to make 5' and 3' RACE specific primers for each species (those labeled with 5' or 3' in Tables 2-2, 2-3, 2-4, 2-5, and 2-6). These primers, along with kit primers that anneal to the ends of the 5' and 3' cDNA, were used in touchdown PCR reactions with the following parameters: 5 cycles of 30 seconds at 94 °C and 1.5 minutes at 72 °C; 5 cycles of 30 seconds at 94 °C and 1.5 minutes at 70 °C; and 25 cycles of 30 seconds at 94 °C, 30 seconds at 60 °C, and 1.5 minutes at 72 °C. As above, a final elongation step of 7 minutes at 72 °C terminated the reaction, which was held at 4 °C. For some reactions, annealing temperatures and elongation times were slightly altered and nested PCR reactions were performed for some reactions using specific primers (Tables 2-2, 2-3, 2-4, 2-5, and 2-6) to increase specificity. Products were visualized, cloned, and sequenced as above.

Sequence Analysis

Initial sequences from degenerate primers, sequence extensions, and 5' and 3' RACE were assembled using GeneTools software (BioTools Inc., Edmonton, Alberta) and the resulting sequences were searched for open reading frames. The predicted amino acids were aligned with other COX proteins using PepTools software (BioTools Inc.) for phylogenetic analysis. Multiple alignments were also generated using ClustalW to search for conserved protein domains across COX sequences. Alignments were manually adjusted and the N and C-terminal areas were realigned using Microsoft Word based on previous COX alignments (Ishikawa *et al*., 2007; Yang *et al*., 2002; Kulmacz *et al*., 2003). For the novel sequences, COX forms were aligned with

other relevant COX proteins and annotated using functional amino acids and domains highlighted from Ishikawa *et al*. (2007), Yang *et al*. (2002), and Kulmacz *et al*. (2003).

Phylogenetic Analysis

To investigate the evolutionary relationships of the different forms of COX in the chordates, phylogenies were generated using the novel amino acid sequences generated in this study and several other COX protein sequences from GenBank and genome databases. All novel protein sequences were included in the data set. COX sequences from teleosts were included in the analysis only if all 3 forms of COX were available. These included the zebrafish, rainbow trout, green spotted puffer, stickleback, euryhaline killifish, and longhorn sculpin. COX sequences from non-teleosts were included in the analysis if at least two forms of COX were available and both sequences were designated as Reference Sequences (RefSeqs) in GenBank. These included the chicken, frog, mouse, rat, human, rabbit, dog, cow, sheep, and opossum. COX sequences were also included if they represented an evolutionarily interesting group. These included the dogfish shark and the sea squirt. In total, 50 protein sequences were included in the analyses (Table 2-7).

Models of evolution to be used in phylogenetic analyses were evaluated to account for amino acid substitutions, among-site variation, and invariable sites. Using likelihood ratio tests, a model using the WAG rate matrix (Whelan and Goldman, 2001) and the gamma (Γ) distribution for among-site variation was chosen (Table 2-8).

A distance phylogeny was first generated using Molecular Evolutionary Genetics Analysis 4 (MEGA) software (Tamura *et al*., 2007) with a minimum evolution (ME) analysis and 1000 bootstrap replicates. In this analysis the α parameter was fixed to the value estimated from maximum likelihood (ML) analysis ($\alpha = 0.737$, see below). Additionally, a JTT rate matrix (Jones *et al*., 1992) was used instead of a WAG matrix because a WAG matrix is not available

for MEGA 4. A complete deletion option was also selected. After comparing results between ML, ME, and Bayesian Phylogenetics (BP), lamprey and hagfish sequences were identified as possible rouges and another distance analysis with identical parameters (but with $\alpha = 0.742$) was performed excluding these two sequences.

A maximum likelihood phylogeny (ML) was generated using the program PhyML (Guindon and Gascuel, 2003). An initial phylogeny was generated using WAG analysis, 1000 bootstrap replicates, 8 substitution rate categories, and a gamma shape parameter with an estimated α. Again, the analysis was repeated with the rouge lamprey and hagfish sequences removed. To evaluate support for monophyletic groups, an approximate likelihood ratio test (aLRT, Anisimova and Gascuel, 2006) was performed both including and excluding the lamprey and hagfish sequences. This test used the minimum of Chi-Squared and Shimodaira-Hasegawa (SH) support values.

Finally, A Bayesian phylogenetics (BP) analysis was also performed using an initial run with 2 million generations and a final run with 10 million generations using the program MrBayes 3.1.2. Once again, analyses were performed with and without hagfish and lamprey sequences. Each run consisted of one cold and three heated chains ($T = 0.2$). The WAG + Γ model was used with an estimated α. After discarding the first 10% of each run as burnin, posterior probabilities were calculated based on trees sampled every 500 generations.

Due to inconsistencies in tree topologies between the three methods used above, alternative topologies were generated in which the positions of the hagfish and lamprey sequences were forced to be included in different clades (Figure 2-15). These alternative topologies were generated and viewed using TreeView 1.6.6. Alternative topologies were

evaluated using Ln *L* scores generated by maximum likelihood criterion with online execution in PhyML (Guindon *et al*., 2005).

Results

Molecular Identification of Cyclooxygenases

Branchiostoma

Initial PCR reactions using the degenerate primers CH COX F3 and CH COX R2 (Table 2-1) amplified two 532 bp products from the lancelet (*Branchiostoma lancelolatum*). These two products (referred to hereafter as COXc and COXd) were found to share the most identity with COXa and COXb from the tunicate. COXc was 50.9% identical to COXa from the tunicate and COXd was 58.8% identical to COXb from the tunicate. All attempts to extend these sequences using specific primers designed against these sequences (Table 2-2) were unsuccessful. After searching for open reading frames, two putative 177 amino acid proteins were predicted from the lancelet.

Myxine

Initial PCR reactions using the degenerate primers CH COX F1, CH COX F2, CH COX R1, and CH COX R2 (Table 2-1) were used to amplify a single 1454 bp product from the hagfish (*Myxine glutinosa*). This product (hereafter referred to as hCOX) was found to be 63.8% identical to COX-1 of the zebrafish (*Danio rerio*). RACE primers (5' and 3', Table 2-3) were designed against this initial product and used with kit primers to completely sequence the 2416 bp product. Searching for open reading frames yielded a putative 610 amino acid protein.

Petromyzon

Initial PCR reactions using degenerate primers CH COX F3 and CH COX R1 (Table 2-1) amplified a single 541 bp product from gill tissue of the lamprey (*Petromyzon marinus*). This product (hereafter referred to as lCOX) was later extended using degenerate primers (Table 2-1)

and specific primers designed against the initial 541 bp product (Table 2-4). Despite all attempts to amplify the complete lCOX product (including RACE) the sequence was only extended to 860 bp. A 286 amino acid open reading frame was predicted from this product.

Fundulus

Because the COX-2 sequence for the euryhaline killifish (*Fundulus heteroclitus*) was previously completed (Choe *et al*., 2006), the degenerate primer CH COX-1 F2 (Table 2-1) designed to amplify COX-1 forms over COX-2 forms was used with the degenerate primer CH COX R1 (Table 2-1) to initially amplify a non-COX-2, 1335 bp product (hereafter referred to as COX-1b *Fundulus*) from killifish gill tissue. This product was then extended and completed using specific primers and RACE primers (Table 2-5). The completed 2226 bp sequence contains a 598 amino acid open reading frame that was nested inside the teleost COX-1b clade in all phylogenetic analyses (see below), prompting the design of degenerate primers (Table 2-1) that were used to amplify COX-1a forms in teleosts over COX-1b forms. Using the degenerate primers CH COX-1a F1 and CH COX-1a R1 (Table 2-1), a 759 bp product was initially amplified in PCR reactions. Using RACE primers designed to complete this sequence (Table 2-5), the 3' end of this product was completed, resulting in a 1655 bp product (hereafter referred to as COX-1a *Fundulus*) that contains a 452 amino acid reading frame. However, the 5' end of this product was not sequenced despite multiple attempts. This putative protein grouped with the COX-1a sequences of other teleosts in all phylogenetic analyses (see below), leading to the conclusion that the COX-1a, COX-1b, and COX-2 sequences included in the analyses all represent different COX products expressed in the gill tissue of the euryhaline killifish.

Myoxocephalus

Using the degenerate primers CH COX F1 and CH COX R2 (Table 2-1), a 977 bp product was initially amplified in PCR reactions using cDNA from the gills of the longhorn

sculpin (*Myoxocephalus octodecemspinosus*). This product (hereafter referred to as COX-2 *Myoxocephalus*) was then extended and completed using RACE and specific primers (Table 2-6), resulting in a 2618 bp product that contains a 605 amino acid open reading frame. This sequence grouped with COX-2 forms from other teleosts in all phylogenetic analyses, inciting the use of COX-1 degenerate primers (Table 2-1) CH COX-1 F2 and CH COX-1 R2 in PCR reactions to amplify a 1000 bp product (hereafter referred to as COX-1a *Myoxocephalus*). This product was then extended and completed using RACE primers (Table 2-6), resulting in a 2633 bp product encoding a 622 amino acid open reading frame. This product grouped with COX-1a forms from other teleosts in all phylogenetic analyses, prompting the design of degenerate primers used to amplify COX-1b forms in teleosts over COX-1a forms (Table 2-1). Using the degenerate primers CH COX-1b F1 and CH COX-1b R0 (Table 2-1), an initial 895 bp product (hereafter referred to as COX-1b *Myoxocephalus*) was amplified in PCR reactions. This product was extended and completed using specific RACE primers (Table 2-6), resulting in a 2332 bp product containing a 600 amino acid open reading frame. This product grouped with other teleost COX-1b forms in all phylogenetic analyses, suggesting that the COX-2, COX-1a, and COX-1b forms sequenced represent 3 different COX forms expressed in the gills of the longhorn sculpin.

Sequence Analyses

Teleosts COX-1a, COX-1b, and COX-2

Three different COX forms are now available from the teleosts studied here: the COX-2 sequenced from the euryhaline killifish (Choe *et al*., 2006), the COX-2 sequenced here from the longhorn sculpin, and two the COX-1 forms sequenced here from both the killifish and sculpin, named as COX-1a and COX-1b (following Ishikawa and Herschman, 2007). COX-1a and COX-1b predicted proteins from the killifish were more similar to *Mus* COX-1 than COX-2 (70%

versus 64% for COX-1a and 68% versus 59% for COX-1b, respectively). Similarly, COX-2 from the sculpin was more similar to *Mus* COX-2 than *Mus* COX-1 (70% versus 60%) and COX-1a and COX-1b were more similar to *Mus* COX-1 than *Mus* COX-2 (53% versus 47% for COX-1a and 67% versus 57% for COX-1b, respectively).

An alignment of COX forms from the killifish (Figure 2-1) shows that all the important amino acid residues for cyclooxygenase function are conserved in all forms. However, because COX-1a is incomplete it is unknown whether the N-terminal characteristics are conserved in this form. These include an active site tyrosine (Tyr-385, using ovine COX-1 numbering), haem-binding histidines (His-207 and His-338), and the aspirin acetylation site (Ser-530) (Ishikawa *et al*., 2007; Ishikawa and Herschman, 2007; Yang *et al*., 2002; Kulmacz *et al*., 2003). In addition the COX-2 form contains a C-terminal amino acid insertion and the COX-1 forms contain an N-terminal amino acid insertion. This is consistent with the COX-1 and COX-2 forms of other vertebrates (Herschman *et al*., 2003; Kulmacz *et al*., 2003), although killifish COX-2 seems to have a shorter than average insertion. In mammals, the amino acid residues thought to give COX-1 and COX-2 their differing affinities for substrates are positions 513 (His in COX-1 and Arg in Cox-2) and 523 (Ile in COX-1 and Val in COX-2) (Guo *et al*., 1996). However, as previously noted in other teleosts (Grosser *et al*., 2002; Choe *et al*., 2006; Ishikawa *et al*., 2007) these differences appear to be absent in teleosts, with Arg and Val present in positions 513 and 523, respectively, in both teleost COX-1 and COX-2. This is also the case in the killifish with Arg in position 513 and Val in position 523 in all COX forms reported here.

As in the killifish, alignment of the three COX forms sequenced from the sculpin (Figure 2-2) shows conserved amino acid residues characteristic of COX function in all three forms. The characteristic terminal amino acid insertions found in mammalian COX forms are also present in

their respective sculpin counterparts. However, COX-1b from the sculpin seems to have both N and C-terminal insertions. Disulfide bonds near the n-terminus of sculpin COX-1a also seem to be altered due to the substitution of other amino acids for cysteine. It also appears that some functional amino acid residues near the C-terminus of sculpin COX-1a are not conserved with the other forms. These include the substitution of Leu for Arg at position 513, Leu for Val at position 523, and Phe for Ser at position 530.

Lamprey, hagfish, and amphioxus COX forms

The one incomplete putative COX protein obtained from the gills of the lamprey shares near equal identity (63%) with *Mus* COX-1 and COX-2. The gene, referred to here as lamprey COX (lCOX) (Yang *et al*., 2002) shares the most identity (70%) with a form from the lancelet (COXc), but shares notable identity (64%) with the form from the hagfish as well. Based on an alignment of COX forms sequenced from select non-mammalian and non-teleost chordates (Figure 2-3), the partial COX form sequenced from the gills of the lamprey has the predicted histidine site (His-207) critical for peroxidase activity as well as conserved haem-binding domains. However, the membrane binding regions near the Arg-277 loop that differ between COX-1 and COX-2 forms appear to be different from both COX-1 and COX-2 in the lamprey form. This is seen when the lamprey form is aligned with COX-1 and COX-2 forms from vertebrates (Figure 2-4).

The putative COX protein from the gills of the hagfish shares slightly more identity with *Mus* COX-2 than *Mus* COX-1 (63% versus 60%). The gene, referred to here as hCOX, shares the most identity (> 60%) with vertebrate COX-2 forms, but also shares notable identity with lCOX (64%) and forms from amphioxus (> 60%). An alignment of hCOX with other non-mammalian, non-teleost COX proteins (Figure 2-3) shows that all important amino acid residues and binding sites critical to cyclooxygenase activity are conserved in hCOX. An alignment of

hCOX with COX-1 and COX-2 proteins from various vertebrates (Figure 2-5) shows that hCOX has an insertion of amino acids near the C-terminal; characteristic of COX-2 proteins in vertebrates. However, there are several regions where COX-1 differs from COX-2 but hCOX shows no identity to COX-1 or COX-2 amino acids (indicated as sites with a + over them in Figure 2-5).

Two putative incomplete COX sequences were obtained from amphioxus. These sequences, referred to here as COXc and COXd, are most identical (81%) to each other, but also share notable identity with hCOX (66%), lCOX (67%), vertebrate COX-1 (> 60%), and vertebrate COX-2 (> 60%). They are the least identical to *Ciona* COXa (48%) and COXb (55%). Based on an alignment of non-mammalian, non-teleost COX proteins (Figure 2-3), it appears that amino acids critical to COX function are conserved in COXc and COXd. However, the first haem-binding domain seems to be very different in the amphioxus forms. This is also the case for *Ciona* COX forms and COXa, COXb, COXc, and COXd all share notable identity in this region as shown by alignment (Figure 2-6).

Phylogenetic analyses

A phylogeny (Figure 2-7) generated under a distance optimality criterion with 1000 bootstrap replicates using MEGA4 shows predicted COX clades. COX-1a and COX-1b forms from teleosts form monophyletic sister clades. The novel COX-1 forms from the killifish and sculpin group together with other teleosts in these clades as expected based on their designation here. Eutherian COX-1 forms group together in a monophyletic clade sister to the teleost COX-1 clade. The COX-1 proteins from *Gallus*, *Monodelphis*, and *Xenopus* are located at the base of this monophyletic vertebrate COX-1 clade, with sCOX being the most basal. Vertebrate COX-2 forms group together in another monophyletic clade that is sister to the vertebrate COX-1 clade. There are two monophyletic clades within the COX-2 group: a teleost clade and a tetrapod clade.

The forms from lamprey and hagfish (lCOX and hCOX) are basal to the vertebrate COX clade, while the forms from amphioxus (COXc and COXd) form a monophyletic group basal to these forms. The forms from *Ciona* (COXa and COXb) form another monophyletic group that is basal to all other COX forms. Bootstrap values give robust support for vertebrate clades, the amphioxus clade, and the *Ciona* clade, but give weak support for placement of hCOX, lCOX, and sCOX. Based on these results and other generated phylogenies (see below), hCOX and lCOX were identified as possible rogue sequences due to their inconsistent positions depending on the type of analysis used and another distance analysis was completed without including these sequences (Figure 2-8). The topology of this phylogeny is very similar to the complete COX phylogeny. However, sCOX is basal to the vertebrate COX clade and not included with the other vertebrate COX-1 forms. Additionally, bootstrap values for most clades are considerably more robust than when hagfish and lamprey sequences are included.

To further test relationships between COX proteins, a phylogeny (Figure 2-9) was generated using the maximum likelihood criterion, 1000 bootstrap replicates, and the program PhyML (Guindon and Gascuel, 2003). The topology of this tree is very similar to the one generated using distance criterion. Briefly, it contains well supported, monophyletic clades for COX-1 (teleost COX-1a, teleost COX-1b, teleost COX-1, mammalian COX-1, and vertebrate COX-1 with sCOX as the most basal sequence) and COX-2 (teleost COX-2, mammalian COX-2, and vertebrate COX-2). The lamprey (lCOX) is also basal to the vertebrate COX clade, the amphioxus COXc and COXd form a basal monophyletic clade, and the most basal clade in the tree is a monophyletic COXa and COXb from the sea squirt. However, in the ML analysis, the hagfish (hCOX) sequence groups at the base of the vertebrate COX-2 clade instead of being basal to the vertebrate COX clade. Again, the analysis was repeated without hCOX and lCOX

(Figure 2-10), causing bootstrap values to increase dramatically. Also, the estimated gamma shape parameter increased slightly from 0.737 to 0.742.

To further evaluate support for COX clades, an aLRT analysis (Anisimova and Gascuel, 2006) was performed. This type of analysis provides values of support similar to Felsenstein's bootstrap support values but is much faster because bootstrap sampling requires many (1000 in the previous analyses) runs while aLRT is run only once. The fundamental difference between the two is that bootstrap support values are based on repeatability whereas the aLRT is a measure of the likelihood gain of including a branch versus collapsing it (Anisimova and Gascuel, 2006). The resulting aLRT statistics can be interpreted using parametric Chi-Squared distributions or a non-parametric Shimodaira-Hasegawa-like (SH) procedure (Anisimova and Gascuel, 2006). The former seems to be more liberal (giving values similar to Bayesian posterior probabilities) and the later more conservative (Anisimova and Gascuel, 2006). Here, the most conservative approach was used and values represent the minimum of Chi-Squared and Shimodaira-Hasegawa (SH) support values. When all species are included (Figure 2-11) clades of interest have robust support (> 0.90) except for the vertebrate COX-2 clade (containing hCOX, 0.683) and the clade containing lCOX (0.87). When hCOX and lCOX are excluded from the analysis (Figure 2-12), support values generally increase, with a notable increase in the vertebrate COX-2 clade (0.883).

A Basesian phylogenetics (BP) analysis was also performed (Figure 2-13) using the program MrBayes 3.1.2, with one initial 2 million generation run, and one final 10 million generation run. The topology of the consensus tree (with burnin = 10%) is similar to those described above, with well-supported aforementioned vertebrate COX clades. However, in BP analysis lCOX groups at the base of the amphioxus COXc/COXd clade instead of being the basal member of the vertebrate COX clade. This grouping is well-supported by posterior probability

scores (0.93). As in ML analysis, hCOX groups with vertebrate COX-2 proteins at the base of the clade. Also similar to previous analyses, when lCOX and hCOX are excluded (Figure 2-14) scores improve and all clades have robust support.

Due to the contrasting topologies generated using distance, ML, and BP methods, 21 different tree topologies (Figure 2-15) were generated by using the most likely tree from ML analysis (Figure 2-9) and relocating hCOX and/or lCOX sequences to other branches of the tree. Positions of all other sequences were not altered from the most likely tree. A Ln likelihood score (Ln *L*) was generated for each topology using online execution of the program PhyML (Guindon *et al*., 2005). Likelihood scores were compared to the most likely tree. These tests indicate that lCOX and hCOX can be moved somewhat freely around the base of the tree without resulting in a noticeably different Ln *L* than the most likely topology (Table 2-9). It appears that moving lCOX to the base of the COX-1 clade causes the smallest change in Ln *L*, whereas moving hCOX or lCOX deeply into COX-1 or COX-2 clades causes the largest changes in Ln *L*.

Discussion

Here, I present the first forms of the enzyme cyclooxygenase (COX) sequenced from the hagfish, lamprey, and amphioxus. I also show that the killifish and sculpin conform to the current hypothesis that teleosts posses additional COX-1 and COX-2 forms. The phylogenetic relationships among the various COX forms are still not definite, although the results presented here allow some conclusions to be drawn and predictions to be made. Specifically, it appears that COX sequences in the chordates form at least 3 well supported phylogenetic groups: the urochordata, cephalochordata, and vertebrata. Hagfish and lamprey positions are variable depending upon the analysis used, but likelihood scores seems to favor placement within the vertebrata clade.

Novel COX Forms

Although the two COX sequences from amphioxus were not able to be extended, they aligned readily with other COX sequences. They show a first haem-binding domain that shares little conservation with vertebrate forms (20%), as in the urochordates. However, the second haem-binding domain is completely conserved in these forms, as is histidine-207 which is crucial for peroxidase activity. The arginine-277 loop region does not resemble COX-1 or COX-2 forms. Due to the truncated nature of these proteins, it is difficult to determine if COX in the cephalochordates plays similar roles as in the vertebrates, but some functions are likely conserved. Following the nomenclature used by Jarving *et al*. (2004) these forms are named COXc and COXd. The COX sequence found in the gills of the hagfish was extended to completion and readily aligned with other COX forms. It has conserved amino acids, binding domains, and bonds critical for COX function. Therefore, it likely plays a similar role in the hagfish. The hagfish COX has an N-terminus deletion and valine-523 characteristic of COX-2 forms, but there are multiple sites where it shares equal or no homology with COX-1 and COX-2 of vertebrates. Therefore, following the nomenclature of Yang *et al*. (2002) this form is named hCOX. Although incomplete, the one COX sequence cloned from the gills of the lamprey readily aligns with other COX sequences and contains amino acids and regions critical to COX function. However, like the hagfish sequence it shares no biased homology with COX-1 or COX-2 forms. This form is therefore named lCOX. As predicted based on phylogenetic position, COX-1a, COX-1b, and COX-2 were found in the gills of the euryhaline killifish and the longhorn sculpin. With the exception of COX-1a in the sculpin, all COX forms from these teleosts contain conserved regions critical to COX function. As has been reported in other teleosts (Ishikawa *et al*., 2007), these forms all contain valine at position 523, unlike in mammals. COX-1a from the sculpin does not have several amino acids critical for COX

function located near the C and N terminus of the sequence. These include disruption of disulfide bonds near the N terminus due to cystine replacements, little conserved homology in the membrane binding domain, and alternate residues for arginie-513 (leucine), valine-523 (leucine), and serine-530 (phenylalanine). This is not the case in the other acanthopterygiians, where all COX forms have these conserved regions. This may indicate that in the sculpin COX-1a has partially lost function, and COX-1b or COX-2 may therefore have additional responsibilities.

Phylogenetic Analyses

As has been seen when tracing the evolutionary histories of other gene families in evolutionary deep time (Abbasi and Grzeschik, 2007; Goudet *et al*., 2007), the pedigree of the COX family is littered with duplications, losses, and uncertainty. Despite utilizing a multi-faceted approach to depict COX evolution in the chordates, the results indicate that COX has a complicated and under-examined history of evolution in the chordates. This is not surprising, considering the complex functions COX has been shown to have in the chordates. Because of these complex functions, duplications, partitioning of roles, differential expression, and multiple forms with multiple functions are to be expected.

In all phylogenetic analyses, the more recent history of COX evolution is more clear and consistent than the relationships of the "ancestral" taxa. The teleosts consistently form monophyletic COX clades nested within the gnathostomes, as expected. The gnathostomes consistently form two COX sister clades in phylogenetic analyses: COX-1 and COX-2. This is expected based on previous phylogenetic analyses in the vertebrates (Gu, 2001; Gu, 2006). The COX-1 clade is consistently rooted with the sequence from the dogfish (sCOX), although support values are not robust for this relationship when hagfish and lamprey sequences are included. In distance analysis excluding hagfish and lamprey sequences, sCOX groups outside

the COX-1 and COX-2 clades, representing another COX gnathostome lineage. These groupings are as expected, based on Yang *et al.*'s (2002) original description of sCOX as neither a COX-1 nor COX-2. These are the first phylogenetic analyses of COX to include sCOX and it is concluded that sCOX is most likely a basal member of the COX-1 clade.

In all analyses, the COX-1 clade contains robustly supported, monophyletic teleost and eutherian mammal clades. In the teleost clade, COX-1b and COX-1a form well-supported, monophyletic clades. Although this is expected based on initial studies (Ishikawa *et al*., 2007; Ishikawa and Herachmann, 2007), this is the first study to use sophisticated tree-building techniques to show the monophyly and legitimacy of the COX-1 forms in teleosts. It is also shown in each analysis that the non-duplicate COX-1 forms from the zebrafish and trout are more related to COX-1b forms from the acanthopterygiians, grouping with them at the base of a monophyletic COX-1/COX-1b clade.

The mammalian COX-1 group is not as robustly supported using all types of analyses. The expected relationships (based on assumed evolutionary relationships in the tetrapods) are shown and supported with some confidence in the maximum likelihood analysis (ML) when the hagfish and lamprey sequences are excluded. In this anlaysis, the tetrapods form a monophyletic group with the frog and chicken at the base of the clade, followed by the opossum sequences, and then the eutherians. Even in this analysis, the frog and chicken sequences group together, contrary to what is biologically expected (the frog should be more basal, representing an evolutionary ancient non-amniote lineage, and the chicken should be a sister to the mammals). However, this grouping is not well-supported in any analysis. When hagfish and lamprey are included in ML analysis, the chicken/frog clade occurs at the base of the mammalian/teleost clade, contrary to biological predictions. However, this relationship is poorly supported. These

low support values are confirmed in the aLRT analysis. In Bayesian and distance analyses, the frog is basal to the chicken, but these sequences are again at the base of a biologically irrelevant teleost/mammal clade. However, unlike distance and ML methods, Bayesian posterior probabilities strongly support this relationship. Bayesian posterior probabilities traditionally overestimate support values for a variety of reasons (Cummings *et al*., 2003: Lewis, pers. comm..) and can give incorrect clades strong posterior probabilities. This may explain why this biologically unexpected relationship is strongly supported only in the Bayesian trees. Therefore, ML analyses (supported by bootstrap and aLRT scores) show the most probable scenario for COX-1 evolution in the gnathostomes: a basal sCOX, followed by a teleost COX-1 clade (as described above) sister to a tetrapod COX-1 clade.

The COX-2 groups within the gnathostomes mirrors the same general trends as the COX-1 clade. Teleosts consistently form a well-supported, monophyletic group. The COX-2a sequences from zebrafish and trout group together to form the most basal members of this clade. The COX-2b sequences group with the non-duplicate COX-2 sequences from the acanthopterygiians to form the other teleost COX-2 clade. Sister to the teleosts, the tetrapods form another COX-2 clade in the gnathostomes. In this tetrapod COX-2 clade, frog and chicken sequences form the most basal (although not well-supported) clade, with the mammals as a sister clade. This biologically relevant relationship between COX-2 sequences is predicted in all analyses.

Basal to the COX-1 and COX-2 clades in the gnathostomes are two clades that are consistently and robustly supported in all phylogenetic analyses: a urochordate COX clade and a cephalochordate COX clade. The cephalochordate clade consists of the two COX sequences obtained in this study (COXc and COXd) and the urochordate clade consists of the two

sequences from *Ciona* (COXa and COXb). The urochordate COX forms always forms the base of the entire tree and the cephalochordate COX forms always group as a sister clade to the gnathostome COX clade. This study is the first to show this biologically relevant relationship between COX forms in these "ancestral" chordates.

The hagfish (hCOX) and lamprey (lCOX) sequences have alternate phylogenetic positions depending on the technique used to reconstruct their evolutionary histories. Also, when these sequences are removed from analysis, bootstrap, aLRT, and posterior probability scores supporting COX clades increase. Because of their uncertain placement in COX evolution, these sequences are designated as "rogues" and analyses excluding them likely show a more accurate representation of COX evolution. In distance analysis, both hCOX and lCOX are placed outside the gnathostome COX clade and form two separate COX lineages between the cephalochordate and gnathostome clades. In ML analysis, lCOX remains basal but hCOX is placed at the base of the gnathostome COX-2 clade. In BP analysis, hCOX remains in the COX-2 clade but lCOX is placed within the cephalochordate clade. Support values for hCOX and lCOX placement are weak in all but BP analyses. Clearly, the placement of hCOX and lCOX in COX evolution is not clear and without further evidence, placing them with any degree of certainty becomes difficult. In an attempt to find the most likely placement of these sequences, 21 different phylogenies were generated based on the most likely tree with hCOX and lCOX placed in variable positions. The trees were then evaluated in a maximum likelihood framework.

It is important to note that although Ln *L* scores were generated for each topology, other biological factors may influence which topology or type of topology is actually the most likely. For example, placing hCOX in the COX-2 clade and lCOX at the base of the tree (technically the most likely tree) implies that another COX lineage exists for all vertebrates (the lineage

represented by lCOX) because the hagfish is biologically ancestral to the lamprey. This would mean that dozens of novel COX forms have yet to be discovered in well-examined groups such as the mammals and teleosts. With genomes of several of these groups available, it is unlikely that such a vast amount of sequence data have yet to be found. Therefore, the most likely scenarios for COX evolution would imply that either hagfish is basal and lamprey is derived, both are basal, or both are derived. However, placing lCOX, hCOX, or sCOX in the COX-1 or COX-2 clade implies that another COX form has yet to be found in these species.

It appears that moving lCOX into the base of the COX-1 clade causes less of a change in Ln *L* scores than moving hCOX outside the gnathostome COX clade. However, it is apparent that either sequence can be moved somewhat freely throughout the tree without resulting in noticeably worse (~10 Ln *L*) tree scores. This leads to the conclusion that although hCOX and lCOX may represent independent, basal lineages of COX, they are most likely basal members of the COX-2 and COX-1 clades (respectively) in the gnathostomes. If this is the case then at least one other form of COX should exist in the hagfish and lamprey, as well as in the dogfish shark.

Summary

Presented here are the first COX sequences from amphioxus, the hagfish, and the lamprey. All predicted COX sequences from the killifish and sculpin are also described. These sequences all likely represent functional forms of COX found in the chordates based on conserved amino acids and domains critical to COX function. For the first time, a sophisticated phylogenetic analysis of COX forms from the majority of representative chordate lineages was attempted. Although still not definitive, a general hypothesis for the evolutionary history of COX in the chordates can be made (Figure 2-16). In this scenario, there are three main COX groups corresponding to the three subphyla found in the phylum Chordata: the urochordata, cephalochordata, and vertebrata. Each contains two main COX groups, with the well-

documented COX-1 and COX-2 forms only found in the vertebrata clade. These forms contain monophyletic groups for the teleosts and the tetrapods, with multiple forms in the teleosts confirming previous hypotheses (Ishikawa *et al*., 2007; Ishikawa and Herschman, 2007). Hagfish and lamprey placement is uncertain, but most likely these sequences represent basal members of the vertebrata clade. This does not support the timing of genomic duplications in the chordates according to the most recent view of the 2R Hypothesis (Kasahara, 2007), indicating two rounds of genome duplication after the origin of the urochordates but prior to the radiation of the jawed vertebrates. However, several developmentally important proteins have been shown to not support this timing and indicate an earlier duplication (Hughes, 1999). Multiple COX-1 and COX-2 genes in the teleosts do support the hypothesis that the teleosts are characterized by another round of genome duplication after the origin of the vertebrates (Li *et al*., 2007). Clearly, this uncertain period representing some 600 million years of evolution is characterized by many losses and duplications and remains to be resolved definitively.

Table 2-1. Degenerate primers used in PCR

Name	Orientation	Nucleotide sequence (5' to 3')
CH COX F1	Sense	ATG GAC GAC TAC CAG TGY GAY TGY AC
CH COX F2	Sense	GAT GTT TGC ATT TTT CGC TCA RCA YTT YAC
CH COX F2.25	Sense	TGG CGT GGA CCT AGG TCA NRT NTA YGG
CH COX F2.5	Sense	CGA GCT GCG GTT CCA TAA ARA YGG NAA RYT
CH COX F3	Sense	CCA CTA TAT GGC TGC GGG ARC AYA AYM G
CH COX F5	Sense	CAT GTG GAA TTC CAT CAC CTG TAY CAY TGG CA
CH COX R1	Antisense	CCC CGA AGG TGG ATG GYT TCC AVY A
CH COX R2	Antisense	GCA TCA GCG GGT GCC ART GRT A
CH COX R3	Antisense	TCC TCT TTT AGT ATG TCG CAG ACT CKR TTR TGY TC
CH COX-1 F4	Sense	TGC CAG ACA GCA TCC ACA THG AYG GNG A
CH COX-1 F5	Sense	CAG CAG ACA ATG CGC AGG NCA RAT HGG
CH COX-1 R2	Antisense	TGG GTG TAT GTT GTG TCC TCC NCC DAT YTG
CH COX-1a F1	Sense	CCC CAC CAA CCT ACA ATA CCA ART AYG GNT A
CH COX-1a F2	Sense	AAA GTG CTG ACT GAR MGN TTY TT
CH COX-1a F3	Sense	GGA CTA ATC TGA TGT TCG CGT TYA TGG CNC A
CH COX-1a F4	Sense	AGC GGA AGG CGG CTT YAC NAA NGC
CH COX-1a F5	Sense	CAA ATA GTA AAT GGG GAR AYN TAY CC
CH COX-1a R1	Antisense	ATA GGG AAT GTC GTC NCC RTC DAT
CH COX-1a R2	Antisense	GGG GCA CGT TTT CCG GRT ANA YCA T
CH COX-1a R3	Antisense	ACC TCA GTG ACA GTG GGA GGR TAN RTY TC
CH COX-1b F1	Sense	CGG ATG GGT GTG GGC TTY ACN AAR GC
CH COX-1b R0	Antisense	GGA GAA GGG AGC ACC CAT YTC NAC CAT
CH COX-1b R3	Antisense	CCA GCG TCC GCG CDA TYT CYT C

Table 2-2. Amphioxus COX specific primers used in PCR

Name	Orientation	Nucleotide sequence (5' to 3')
COXa F1	Sense	TAC TCC ACC GTG TGG CTG C
COXa F2	Sense	TGG GAC GAC GAG AGG CTC
COXa F2.5	Sense	ACC GGT GAG ACC ATC AAC ATC AT
COXa F3	Sense	CAC CTG AGC GGC TAC AAC TTT
COXa F4	Sense	GAC CTG TTC TGG GAC CCT GAG
COXa F5	Sense	CCA GTA CCA GAA CCG CAT CTT C
COXa R1	Antisense	CGT ACG GCG GGT AGA TCA TG
COXa R2	Antisense	GCT GCC GCT CCA CGG
COXa R3	Antisense	CCC GTA GAT ATG GCT CAG GTC A
COXa 3' F1	Sense	GAG GCT CTT CCA GAC AGC CA
COXa 3' F2	Sense	CAC CTG AGC GGC TAC AAC TTT
COXa 5' R1	Antisense	TGG CTG TCT GGA AGA GCC TC
COXa 5' R2	Antisense	TGG AGT AGA CGA ACA GGC CC
COXa 5' R3	Antisense	CCC CGT AGA TAT GGC TCA GGT
COXb F0	Sense	CAG ACA GCT AGA CTC ATT CTT ATC AGT GA
COXb F1	Sense	TAT CAA CAT CGT CAT TGG AGA GTA TG
COXb F1.5	Sense	GGC TGG CAA AAA CTT CCA ACT
COXb F2	Sense	TGC AGT ACC AGA ACA GCA TAT TTG
COXb F3	Sense	AGC ATA TTT GTG GAG TTT AAC CAC TTG
COXb R1	Antisense	CGT CCG GAG GGT AAA TCA TGT
COXb R2	Antisense	ACT TCA GTT TCC CGT CCT GGA
COXb R3	Antisense	CGT AAA CAT GGC TCA TGT CCA CT
COXb 3' F1	Sense	GTC CCC GAG AAG AAG CGA TT
COXb 3' F2	Sense	GAA CGG CTC TAC CAG ACA GCT AG
COXb 3' F3	Sense	TGT TCT GGG ACC CTG AGC TG
COXb 5' R1	Antisense	TGG TAG AGC CGT TCG TCG TC
COXb 5' R2	Antisense	GGA GGG TAA ATC ATG TGT ACG GA
COXb 5' R3	Antisense	ACA TGG CTC ATG TCC ACT GC

Table 2-3. Hagfish COX specific primers used in PCR

Name	Orientation	Nucleotide sequence (5' to 3')
hCOX 3' F1	Sense	TTA ACC ACC TGT ACC ACT GGC AC
hCOX 3' F2	Sense	CTC AAC CAT GGA GTA CGA GGT CT
hCOX 3' F3	Sense	CAA TGC TAT GGA GTT CTA CCT GGG
hCOX 3' F0.8	Sense	CAG TAC AGC AAC CGC ATC TCA
hCOX 3' F0.6	Sense	AAT GGG ACG ACG AGA GAA TCT TT
hCOX 3' F0.4	Sense	GGT TTG CTG TTG GTC ACG AAG
hCOX 3' F0.2	Sense	AGT CCA CCC ACA TAC AAT GCC
hCOX 3' F0.1	Sense	CTC ACG CAC TTT GCT CCC TT
hCOX 3' F0	Sense	GAA CTG CAC CTA CCC CGA GAC
hCOX mid F1	Sense	GTT TCG AGT CCA CCC ACA TAC AA
hCOX mid F2	Sense	CAT ACT CCA ACC TCA GCT ACT TCA CT
hCOX mid R1	Antisense	CAC ATC GAG TAC GTC ACA CAC G
hCOX mid R2	Antisense	AAG GGT CCC GTA GAG CAT GA
hCOX 5' R1	Antisense	TCT CCG AAT AAA GCT CCC TGT C
hCOX 5' R2	Antisense	TGC TGC CAT TTC CGT TTC TC
hCOX 5' R3	Antisense	CAT GTT TGA TGG ATG CTG TTG C
hCOX 5' R4	Antisense	AGG CGA GTG AAG TAG CTG AGG TT
hCOX 5' R5	Antisense	CCT CCC AGG ACT TGT ACC GTA AT
hCOX 5' R6	Antisense	GAG GAA GTA ATG CAC GGT ATC TGG

Table 2-4. Lamprey COX specific primers used in PCR

Name	Orientation	Nucleotide sequence (5' to 3')
lCOX F1	Sense	GAG GAA CGC TTG AGA GAC AAC A
lCOX R1	Antisense	TCT TGT TGA TGT TTC GAC CGC
lCOX R2	Antisense	CCA CCG CCA ATC TGG C
lCOX R3	Antisense	AAT CAG GAA GCT CTC AGG CAT C
lCOX 3' F1	Sense	GAG TTC CTC TTC AAC CCC GG
lCOX 3' F2	Sense	TCG AAA CAT CAA CAA GAA CCT CC
lCOX 3' F3	Sense	TTC ACC TCC TTC CTG GAG CTC
lCOX 5' R1	Antisense	TGC TGC ACG TAC TCC TCG ATC
lCOX 5' R2	Antisense	GCA GCT GGT GTT GTC TCT CAA

Table 2-5. Killifish COX specific primers used in PCR

Name	Orientation	Nucleotide sequence (5' to 3')
Fh COX-1b 3' F1	Sense	CTT CAC CGA TAG CGA GGA GAT AG
Fh COX-1b 3' F2	Sense	AGC TCT ACG GTG ACA TCG ACA CT
Fh COX-1b 3' F3	Sense	GGG TAA CCC CAT ATG TTC TCC AC
Fh COX-1b 5' R1	Antisense	CCC ATG TGA GAG AGC CTT AGT GA
Fh COX-1b 5' R2	Antisense	CAG CCT CTC AAA CAA CAC CTG AG
Fh COX-1b 5' R3	Antisense	GTA GTA GGA TTC CCA GCT GAG GTA GT
Fh COX-1a 3' F1	Sense	GGT GTA CCC CGA AGG TTT CC
Fh COX-1a 3' F2	Sense	GAG TAC GTG CAG CAC CTG AGC
Fh COX-1a 3' F3	Sense	AGT ACA CCA ATC GCA TCG CC
Fh COX-1a 5' R1	Antisense	GAT GAT AAG TCT GGC GGT CTG G
Fh COX-1a 5' R2	Antisense	GAT GGT GGC ATA CAC GGT GA
Fh COX-1a 5' R3	Antisense	TTT ATG AAG CCG AAG CTG GTG

Table 2-6. Sculpin COX specific primers used in PCR

Name	Orientation	Nucleotide sequence (5' to 3')
Mo COX-2 3' F1	Sense	CCC CGG TCT GAT GAT GTA CG
Mo COX-2 3' F2	Sense	ACC GAG TGT GTG ACG TGT TGA
Mo COX-2 3' F3	Sense	TCG AGG ACT ACG TGC AGC AC
Mo COX-2 3' F4	Sense	CAG GTC TAA CGC AAT CTT TGG G
Mo COX-2 3' F5	Sense	CCT CAA GGG CTT AAT GGG AAA C
Mo COX-2 3' F6	Sense	AAC ATC GTC AAC ACC GCC TC
Mo COX-2 mid F1	Sense	ACA GTT CAT TCC GGA TCC ACA
Mo COX-2 mid F2	Sense	CAC CAG CCT GAT GTT TGC ATT
Mo COX-2 mid R1	Antisense	CGA AGC GGT GAG ATT CAG GA
Mo COX-2 mid R2	Antisense	CTC CAT CCA GGA CCT GAT ATT TAA G
Mo COX-2 5' R1	Antisense	TCC GGA ATG AAC TGT CTT CTC A
Mo COX-2 5' R2	Antisense	AGG TCG GAG GAC TAT CAA TCA AGT
Mo COX-2 5' R3	Antisense	GAA GGA GAT GGA GTT AAT GAT GTT CC
Mo COX-1a 3' F1	Sense	CAT CAC CTC CGG CTT CAT AAA
Mo COX-1a 3' F2	Sense	GAG GGA GCA TAA CAG ACT CTG TGA
Mo COX-1a 3' F3	Sense	CTG GAG TTC TGC CAC CTC TAC C
Mo COX-1a 3' F4	Sense	GCA GCC CTT CAA TGA ATA CAG G
Mo COX-1a 3' F5	Sense	CAT GCT GGA GAA GAC CCT TCC
Mo COX-1a 3' F6	Sense	GAG TAT GTT GGA GAT GGG TGC TC
Mo COX-1a 5' R1	Antisense	GCC GAA ATG TCT TCC TCC TAA A
Mo COX-1a 5' R2	Antisense	CAT CGG TAA AGG GCA GTC CTC
Mo COX-1a 5' R3	Antisense	TGA CCG TCA GCA CTA ATC TCA TG
Mo COX-1b 3' F1	Sense	TCA CAC ACT ACG GCA TCG AGA
Mo COX-1b 3' F2	Sense	TTG GCG GTG GCT TTA ACA TC
Mo COX-1b 3' F3	Sense	CAG CCC TTC AAC GAG TAC AGG
Mo COX-1b 5' R1	Antisense	GAG CGA TAC GGT TCC CAT ACT G
Mo COX-1b 5' R2	Antisense	GTT GTG CTC CCT GAG CCA GA
Mo COX-1b 5' R3	Antisense	AAT AGC CAT CTG AGC CTC AGG A

Table 2-7. Sequences included in phylogenetic analyses

#	Name	Organism	Size (AA)
1	COX1a Fundulus	*Fundulus heteroclitus* (killifish)	452
2	COX1b Fundulus	*Fundulus heteroclitus* (killifish)	598
3	COX2 Fundulus	*Fundulus heteroclitus* (killifish)	610
4	COX1a Myoxocephalus	*Myoxocephalus octodecimspinosus* (lh. Sculpin)	622
5	COX1b Myoxocephalus	*Myoxocephalus octodecimspinosus* (lh. Sculpin)	600
6	COX2 Myoxocephalus	*Myoxocephalus octodecimspinosus* (lh. Sculpin)	605
7	COXa Ciona	*Ciona intestinalis* (sea squirt)	653
8	COXb Ciona	*Ciona intestinalis* (sea squirt)	600
9	COXc Branchiostoma	*Branchiostoma lancelolatum* (amphioxus)	177
10	COXd Branchiostoma	*Branchiostoma lancelolatum* (amphioxus)	177
11	hCOX Myxine	*Myxine glutinosa* (Atlantic Hagfish)	610
12	lCOX Petromyzon	*Petromyzon marinus* (Sea Lamprey)	286
13	COX1a Oryzias	*Oryzias latipes* (Japanese medaka)	605
14	COX1b Oryzias	*Oryzias latipes* (Japanese medaka)	606
15	COX2 Oryzias	*Oryzias latipes* (Japanese medaka)	609
16	COX1a Gasterosteus	*Gasterosteus aculeatus* (stickleback)	598
17	COX1b Gasterosteus	*Gasterosteus aculeatus* (stickleback)	598
18	COX2 Gasterosteus	*Gasterosteus aculeatus* (stickleback)	620
19	COX1a Tetraodon	*Tetraodon nigroviridis* (Green Spotted Puffer)	1023
20	COX1b Tetraodon	*Tetraodon nigroviridis* (Green Spotted Puffer)	589
21	COX2 Tetraodon	*Tetraodon nigroviridis* (Green Spotted Puffer)	456
22	COX1 Oncorhynchus	*Oncorhynchus mykiss* (rainbow trout)	624
23	COX2a Oncorhynchus	*Oncorhynchus mykiss* (rainbow trout)	607
24	COX2b Oncorhynchus	*Oncorhynchus mykiss* (rainbow trout)	609
25	COX1 Danio	*Danio rerio* (zebrafish)	597
26	COX2a Danio	*Danio rerio* (zebrafish)	601
27	COX2b Danio	*Danio rerio* (zebrafish)	606
28	COX1 Monodelphis	*Monodelphis domestica* (gr. S.t. opossum)	625
29	COX1b Monodelphis	*Monodelphis domestica* (gr. S.t. opossum)	627
30	COX1b2 Monodelphis	*Monodelphis domestica* (gr. S.t. opossum)	729
31	COX2 Monodelphis	*Monodelphis domestica* (gr. S.t. opossum)	608
32	COX1 Gallus	*Gallus gallus* (rd. jungle fowl)	649
33	COX2 Gallus	*Gallus gallus* (rd. jungle fowl)	603
34	COX1 Bos	*Bos taurus* (cattle)	600
35	COX2 Bos	*Bos taurus* (cattle)	604
36	COX1 Xenopus l	*Xenopus laevis* (Afr. Claw. Frog)	587
37	COX2 Xenopus l	*Xenopus laevis* (Afr. Claw. Frog)	604
38	COX1 Canis	*Canis lupus familiaris* (dog)	633
39	COX2 Canis	*Canis lupus familiaris* (dog)	604
40	COX1 Homo	*Homo sapiens* (human)	599
41	COX2 Homo	*Homo sapiens* (human)	604
42	COX1 Rattus	*Rattus norvegicus* (norway rat)	602
43	COX2 Rattus	*Rattus norvegicus* (norway rat)	604
44	COX1 Mus	*Mus musculus* (house mouse)	602

Table 2-7. Continued.

#	Name	Organism	Size (AA)
45	COX2 Mus	*Mus musculus* (house mouse)	604
46	COX1 Oryctolagus	*Oryctolagus cuniculus* (rabbit)	606
47	COX2 Oryctolagus	*Oryctolagus cuniculus* (rabbit)	604
48	COX1 Ovis	*Ovis aries* (sheep)	600
49	COX2 Ovis	*Ovis aries* (sheep)	603
50	sCOX Squalus	*Squalus acanthias* (spiny dogfish)	593

Table 2-8. LRTs between amino acid based models of COX evolution

Evolutionary Model*	Ln liklihood (Ln L) Score	(Ln L_1—LnL_2) ·2	Δ d.f.[@]	P[#]
WAG	-26632			
WAG + I	-26315	317	1	< 0.01
WAG + Γ	-25294	1021	1	< 0.01
WAG + I + Γ	-25294	0	2	> 0.05

*Evolutionary models are arranged from the most simple to the most complex. Only models using the WAG matrix were evaluated. I represents an invariable sites parameter and Γ represents an among site variation parameter

[@] Δ d.f. indicates the difference in free parameters between the complex and simple models

[#]P values represent the comparison of simple to complex models and were obtained using a chi-squared distribution test

Table 2-9. LRTs between tree topologies with altered positions of hCOX and lCOX

Tree Topology Name*	Ln liklihood (Ln *L*) Score	Estimated $\alpha^{@}$	(Ln L_1—LnL_2)·$2^{\#}$
COX2 Hag (most likely)	-25294.22	0.737	
COX1Lamp/COX2Hag	-25295.57	0.737	2.70
COX2 Agnatha	-25299.09	0.737	9.74
Lamp/Amp	-25299.27	0.737	10.1
COX2 Vert	-25299.61	0.736	10.7
COX1 Hag	-25300.92	0.735	13.4
COX1 Vert	-25301.22	0.735	14.0
Ancestral Hag	-25301.56	0.735	14.6
COX1 Agnatha	-25301.78	0.735	15.1
COX1Hag/COX2Lamp	-25302.19	0.735	15.9
Ancestral agnatha	-25302.55	0.735	16.7
COX1 Lamp	-25303.25	0.735	18.1
COX2 Lamp	-25303.85	0.735	19.3
Agnatha/Amp	-25308.82	0.735	29.2
Hag/Amp	-25308.90	0.735	29.4
Hag Internal	-25321.15	0.737	53.9
Lamp Internal	-25322.77	0.736	57.1
Lamp Mammal	-25341.41	0.732	94.4
Lamp Teleost	-25344.68	0.735	101
Hag Teleost	-25350.30	0.734	112
Hag mammal	-25408.58	0.731	229

*Each tree has the same topology as the most likely tree (Figure 2-9) but with alternative placements of hagfish (hCOX) and lamprey (lCOX) sequences as noted in Figure 2-15.

$^{@}\alpha$ represents the shape value under the Γ model

$^{\#}$Where Ln_1 is the most likely tree score and Ln_2 is the tree score of the topology in question

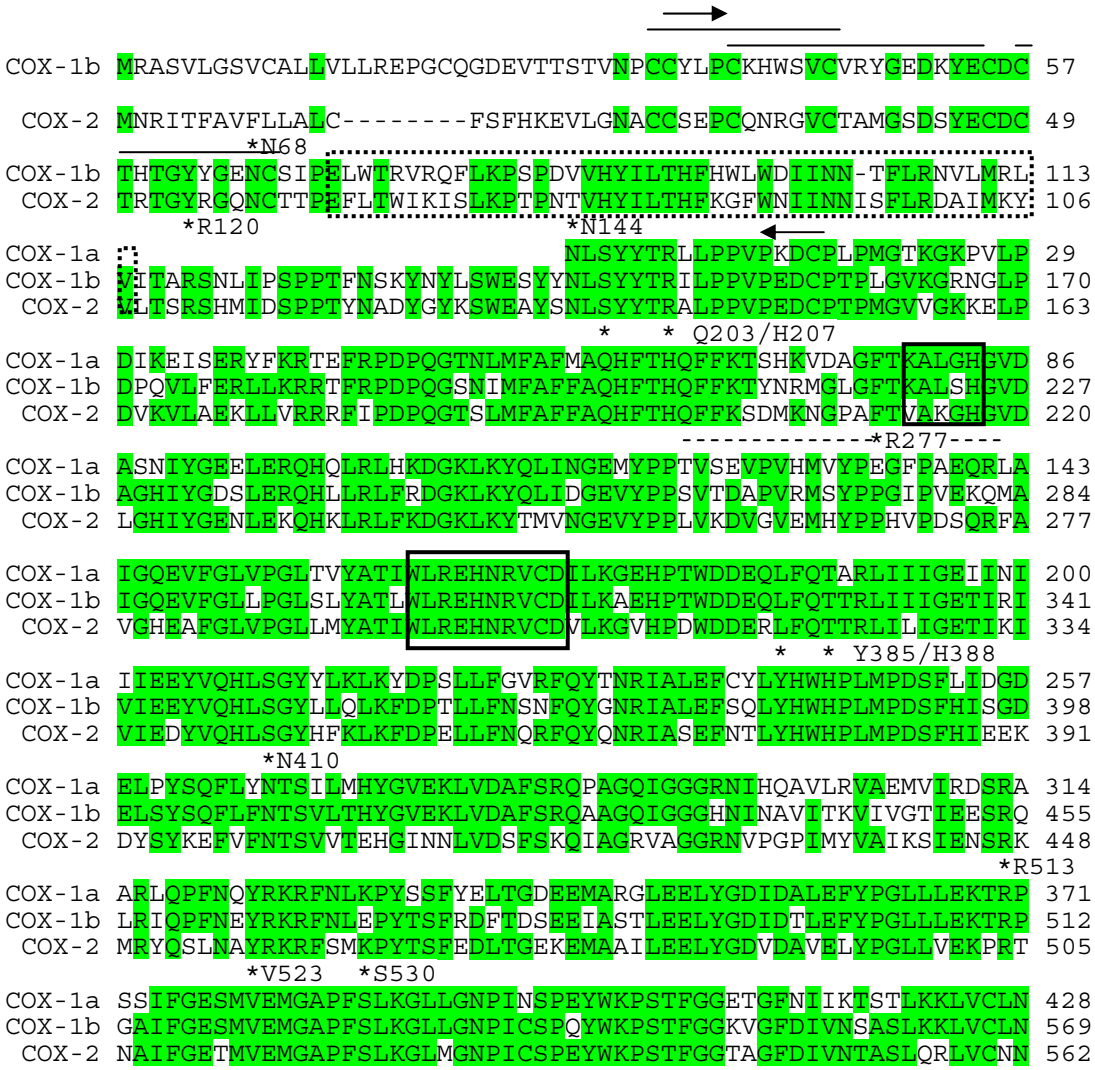

Figure 2-1. Amino acid alignment of the forms of COX sequenced from the gills of the euryhaline killifish (*Fundulus heteroclitus*). The COX-2 sequence is from GenBank (Accession #AAS21313) and the others were obtained in this study. Amino acids that share at least 60% identity across sequences are highlighted. *Indicates amino acids (numbered based on ovine COX-1) involved in COX function: active site of COX (tyrosine-385, histidine-388, and serine-530), substrate binding site (arginine-120), *N*-glycosylation sites (asparagine-68, 144, and 410), sites for peroxidase activity (glutamine-203 and histidine-207), and the two sites which define conformational differences in channels between COX-1 and COX-2 in mammals (arginine-513 and valine-523) (Ishikawa *et al*., 2007). The black boxes indicate the two domains that define the haem-binding sites and the dashed box indicates the membrane binding domain (Kulmacz *et al*., 2003; Ishikawa *et al*., 2007). The solid lines represent disulfide bonds, with the beginning and end of the longest bond represented by arrows (Kulmacz *et al*., 2003). The dashed line represents the arginine-277 loop region, which has been shown to differ between COX-1 and COX-2 forms in mammals (Yang *et al*., 2003). The numbers to the right of the sequences indicate the amino acid number of each sequence.

```
COX-1a TK-WCPYVDFHVPRNEEERKPS----------------------TEL          452
COX-1b SR-TCPYVAFSVPAEEEPGRNDGKERS-----------------SEL          598
 COX-2 VRGPCPVASFYVPDVKETGSMTINSSTSHSRDSNINPTVILKERTSEL          610
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Figure 2-1. Continued.

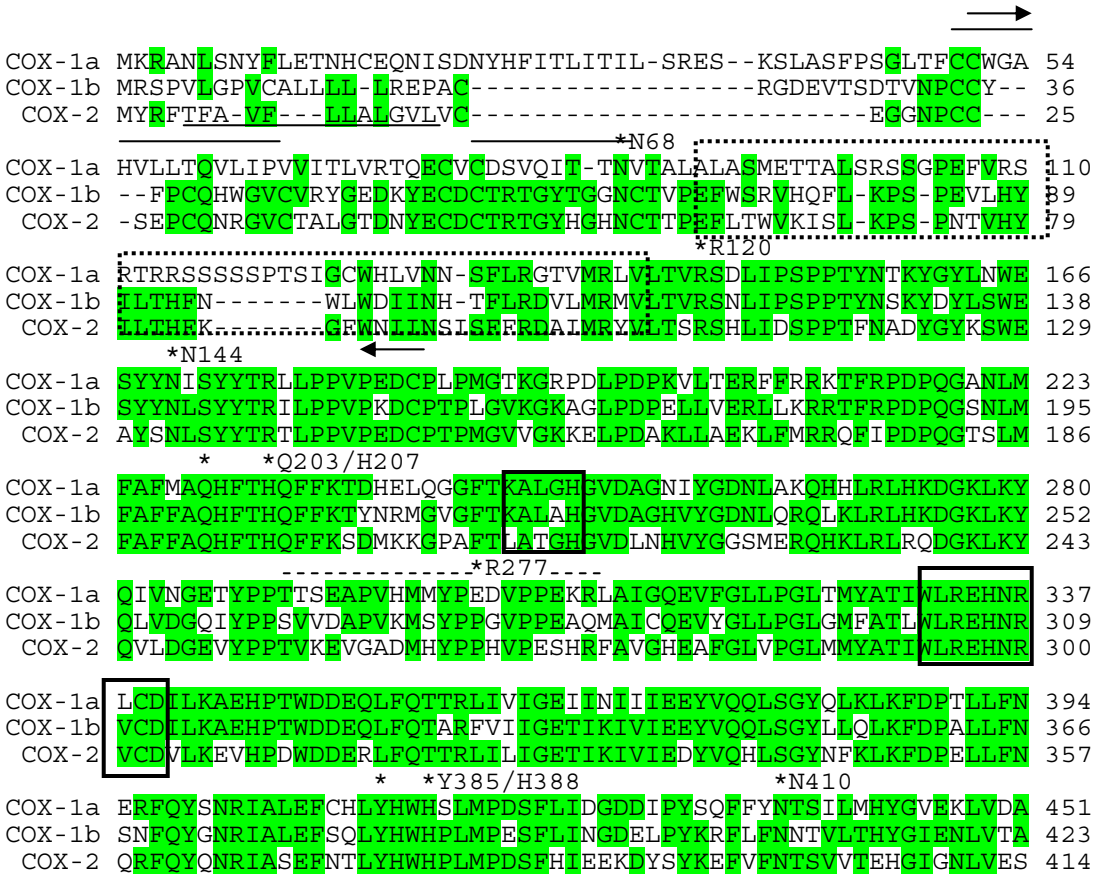


Figure 2-2. Amino acid alignment of the three forms of COX sequenced from the gills of the longhorn sculpin (*Myoxocephalus octodecemspinosus*): COX-1a, COX-1b, and COX-2. All sequences were obtained during this study. Amino acids that share at least 60% identity across sequences are highlighted. *Indicates amino acids (numbered based on ovine COX-1) involved in COX function: active site of COX (tyrosine-385, histidine-388, and serine-530), substrate binding site (arginine-120), *N*-glycosylation sites (asparagine-68, 144, and 410), sites for peroxidase activity (glutamine-203 and histidine-207), and the two sites which define conformational differences in channels between COX-1 and COX-2 in mammals (arginine-513 and valine-523) (Yang *et al*., 2002; Ishikawa *et al*., 2007). The black boxes indicate the two domains that define the haem-binding sites and the dashed box indicates the membrane binding domain (Kulmacz *et al*., 2003; Ishikawa *et al*., 2007). The solid lines represent disulfide bonds, with the beginning and end of the longest bond represented by arrows (Kulmacz *et al*., 2003). The dashed line represents the arginine-277 loop region, which has been shown to differ between COX-1 and COX-2 forms in mammals (Yang *et al*., 2003; Kulmacz *et al*., 2003). The numbers to the right of the sequences indicate the amino acid number of each sequence.

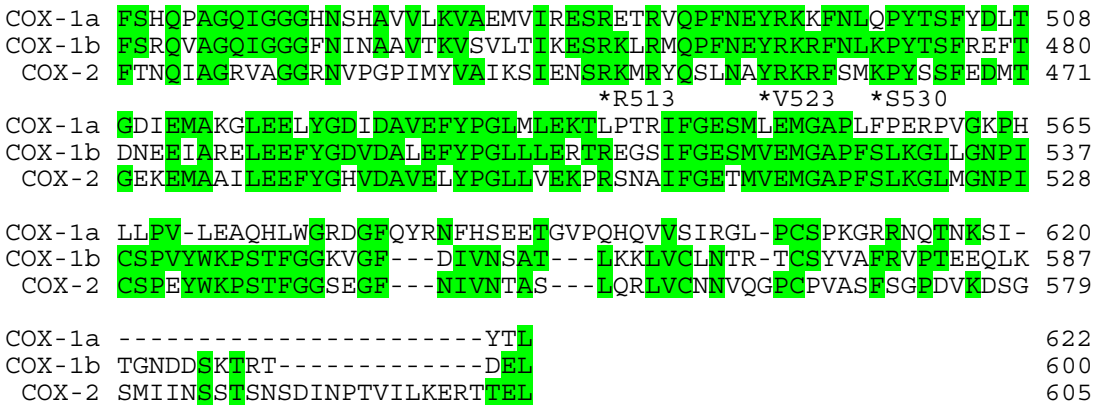


Figure 2-2. Continued.

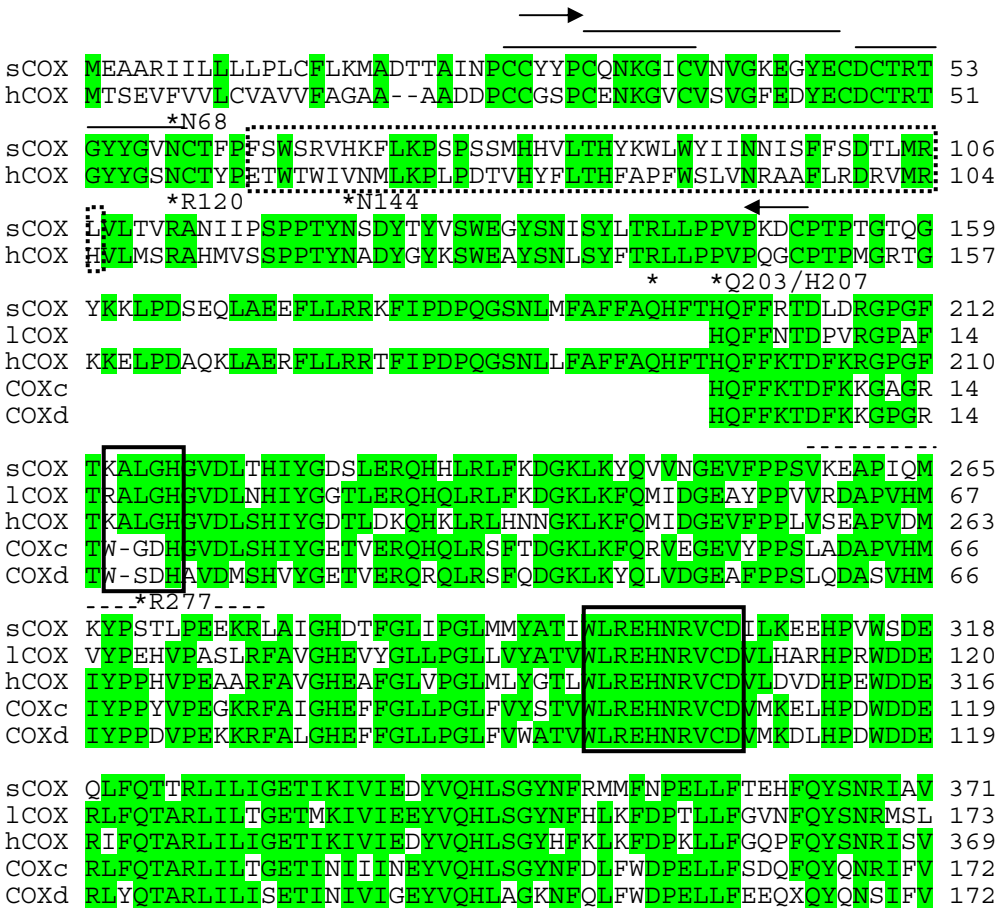


Figure 2-3. Amino acid alignment of the forms of COX sequenced from the gills of the lamprey (*Petromyzon marinus*), hagfish (*Myxine glutinosa*), and amphioxus (*Branchiostoma lancelolatum*), along with sCOX from the dogfish (Squalus acanthias, Accession #AAL37727). Amino acids that share at least 60% identity across sequences are highlighted. *Indicates amino acids (numbered based on ovine COX-1) involved in COX function: active site of COX (tyrosine-385, histidine-388, and serine-530), substrate binding site (arginine-120), *N*-glycosylation sites (asparagine-68, 144, and 410), sites for peroxidase activity (glutamine-203 and histidine-207), and the two sites which define conformational differences in channels between COX-1 and COX-2 in mammals (arginine-513 and valine-523) (Yang *et al*., 2002; Ishikawa *et al*., 2007). The black boxes indicate the two domains that define the haem-binding sites and the dashed box indicates the membrane binding domain (Kulmacz *et al*., 2003; Ishikawa *et al*., 2007). The solid lines represent disulfide bonds, with the beginning and end of the longest bond represented by arrows (Kulmacz *et al*., 2003). The dashed line represents the arginine-277 loop region, which has been shown to differ between COX-1 and COX-2 forms in mammals (Yang *et al*., 2003; Kulmacz *et al*., 2003). The numbers to the right of the sequences indicate the amino acid number of each sequence.

```
          *  *Y385/H388             *N410
sCOX EFDHLYHWHPLMPDSFIVKGQDFSYKDFLFNTDILLNLGVDALVESFSKQIAG 424
lCOX EFNHLYHWHPLMPDSLLIDGRNYSYDEFLFNPGLLADKKLMPLVRSFMRQRAG 226
hCOX EFNHLYHWHGLNPDAFRVGTQEYQYSQFLFNNTILLNHGVRGLLEAFNVQQAG 422
COXc EFNHL                                                 177
COXd EFNHL                                                 177

sCOX RIGGGRNIHQSLLHIAIATIEHGRLLRFQPYNEYRKKLGLTPYKSFQELTGER 477
lCOX TVSGGRNINKNLLHVATSIIEHGRTLRLQSLNQYRHRFNMRPFTSFLELTGDE 279
hCOX RIGGGQNIHGALLHVATASIKHGRKMRFQSLNQYRKQFGLQPYQSFEQLTGET 475
                                          *V523  *S530
sCOX EVAARLEKLYGHIDAMEFYPALLLEAPNKNSIFGESMVEMGAPFSLKGLMGNP 530
lCOX AMAAEME                                               286
hCOX EMAADLAELYSDINAMEFYLGLMVEKPRQGALFGETMVEAGAPFSLKGLMGNA 528

sCOX ICSPDYWKPSTFGGKTGFDIVNTATFEKLICLNVK-KCPYVGFHVPY------ 582
hCOX ICSPEYWKPSTFGGNRGFEIVNSASLRRLVCLNLQGPCPDVAFHVPRDNQQDV 581

sCOX NVDNDYEREKGKP------------STEL                          593
hCOX VVNVTGSQGGSDGVTTTPHYVADQQSREL                          610
```

Figure 2-3. Continued.

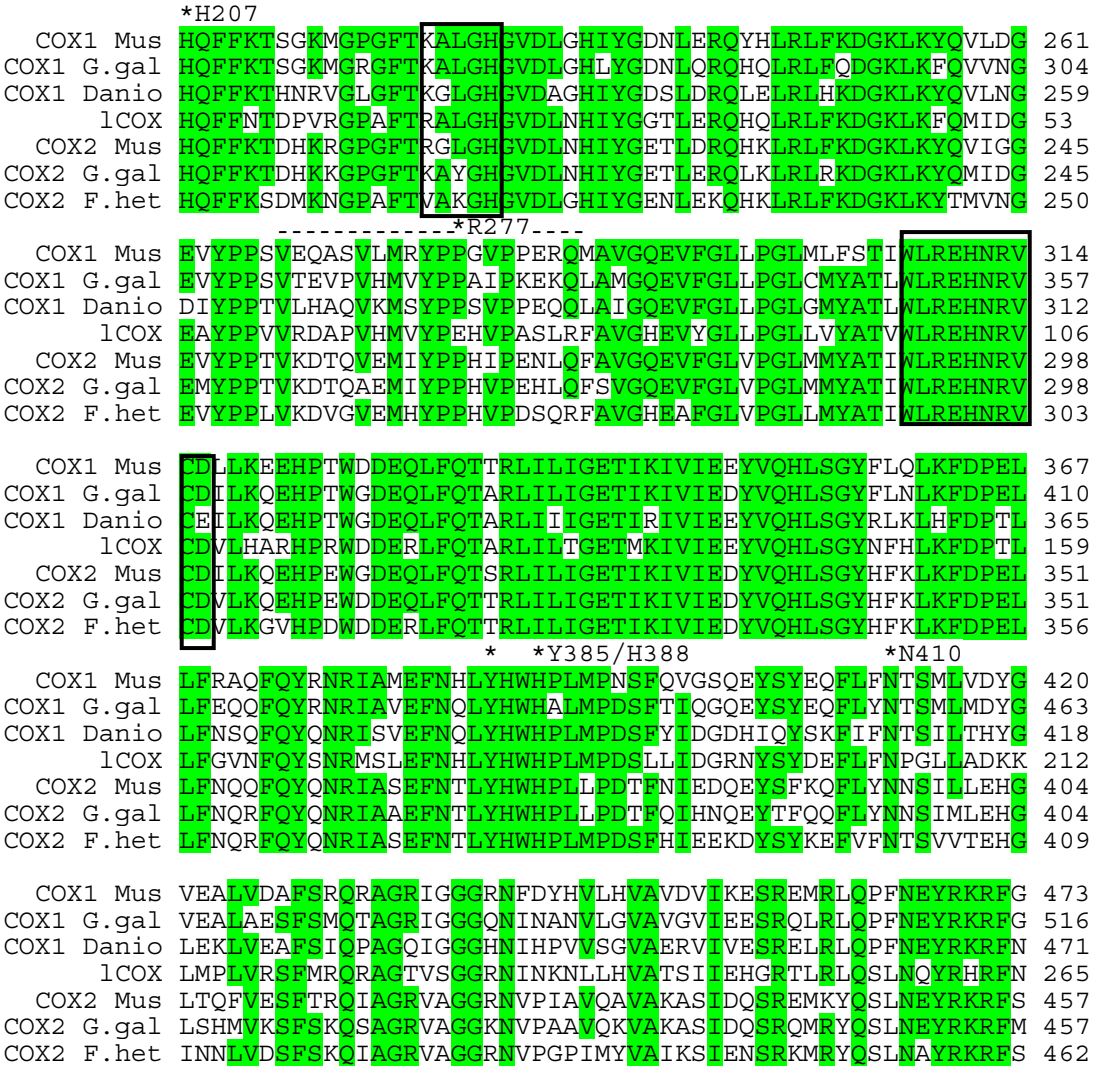


Figure 2-4. Amino acid alignment lCOX sequenced from the gills of the lamprey (*Petromyzon marinus*), with COX-1 and COX-2 forms from vertebrates (Accession # NP_032995, NP_035328, XP_425326, XP_422297, AAS21313, and NP_705942). Amino acids that share at least 60% identity across sequences are highlighted. *Indicates amino acids (numbered based on ovine COX-1) involved in COX function: active site of COX (tyrosine-385 and histidine-388), *N*-glycosylation site (asparagine-410), and sites for peroxidase activity (glutamine-203 and histidine-207) (Yang *et al*., 2002; Ishikawa *et al*., 2007). The black boxes indicate the two domains that define the haem-binding sites (Ishikawa *et al*., 2007). The dashed line represents the arginine-277 loop region, which has been shown to differ between COX-1 and COX-2 forms in mammals (Yang *et al*., 2003; Kulmacz *et al*., 2003). The numbers to the right of the sequences indicate the amino acid number of each sequence.

```
  COX1 Mus LKPYTSFQELTGEKEMAAEL 493
COX1 G.gal LKPYTSFQELTGEEDKAAEL 536
COX1 Danio LKPYTSFAELTGEQEMSKEL 491
      lCOX MRPFTSFLELTGDEAMAAEM 285
  COX2 Mus LKPYTSFEELTGEKEMAAEL 477
COX2 G.gal LKPFKSFEELTGEKEMAAEL 477
COX2 F.het MKPYTSFEDLTGEKEMAAIL 482
```

Figure 2-4. Continued.

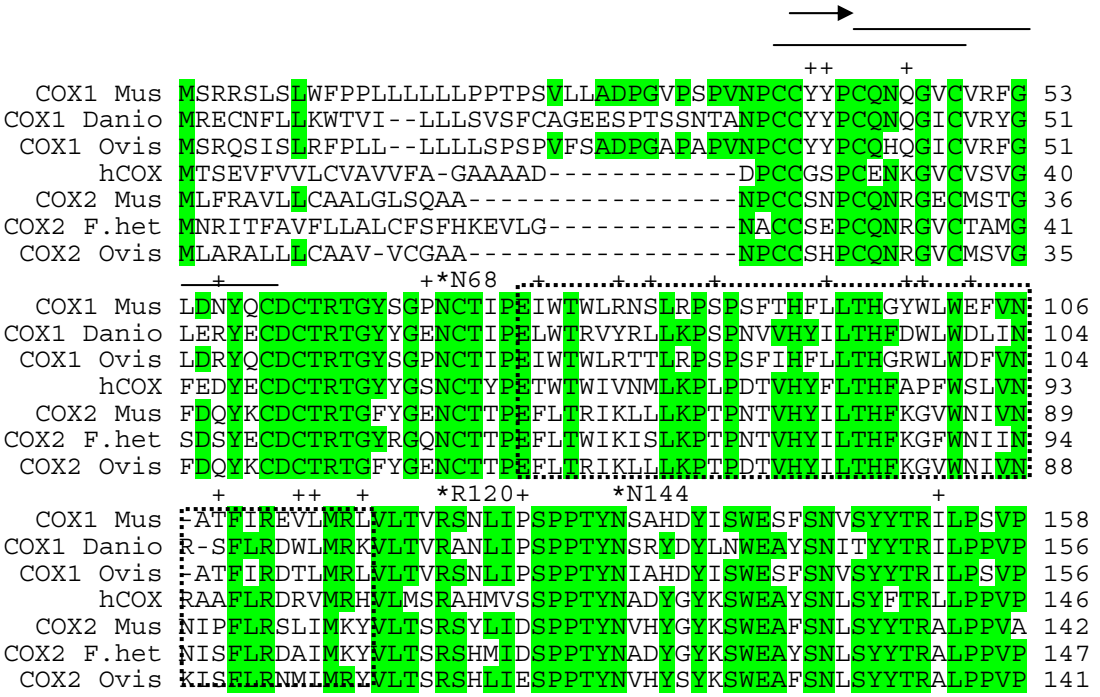


Figure 2-5. Amino acid alignment of lCOX sequenced from the gills of the lamprey (*Petromyzon marinus*), with COX-1 and COX-2 forms from vertebrates (Accession # NP_032995, NP_035328, XP_425326, XP_422297, AAS21313, and NP_705942). Amino acids that share at least 60% identity across sequences are highlighted. *Indicates amino acids (numbered based on ovine COX-1) involved in COX function: active site of COX (tyrosine-385, histidine-388, and serine-530), substrate binding site (arginine-120), *N*-glycosylation sites (asparagine-68, 144, and 410), sites for peroxidase activity (glutamine-203 and histidine-207), and the two sites which define conformational differences in channels between COX-1 and COX-2 in mammals (arginine-513 and valine-523) (Yang *et al*., 2002; Ishikawa *et al*., 2007). +Indicates amino acids unique to hCOX. The black boxes indicate the two domains that define the haem-binding sites and the dashed box indicates the membrane binding domain (Kulmacz *et al*., 2003; Ishikawa *et al*., 2007). The solid lines represent disulfide bonds, with the beginning and end of the longest bond represented by arrows (Kulmacz *et al*., 2003). The dashed line represents the arginine-277 loop region, which has been shown to differ between COX-1 and COX-2 forms in mammals (Yang *et al*., 2003; Kulmacz *et al*., 2003). The numbers to the right of the sequences indicate the amino acid number of each sequence.

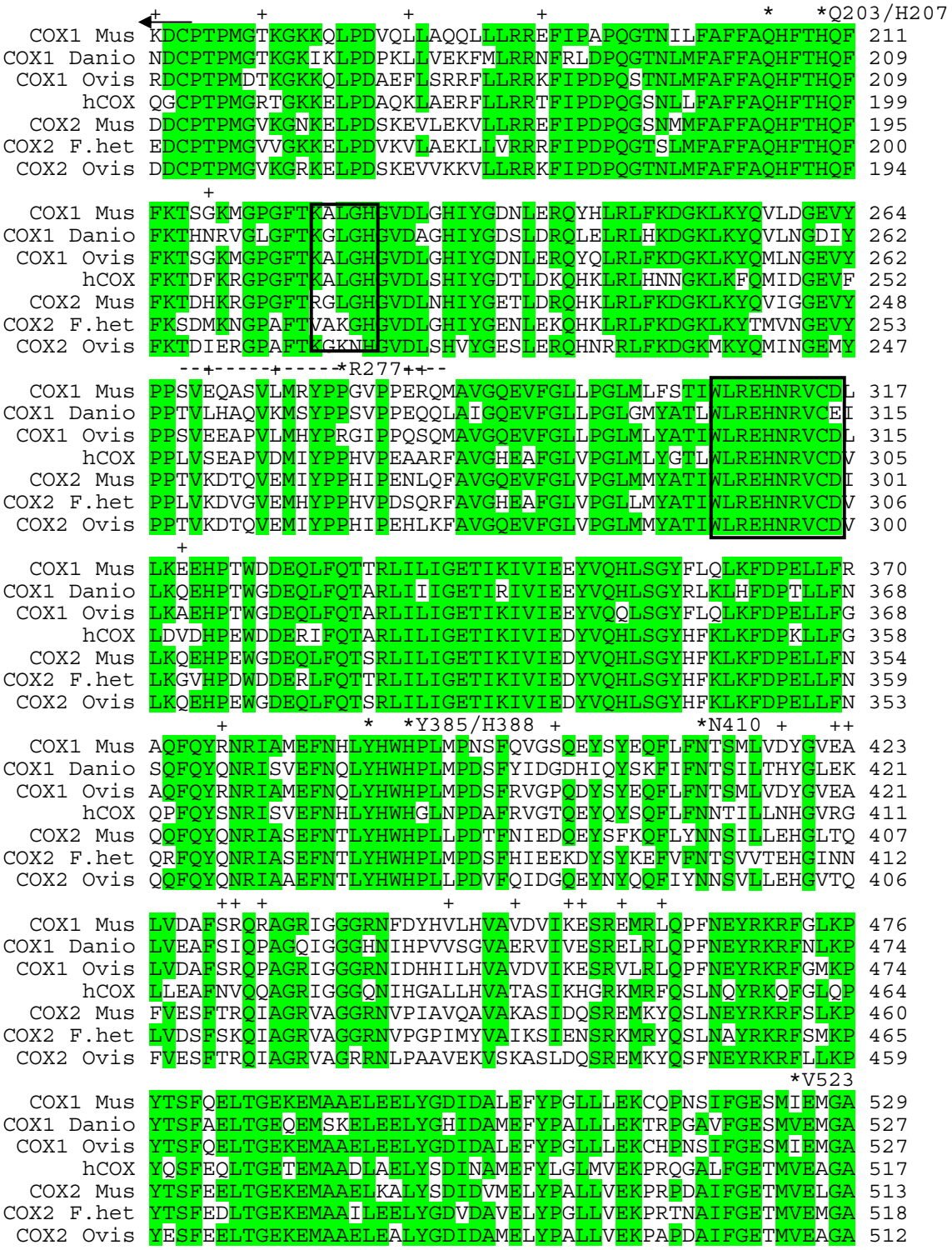


Figure 2-5. Continued

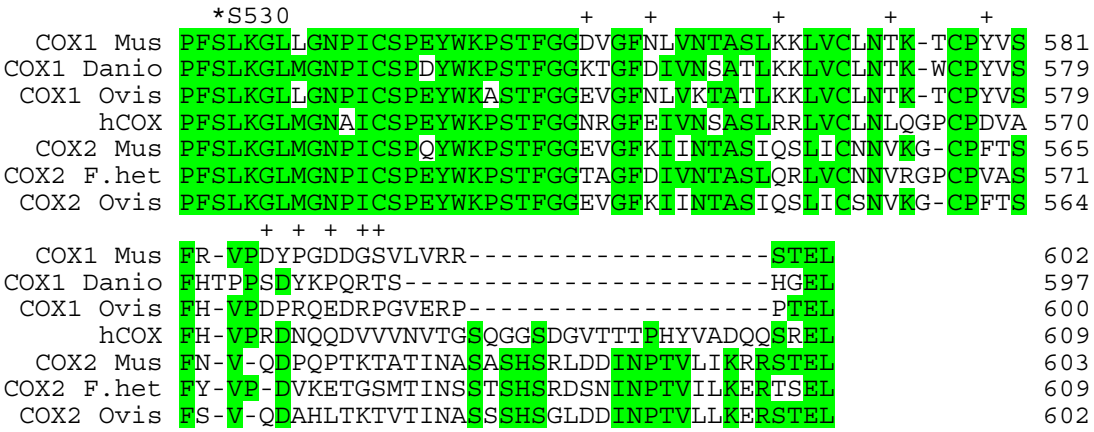


Figure 2-5. Continued

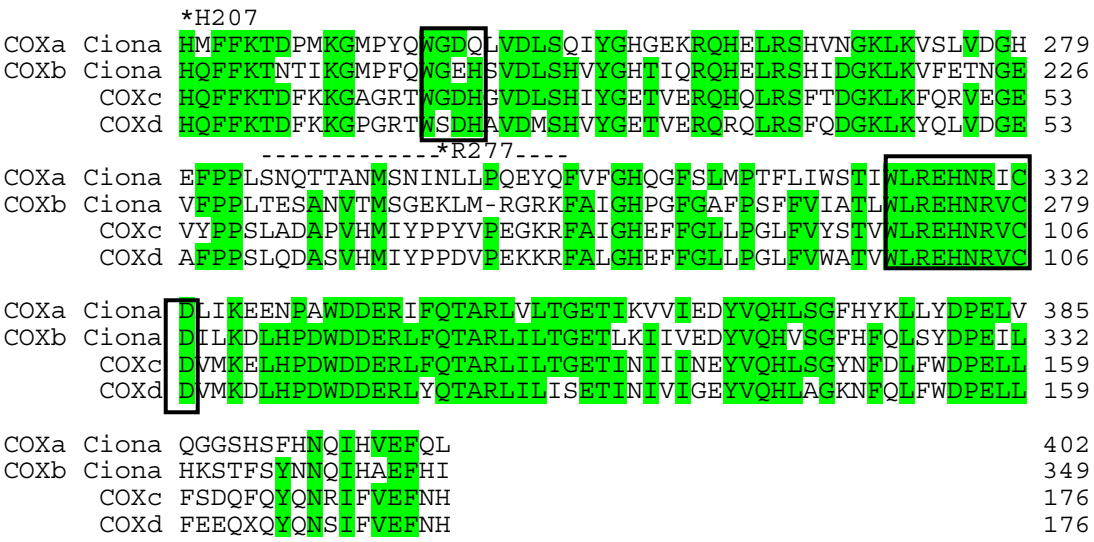


Figure 2-6. Amino acid alignment of COXc and COXd sequences from the lancelet (*Branchiostoma lancelolatum*) with COXa and COXb sequences from the sea squirt (*Ciona intestinalis*). Amino acids that share at least 60% identity across sequences are highlighted. *Indicates the amino acid (numbered based on ovine COX-1) involved in peroxidase activity (histidine-207) (Yang *et al*., 2002; Ishikawa *et al*., 2007). The black boxes indicate the two domains that define the haem-binding sites (Ishikawa *et al*., 2007). The dashed line represents the arginine-277 loop region, which has been shown to differ between COX-1 and COX-2 forms in mammals (Yang *et al*., 2003; Kulmacz *et al*., 2003). The numbers to the right of the sequences indicate the amino acid number of each sequence.

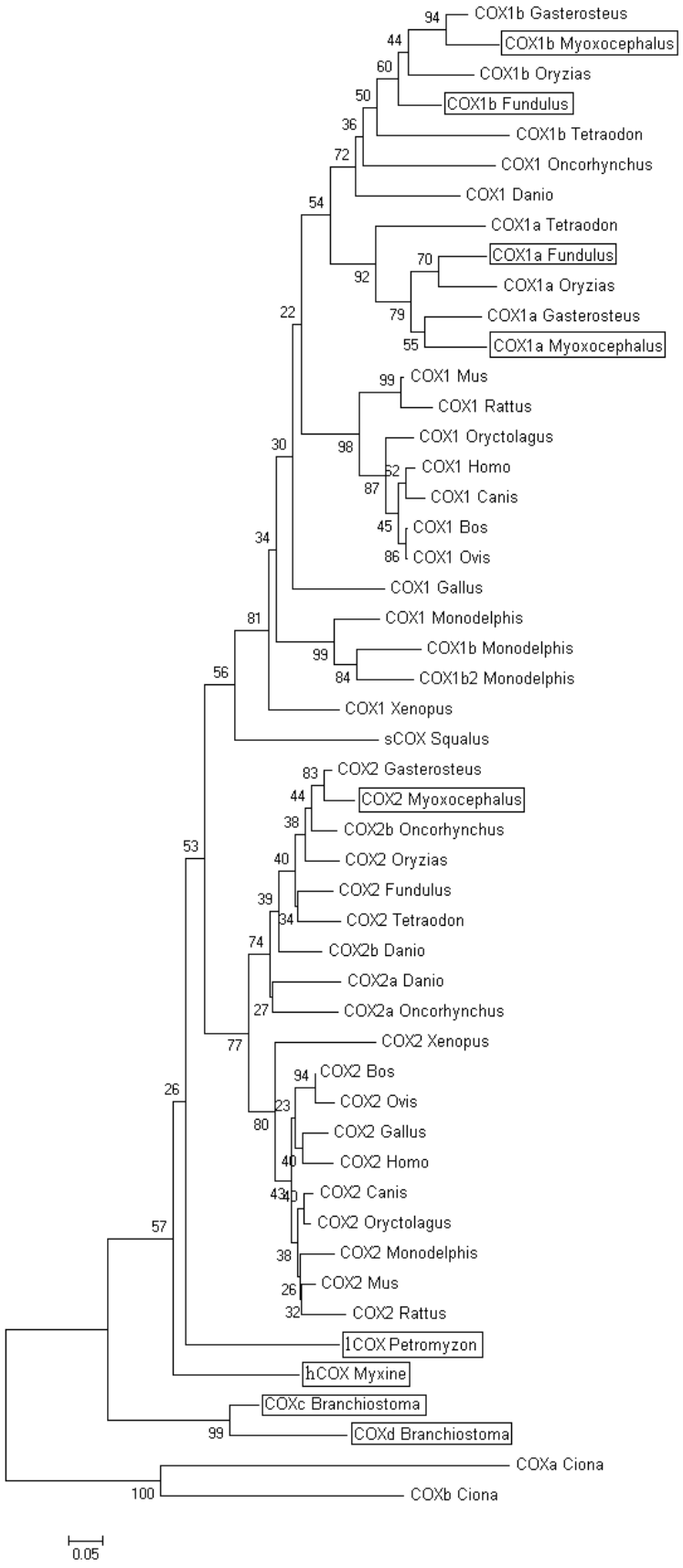

COX1b Gasterosteus
COX1b Myoxocephalus
COX1b Oryzias
COX1b Fundulus
COX1b Tetraodon
COX1 Oncorhynchus
COX1 Danio
COX1a Tetraodon
COX1a Fundulus
COX1a Oryzias
COX1a Gasterosteus
COX1a Myoxocephalus
COX1 Mus
COX1 Rattus
COX1 Oryctolagus
COX1 Homo
COX1 Canis
COX1 Bos
COX1 Ovis
COX1 Gallus
COX1 Monodelphis
COX1b Monodelphis
COX1b2 Monodelphis
COX1 Xenopus
sCOX Squalus
COX2 Gasterosteus
COX2 Myoxocephalus
COX2b Oncorhynchus
COX2 Oryzias
COX2 Fundulus
COX2 Tetraodon
COX2b Danio
COX2a Danio
COX2a Oncorhynchus
COX2 Xenopus
COX2 Bos
COX2 Ovis
COX2 Gallus
COX2 Homo
COX2 Canis
COX2 Oryctolagus
COX2 Monodelphis
COX2 Mus
COX2 Rattus
1COX Petromyzon
hCOX Myxine
COXc Branchiostoma
COXd Branchiostoma
COXa Ciona
COXb Ciona
0.05

Figure 2-7. Evolutionary history of COX sequences inferred using the minimum evolution method with a distance optimality criterion. The optimal tree with the sum of branch length = 6.63322594 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the JTT matrix-based method (Jones *et al*., 1992) and are in the units of the number of amino acid substitutions per site (scale bar = 0.05 amino acid substitutions per site). The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.737). The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1. The Neighbor-Joining algorithm was used to generate the initial tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 147 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al*., 2007). Sequences enclosed in a box are novel and reported here for the first time.

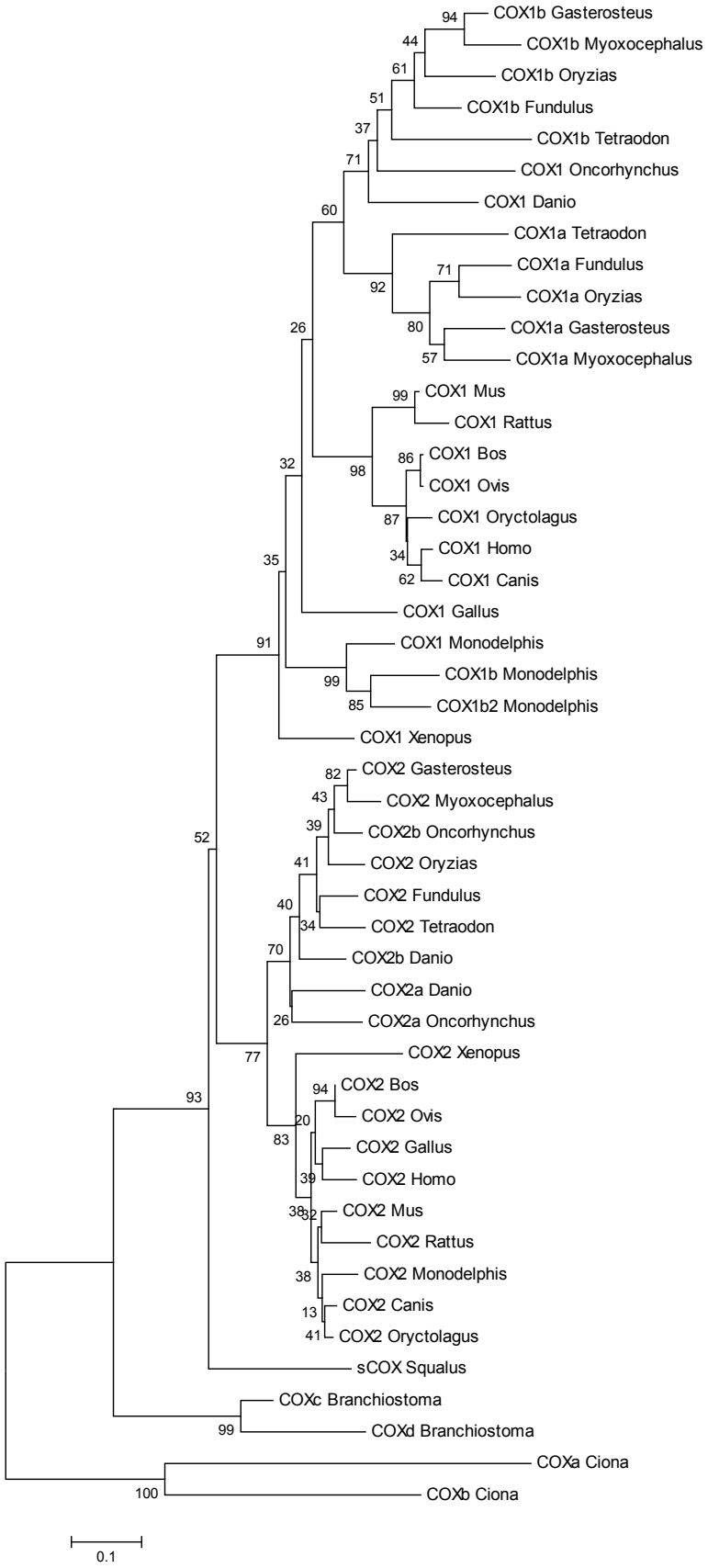

COX1b Gasterosteus
COX1b Myoxocephalus
COX1b Oryzias
COX1b Fundulus
COX1b Tetraodon
COX1 Oncorhynchus
COX1 Danio
COX1a Tetraodon
COX1a Fundulus
COX1a Oryzias
COX1a Gasterosteus
COX1a Myoxocephalus
COX1 Mus
COX1 Rattus
COX1 Bos
COX1 Ovis
COX1 Oryctolagus
COX1 Homo
COX1 Canis
COX1 Gallus
COX1 Monodelphis
COX1b Monodelphis
COX1b2 Monodelphis
COX1 Xenopus
COX2 Gasterosteus
COX2 Myoxocephalus
COX2b Oncorhynchus
COX2 Oryzias
COX2 Fundulus
COX2 Tetraodon
COX2b Danio
COX2a Danio
COX2a Oncorhynchus
COX2 Xenopus
COX2 Bos
COX2 Ovis
COX2 Gallus
COX2 Homo
COX2 Mus
COX2 Rattus
COX2 Monodelphis
COX2 Canis
COX2 Oryctolagus
sCOX Squalus
COXc Branchiostoma
COXd Branchiostoma
COXa Ciona
COXb Ciona
0.1

Figure 2-8. Evolutionary history of COX sequences excluding hagfish (hCOX) and lamprey (lCOX) sequences inferred using the minimum evolution method with a distance optimality criterion. The optimal tree with the sum of branch length = 6.18990857 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the JTT matrix-based method (Jones *et al*., 1992) and are in the units of the number of amino acid substitutions per site (scale bar = 0.1 amino acid substitutions per site). The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.742). The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1. The Neighbor-Joining algorithm was used to generate the initial tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 147 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al*., 2007).

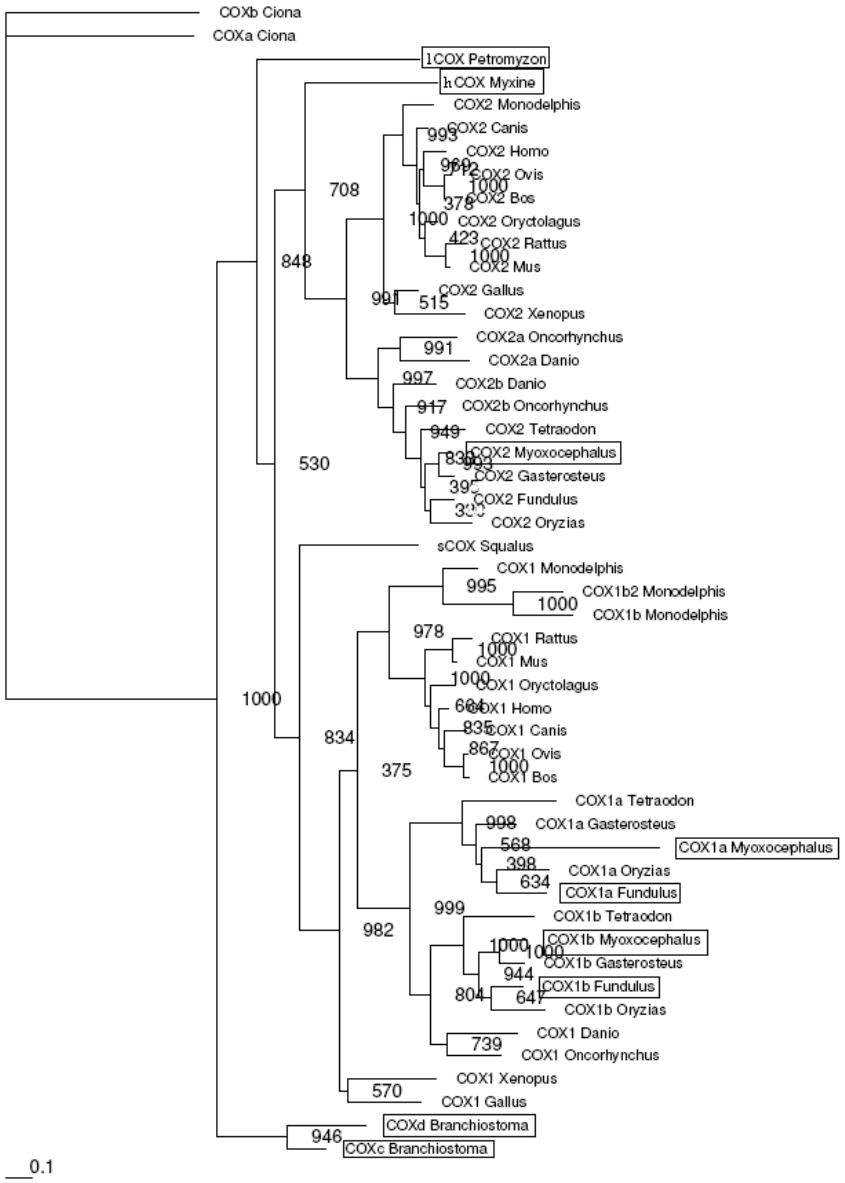


Figure 2-9. Evolutionary history of COX sequences inferred using a maximum liklihood optimality criterion. The most likely tree with an Ln *L* score = -25294.222410 is shown. The number of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the WAG matrix-based method (Whelan and Goldman, 2001) and are in the units of the number of amino acid substitutions per site (scale bar = 0.1 amino acid substitutions per site). The rate variation among sites was modeled with a gamma distribution (shape parameter estimated = 0.737). Phylogenetic analyses were conducted using PhyML (Guindon and Gascuel, 2003). Sequences enclosed in a rectangle are novel and reported here for the first time.

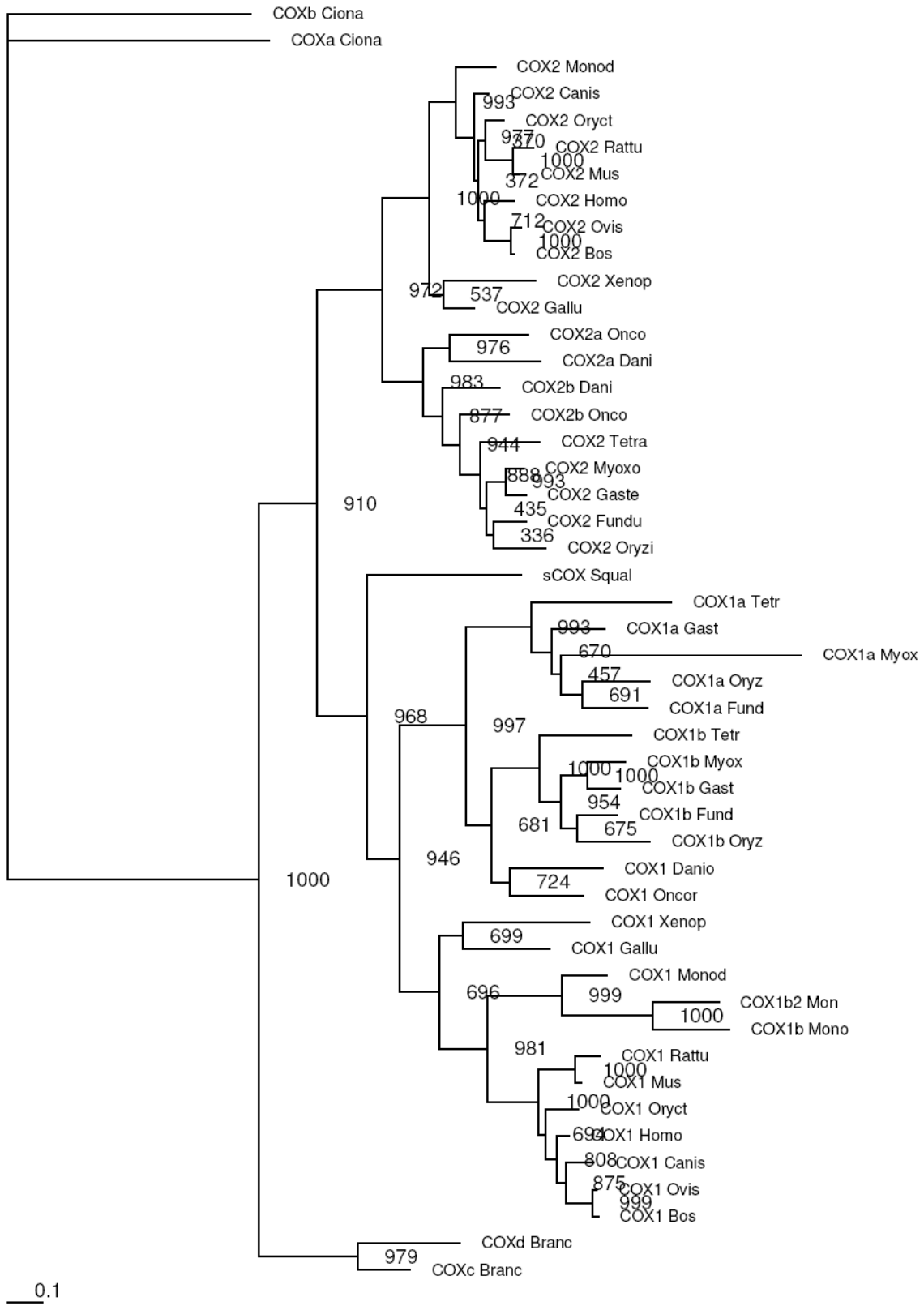

COXb Ciona
COXa Ciona
COX2 Monod
COX2 Canis
993
COX2 Oryct
937
370
COX2 Rattu
1000
COX2 Mus
372
1000
COX2 Homo
712
COX2 Ovis
1000
COX2 Bos
COX2 Xenop
972
537
COX2 Gallu
COX2a Onco
976
COX2a Dani
983
COX2b Dani
877
COX2b Onco
944
COX2 Tetra
COX2 Myoxo
888
993
COX2 Gaste
910
435
COX2 Fundu
336
COX2 Oryzi
sCOX Squal
COX1a Tetr
993
COX1a Gast
670
COX1a Myox
457
COX1a Oryz
691
COX1a Fund
968
997
COX1b Tetr
1000
COX1b Myox
1000
COX1b Gast
954
COX1b Fund
681
675
COX1b Oryz
946
COX1 Danio
1000
724
COX1 Oncor
COX1 Xenop
699
COX1 Gallu
COX1 Monod
696
999
COX1b2 Mon
1000
COX1b Mono
981
COX1 Rattu
1000
COX1 Mus
1000
COX1 Oryct
694
COX1 Homo
808
COX1 Canis
875
COX1 Ovis
999
COX1 Bos
COXd Branc
979
COXc Branc
0.1

Figure 2-10. Evolutionary history of COX sequences excluding sequences from the hagfish (hCOX) and lamprey (lCOX) inferred using a maximum liklihood optimality criterion. The most likely tree with an Ln *L* score = -24242.30059 is shown. The number of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the WAG matrix-based method (Whelan and Goldman, 2001) and are in the units of the number of amino acid substitutions per site (scale bar = 0.1 amino acid substitutions per site). The rate variation among sites was modeled with a gamma distribution (shape parameter estimated = 0.742). Phylogenetic analyses were conducted using PhyML (Guindon and Gascuel, 2003). Sequence names are abbreviated from Figure 2-9.

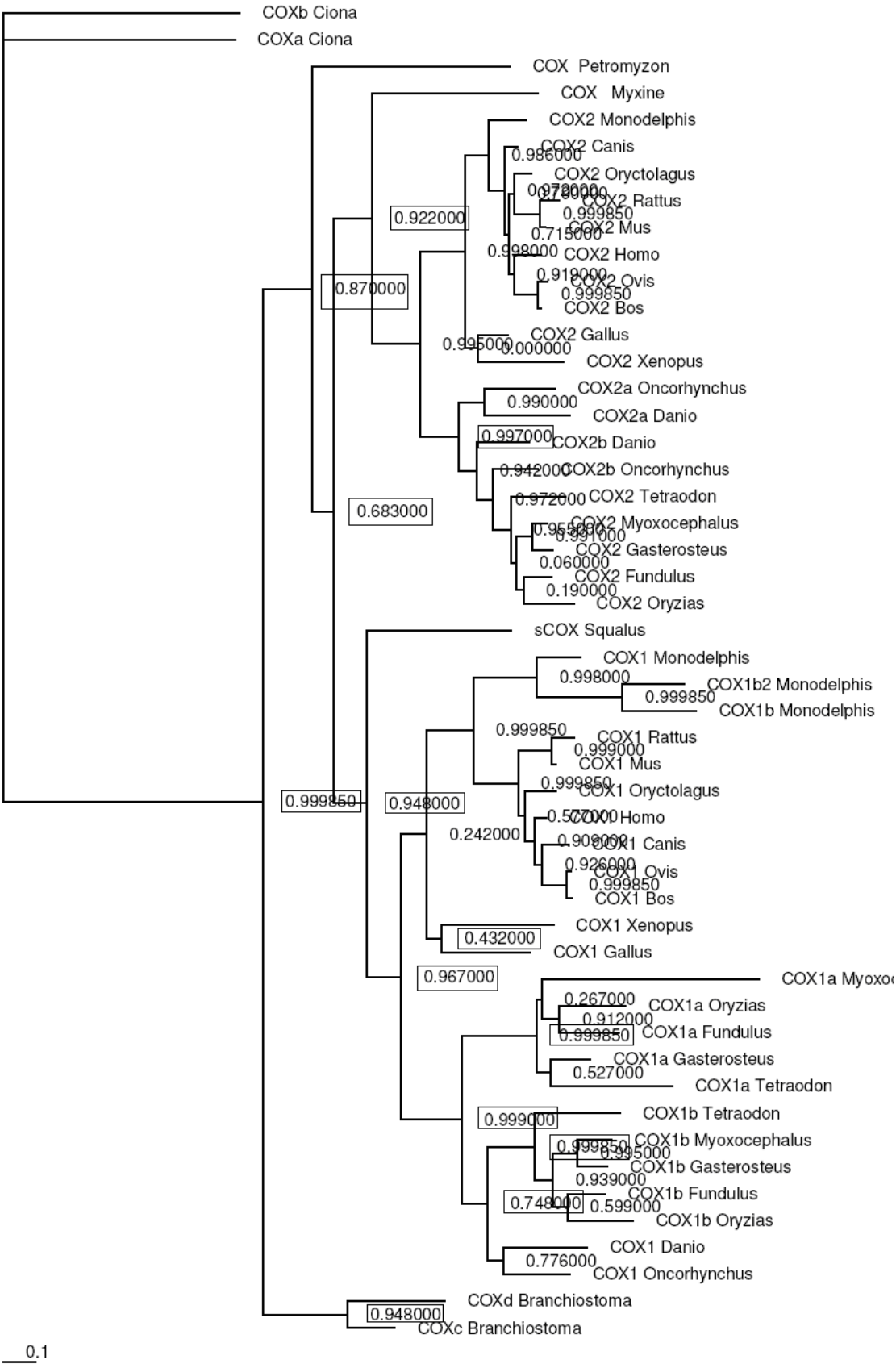
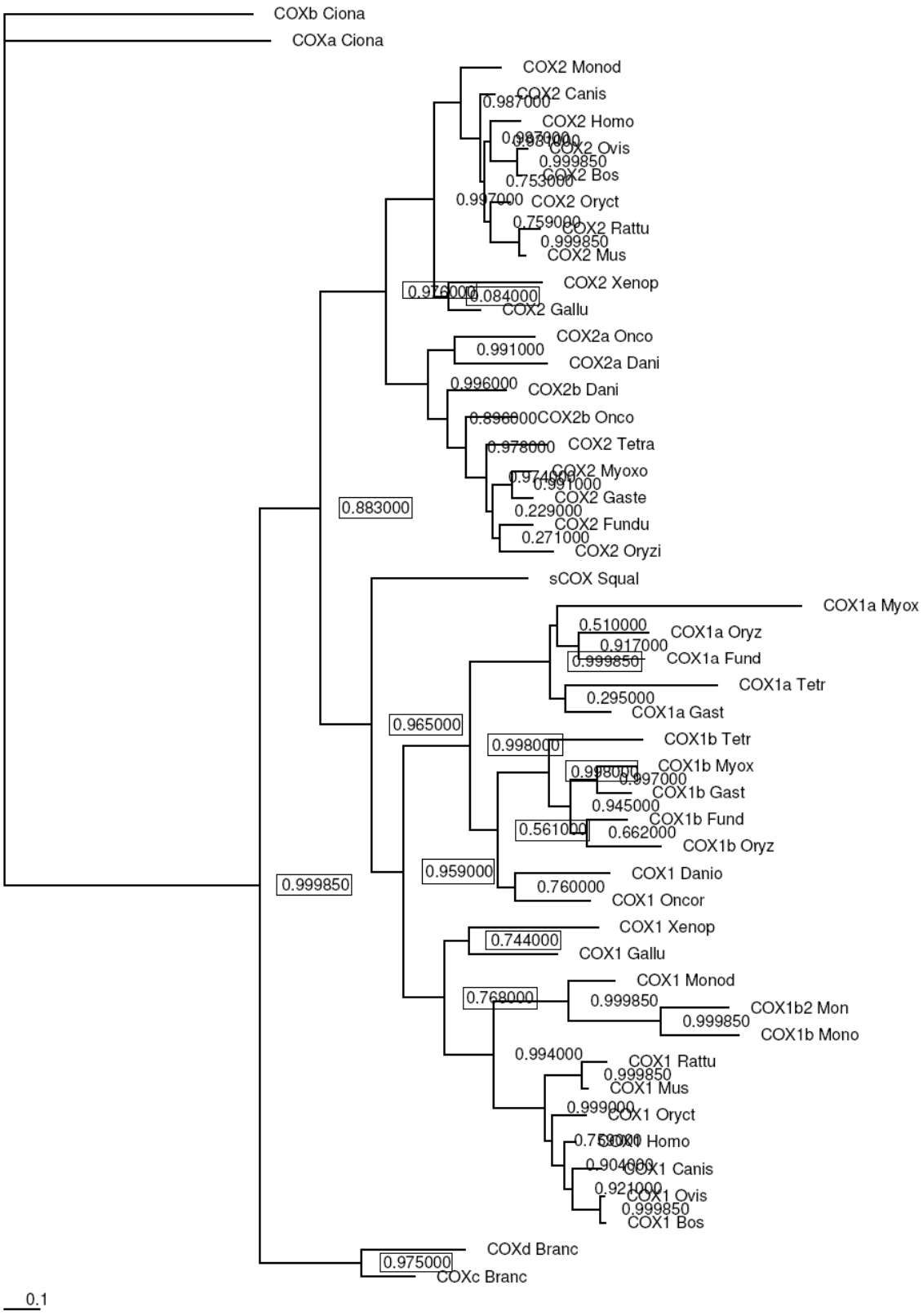

COXb Ciona
COXa Ciona
COX Petromyzon
COX Myxine
COX2 Monodelphis
COX2 Canis
0.986000
COX2 Oryctolagus
COX2 Rattus
0.999850
COX2 Mus
0.715000
0.922000
COX2 Homo
0.919000
COX2 Ovis
0.999850
COX2 Bos
0.870000
COX2 Gallus
0.995000
0.000000
COX2 Xenopus
COX2a Oncorhynchus
0.990000
COX2a Danio
0.997000
COX2b Danio
0.942000
COX2b Oncorhynchus
0.972000
COX2 Tetraodon
0.683000
COX2 Myxoocephalus
0.991000
COX2 Gasterosteus
0.060000
COX2 Fundulus
0.190000
COX2 Oryzias
sCOX Squalus
COX1 Monodelphis
0.998000
COX1b2 Monodelphis
0.999850
COX1b Monodelphis
0.999850
COX1 Rattus
0.999000
COX1 Mus
0.999850
COX1 Oryctolagus
0.999850
0.948000
COX1 Homo
0.242000
COX1 Canis
0.926000
COX1 Ovis
0.999850
COX1 Bos
COX1 Xenopus
0.432000
COX1 Gallus
0.967000
COX1a Myoxo
0.267000
COX1a Oryzias
0.912000
0.999850
COX1a Fundulus
COX1a Gasterosteus
0.527000
COX1a Tetraodon
0.999000
COX1b Tetraodon
COX1b Myoxocephalus
0.995000
COX1b Gasterosteus
0.939000
COX1b Fundulus
0.748000
0.599000
COX1b Oryzias
COX1 Danio
0.776000
COX1 Oncorhynchus
COXd Branchiostoma
0.948000
COXc Branchiostoma
0.1

Figure 2-11. Alternate likelihood ratio test (aLRT) scores for branch support of COX sequences. The most likely tree is shown and the topology, branch lengths, and model of evolution are the same as in Figure 2-9. Scores represent the gain in likelihood when collapsing a particular branch and are independent of bootstrap scores (Felsenstein, 1985). Scores were interpreted using a conservative estimate of the minimum of parametric chi-squared and non-parametric Shimodaira-Hasegawa-like (SH) support value interpretations (Anisimova and Gascuel, 2006). Analysis was performed using PhyML (Guindon and Gascuel, 2003). Support values of interest to the present study are enclosed in boxes.


COXb Ciona
COXa Ciona
COX2 Monod
COX2 Canis
0.987000
COX2 Homo
COX2 Ovis
0.999850
COX2 Bos
0.753000
0.997000
COX2 Oryct
0.759000
COX2 Rattu
0.999850
COX2 Mus
COX2 Xenop
0.084000
COX2 Gallu
COX2a Onco
0.991000
COX2a Dani
0.996000
COX2b Dani
COX2b Onco
0.978000
COX2 Tetra
COX2 Myoxo
0.991000
COX2 Gaste
0.883000
0.229000
COX2 Fundu
0.271000
COX2 Oryzi
sCOX Squal
COX1a Myox
0.510000
COX1a Oryz
0.917000
COX1a Fund
COX1a Tetr
0.295000
COX1a Gast
0.965000
COX1b Tetr
0.998000
COX1b Myox
0.997000
COX1b Gast
0.945000
COX1b Fund
0.561000
0.662000
COX1b Oryz
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COX1 Danio
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0.760000
COX1 Oncor
COX1 Xenop
0.744000
COX1 Gallu
COX1 Monod
0.768000
0.999850
COX1b2 Mon
0.999850
COX1b Mono
0.994000
COX1 Rattu
0.999850
COX1 Mus
0.999000
COX1 Oryct
COX1 Homo
COX1 Canis
0.921000
COX1 Ovis
0.999850
COX1 Bos
COXd Branc
0.975000
COXc Branc
0.1

Figure 2-12. Alternate likelihood ratio test (aLRT) scores for branch support of COX sequences excluding hagfish (hCOX) and lamprey (lCOX) sequences. The most likely tree is shown and the topology, branch lengths, and model of evolution are the same as in Figure 2-11. Scores represent the gain in likelihood when collapsing a particular branch and are independent of bootstrap scores (Felsenstein, 1985). Scores were interpreted using a conservative estimate of the minimum of parametric chi-squared and non-parametric Shimodaira-Hasegawa-like (SH) support value interpretations (Anisimova and Gascuel, 2006). Analysis was performed using PhyML (Guindon and Gascuel, 2003). Support values of interest to the present study are enclosed in boxes.

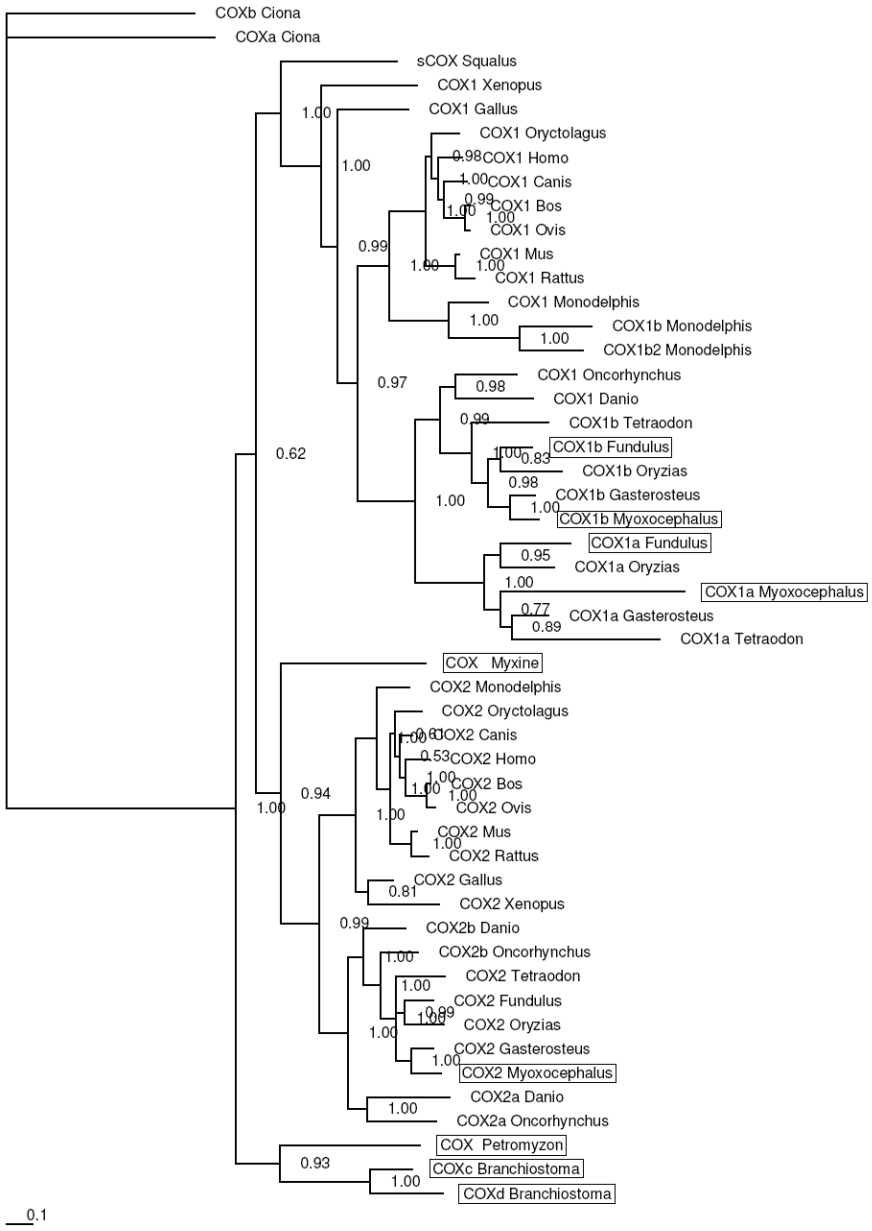

Figure 2-13. Evolutionary history of COX sequences inferred using a Bayesian optimality criterion. The consensus tree is shown. The posterior probabilities (10 million generations) are shown next to the branches. Trees were sampled every 500 generations with a burnin of 10% (401 trees). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the WAG matrix-based method (Whelan and Goldman, 2001) and are in the units of the number of amino acid substitutions per site (scale bar = 0.1 amino acid substitutions per site). The rate variation among sites was modeled with a gamma distribution (shape parameter estimated = 0.672275). Phylogenetic analyses were conducted using MrBayes 3.1.2. Sequences enclosed in a rectangle are novel and reported here for the first time.

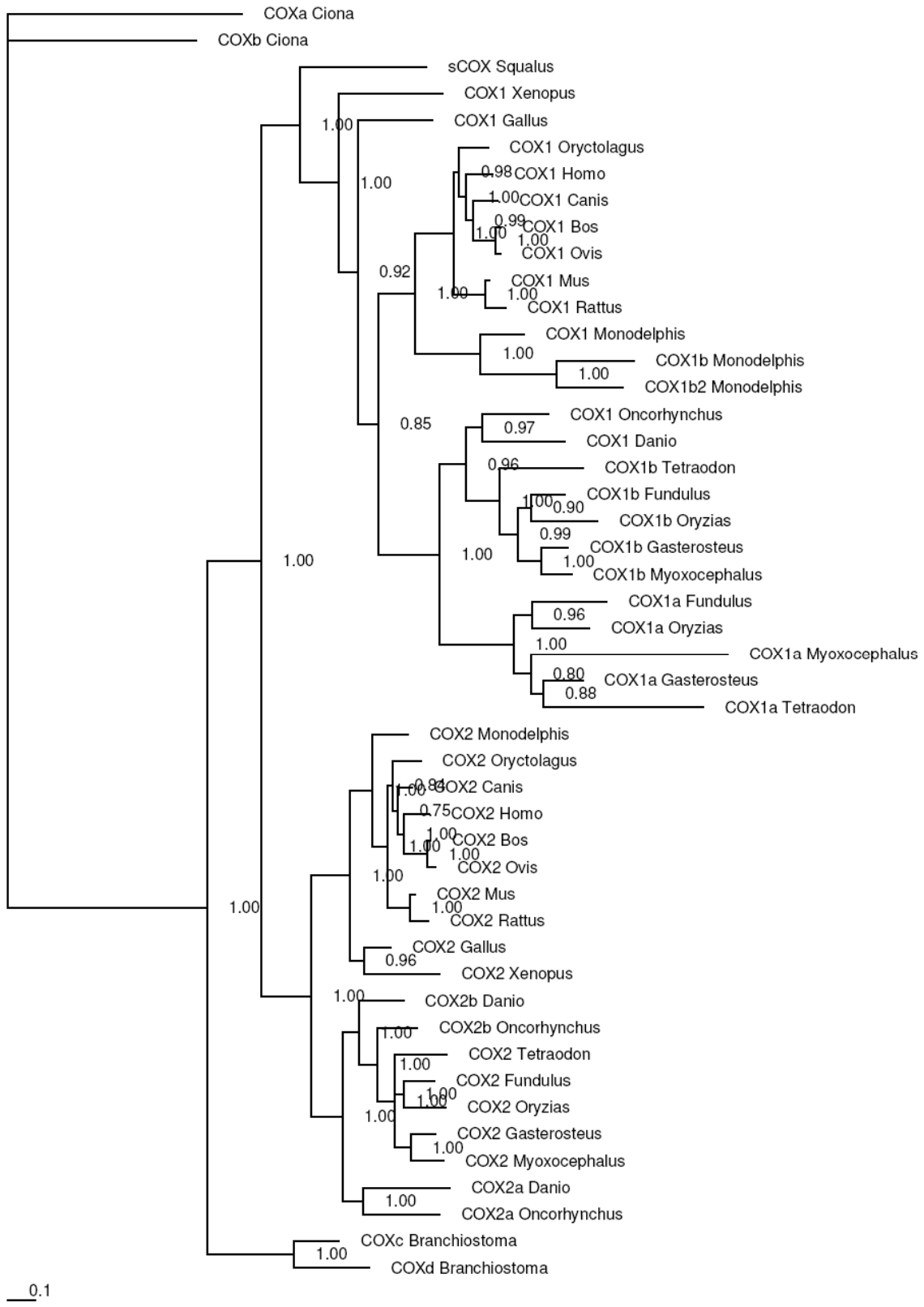
COXa Ciona
COXb Ciona
sCOX Squalus
COX1 Xenopus
COX1 Gallus
COX1 Oryctolagus
COX1 Homo
COX1 Canis
COX1 Bos
COX1 Ovis
COX1 Mus
COX1 Rattus
COX1 Monodelphis
COX1b Monodelphis
COX1b2 Monodelphis
COX1 Oncorhynchus
COX1 Danio
COX1b Tetraodon
COX1b Fundulus
COX1b Oryzias
COX1b Gasterosteus
COX1b Myoxocephalus
COX1a Fundulus
COX1a Oryzias
COX1a Myoxocephalus
COX1a Gasterosteus
COX1a Tetraodon
COX2 Monodelphis
COX2 Oryctolagus
COX2 Canis
COX2 Homo
COX2 Bos
COX2 Ovis
COX2 Mus
COX2 Rattus
COX2 Gallus
COX2 Xenopus
COX2b Danio
COX2b Oncorhynchus
COX2 Tetraodon
COX2 Fundulus
COX2 Oryzias
COX2 Gasterosteus
COX2 Myoxocephalus
COX2a Danio
COX2a Oncorhynchus
COXc Branchiostoma
COXd Branchiostoma
0.1

Figure 2-14. The evolutionary history of COX sequences excluding hagfish (hCOX) and lamprey (lCOX) sequences inferred using a Bayesian optimality criterion. The consensus tree is shown. The posterior probabilities (10 million generations) are shown next to the branches. Trees were sampled every 500 generations with a burnin of 10% (401 trees). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the WAG matrix-based method (Whelan and Goldman, 2001) and are in the units of the number of amino acid substitutions per site (scale bar = 0.1 amino acid substitutions per site). The rate variation among sites was modeled with a gamma distribution (shape parameter estimated = 0.667978). Phylogenetic analyses were conducted using MrBayes 3.1.2.

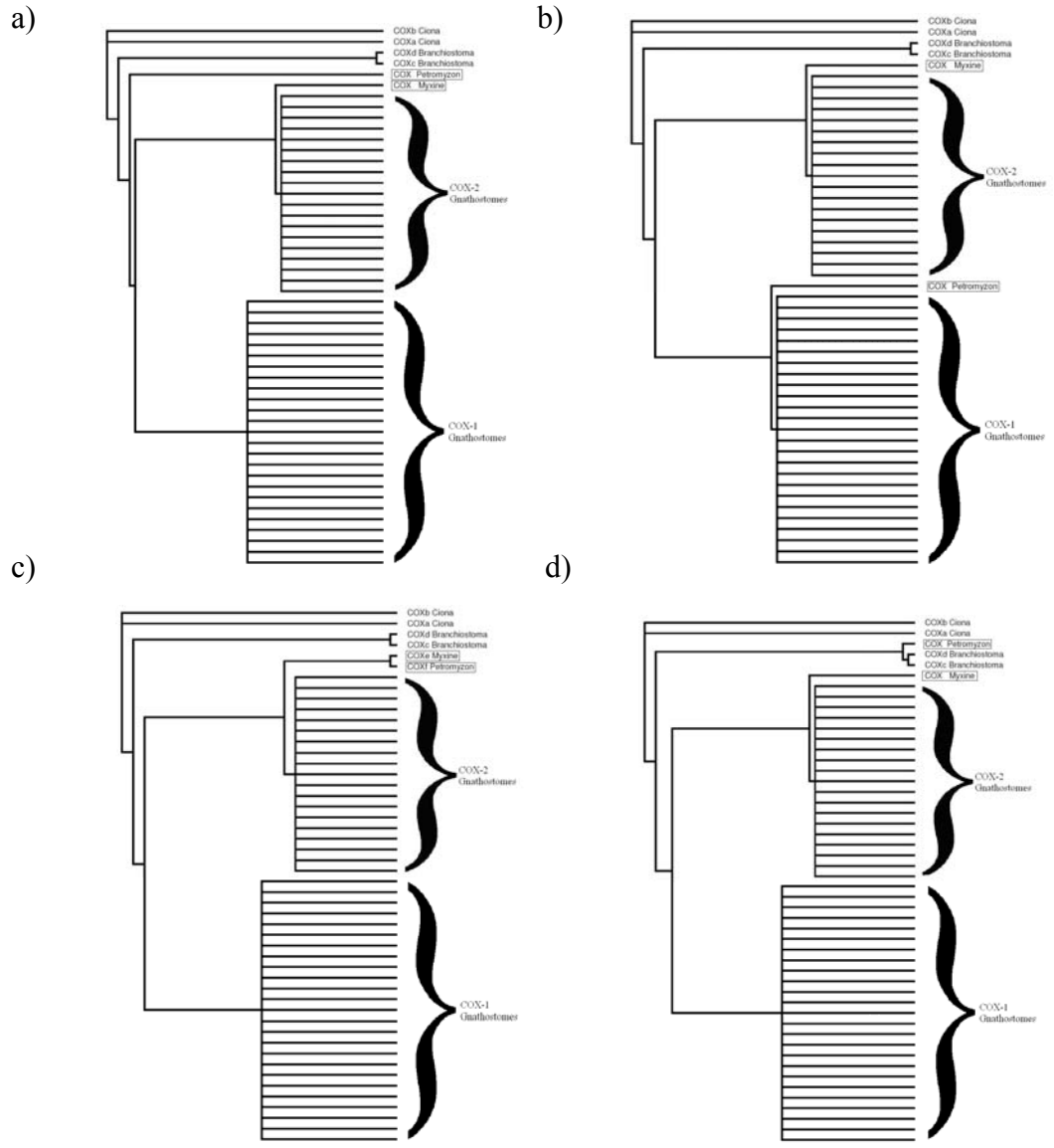

Figure 2-15. The 21 alternate tree topologies generated to test the effects of relocating the hagfish (hCOX) and lamprey (lCOX) sequences on the likelihood score (Ln *L*). Each topology is simplified, but all relationships remain the same as in the most likely topology (Figure 2-9) with the exception of hCOX and lCOX. Each topology was generated using TreeView1.6.6 based on the most likely topology. Ln *L* was calculated for each topology online using PhyML (Guindon *et al*., 2005), branch lengths and model parameters were estimated. The topologies are named in Table 2-9 as follows (from most to least likely): a) COX2 Hag (most likely) b) COX1 Lamp/COX2 Hag c) COX2 Agnatha d) Lamp/Amp e) COX2 Vert f) COX1 Hag g) COX1 Vert h) Ancestral Hag i) COX1 Agnatha j) COX1 Hag/COX2 Lamp k) Ancestral Agnatha l) COX1 Lamp m) COX2 Lamp n) Agntha/Amp o) Hag/Amp p) Hag Internal q) Lamp Internal r) Lamp Mammal s) Lamp Teleost t) Hag Teleost u) Hag Mammal.

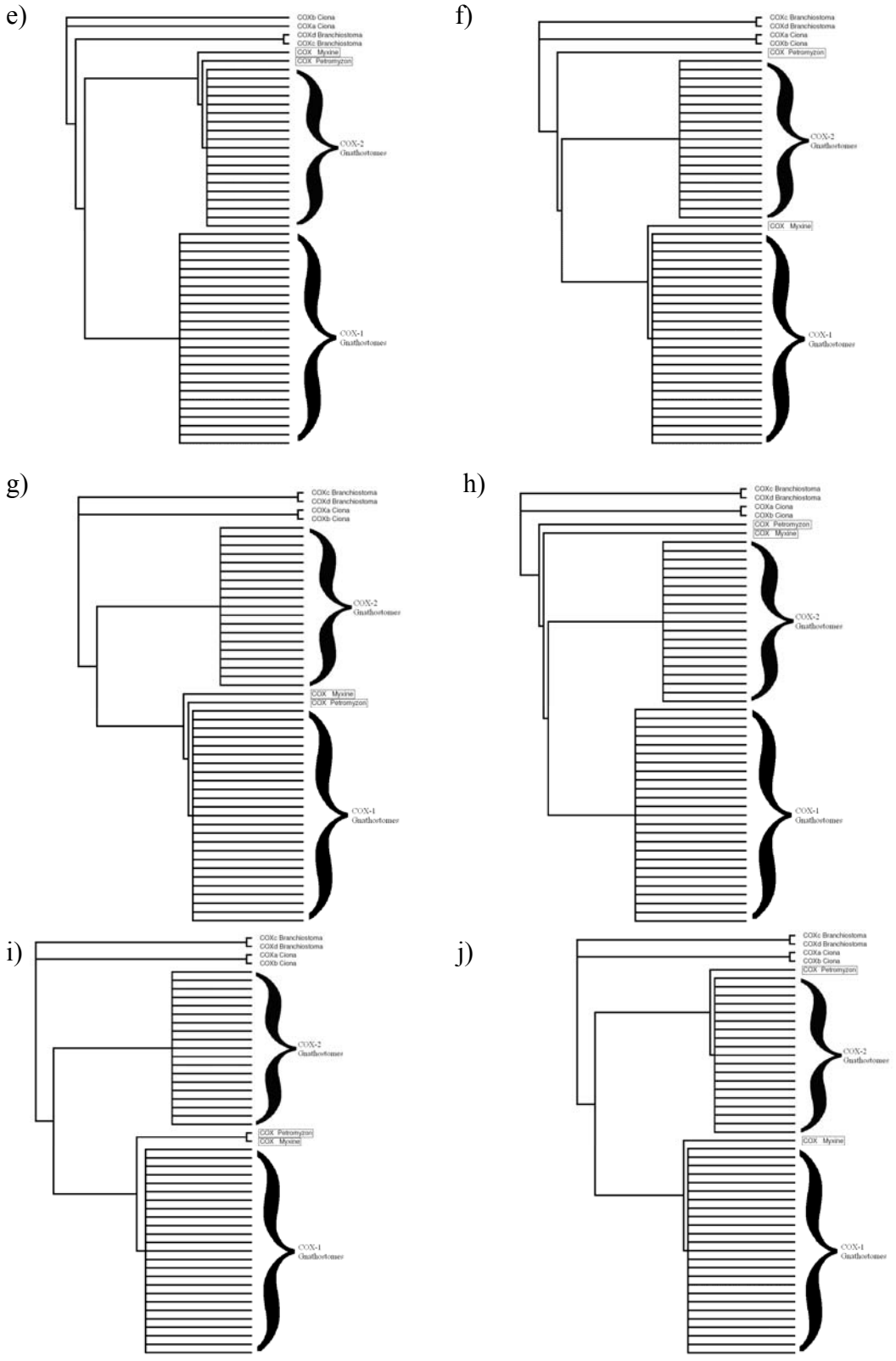

Figure 2-15. Continued.

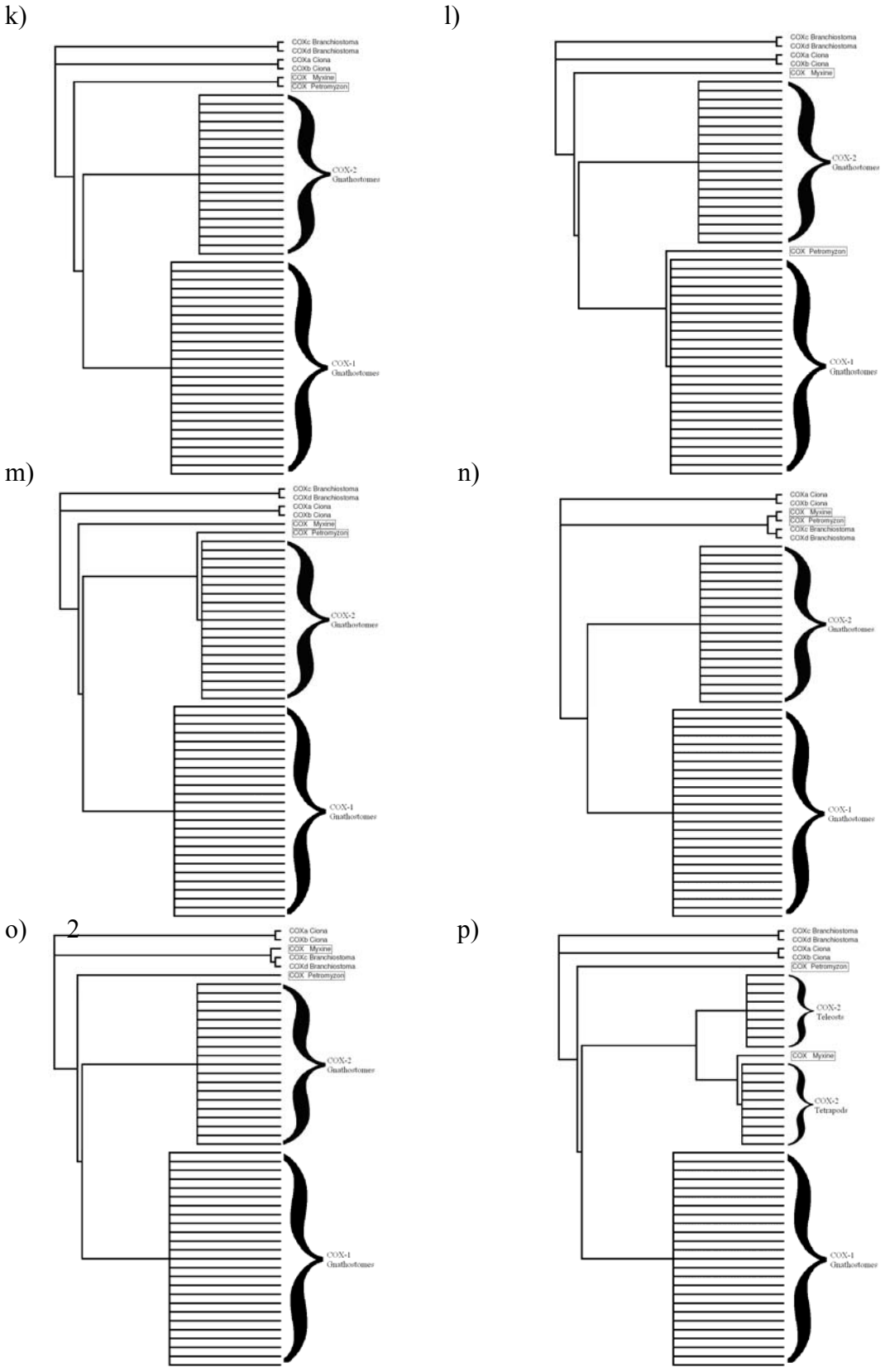

Figure 2-15. Continued.

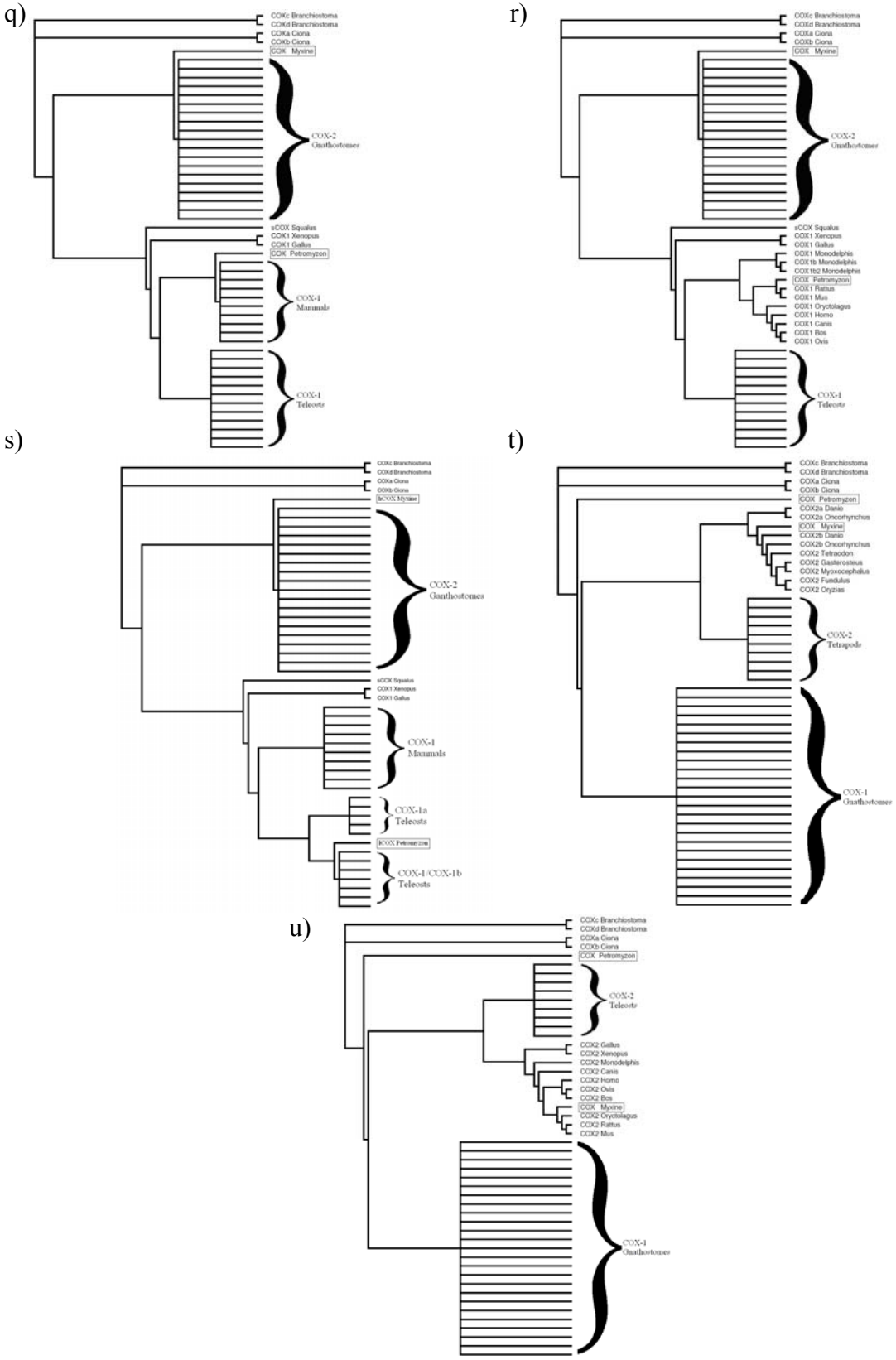

Figure 2-15. Continued.

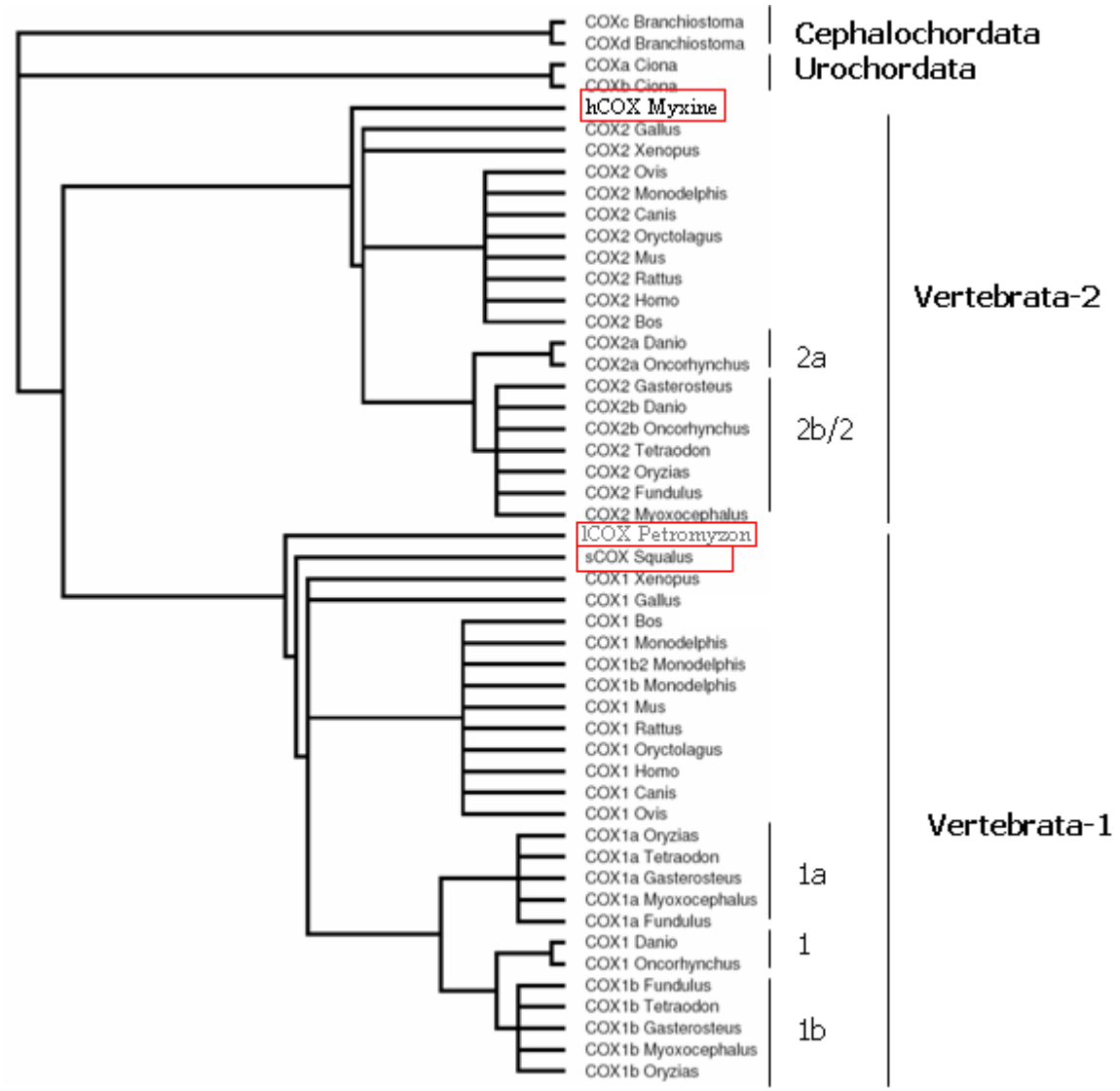

Figure 2-16. Unrooted phylogeny showing the most likely scenario for COX evolution in the chordates based on several other analyses. Clades of interest are highlighted on the right and sequences that represent unclear placements are highlighted in red boxes.

CHAPTER 3
SUMMARY AND FUTURE DIRECTIONS

Summary

This thesis is the first attempt to characterize the evolution of the enzyme cyclooxygenase in the phylum chordata. Traditional molecular protocols such as cloning and polymerase chain reactions were used to search for cyclooxygenase sequences in a wide range of evolutionarily key chordates. Using these novel sequences and pre-existing sequences from GenBank and genomic databases, phylogenetic analyses were performed to reveal the identity of the novel sequences and create the most likely scenario for the evolutionary history of cyclooxygenase in the chordates.

Novel COX Sequences

Chapter 2 describes the process of obtaining and characterizing novel cyclooxygenase (COX) sequences in amphioxus (*Branchiostoma lancelolatum*), the Atlantic hagfish (*Myxine glutinosa*), the sea lamprey (*Petromyzon marinus*), the euryhaline killifish (*Fundulus heteroclitus*), and the longhorn sculpin (*Myoxocephalus octodecemspinosus*). Total RNA was isolated and reverse transcribed from gill tissue (or whole animals in amphioxus) and the resulting cDNA was exposed to PCR in the presence of degenerate and/or specific primers. The products were then visualized and cloned. The resulting plasmids were sequenced at the Marine DNA Sequencing Center at the MDIBL. Complete amino acid sequences of putative COX proteins from the hagfish, killifish, and sculpin and incomplete amino acid sequences from amphioxus and the lamprey were predicted based on the sequences found. These putative amino acid sequences were aligned with known COX protein sequences and conserved domains, regions, and residues critical to COX function were described. Based on the novel sequences' identity to known COX forms and phylogenetic analyses (see below), it was concluded that

COX-1a, COX-1b, and COX-2 are found in the sculpin; COX-1a and COX-1b are found in the killifish; a new COX form named lCOX is found in the lamprey; a new COX form named hCOX is found in the hagfish; and two ancestral forms named COXc and COXd are found in amphioxus.

Phylogenetic Analyses

Chapter 2 also describes the phylogenetic analyses used to reconstruct the evolutionary history of cyclooxygenase in the chordates. The nine novel COX protein sequences described here, as well as 41 additional COX protein sequences from GenBank and genomic databases, were subjected to phylogenetic analyses using three optimality criteria: minimum evolution/distance, maximum likelihood, and Bayesian phylogenetics. In each analysis, two clades were well-supported in the gnathostomes: a COX-1 clade and a COX-2 clade. Each of these clades contained a well supported teleost and tetrapod clade. In the teleost clades, clades for COX-1a, COX-1b, COX-2a, and COX-2b were well-supported, confirming the findings of Ishikawa *et al*. (2007) and Ishikawa and Herschman (2007). The placement of the more evolutionarily ancestral chordates was ultimately uncertain, although it appears that the urochordates, cephalochordates, and vertebrates (containing COX-1 and COX-2) each represent an independent origin of COX proteins. The hagfish and lamprey sequences most likely represent basal members of the COX-2 and COX-1 clades of vertebrates, respectively; however, these forms may belong closer to the root of the tree.

Future Directions

Although here I report 9 novel cyclooxygenase (COX) sequences from a range of chordates and present a likely scenario for the evolution of this gene family in the chordates, many questions remain regarding COX evolution and function in the chordates. These include determining the true history of COX evolution in the earliest chordates, pinpointing the origin of

COX-1 and COX-2, and investigating the history of COX duplication and loss in the teleosts. Finally, the physiological function of COX in the earliest chordates has only been preliminarily investigated.

Completion of novel sequences

Several of the novel sequences documented here could not be completed despite multiple efforts using many primer sets and PCR techniques. Notably, only 532 bp were obtained of the sequences from amphioxus (COXc and COXd). Compared to the complete open reading frame of COX transcripts in other chordates (ranging from about 1700-2500 bp), this indicates that about 75% of these sequences remain unknown. Completing the rest of these sequences or thoroughly searching the recently complete genome of *Branchiostoma floridae* may yield putative proteins that provide novel groupings in phylogenetic analyses. Based on the available sequence data, however, COXc and COXd are well-supported as being near the base of the chordate COX lineage, sister to the vertebrate COX-1 and COX-2 clade. The lCOX sequence obtained from the lamprey is also only about 40% complete. Completing the open-reading frame of this putative protein or searching genomic databases could possibly give a more definite view of its identity as this sequence groups with different clades depending on the phylogenetic analysis used. In actuality, the short size of lCOX compared to the other COX sequences used in the phylogenetic analyses may have contributed to its tendency to appear in different clades in different analyses. Finally, the COX-1a sequence obtained from the sculpin may warrant further investigation and reaffirmation *via* additional sequencing since many residues critical to COX function are missing.

Additional Phylogenetic Analysis

Unfortunately, the evolutionary relationship of the COX proteins in the chordates was not able to be unequivocally resolved, despite the use of three different phylogenetic approaches.

This may be due to the incompleteness of some sequences and a lack of sufficient sampling across taxa or may represent a history that is irresolvable under the current circumstances. However, the phylogenetic techniques used here may also be improved upon in future analyses. Phylogenetically uninformative areas of the sequences (such as near the C and N terminus, which show little homology across sequences) could be removed. Here, protein sequences were used to gain insight into the evolution of COX functionality, but insights could also be gained by performing similar analyses using DNA sequence data instead of using their inferred protein amino acid sequences. This would allow for greater complexity of models to estimate substitution rates and could take additional factors into account such as codon bias. Using nucleotide versus amino acid would also enable additional search strategies and phylogenetic programs to be used. Choosing the optimum search strategy and computer program may be critical in finding the optimum evolutionary reconstruction. For example, maximum likelihood analyses PhyML (the program used in this study) may find suboptimal likelihood scores when the data-set contains little phylogenetic information (Morrison, 2007). However, programs such as RAxML (Stamatakis, 2006), GARLI (Brauer *et al*., 2002), and PAUP* (Rogers and Swafford, 1998) may perform better under the same circumstances (Morrison, 2007). Also, evaluating alternative tree topologies using frameworks other than maximum likelihood may yield alternative results.

Additional Sequences

Based on the most likely scenario of COX evolution in the chordates, there are several COX sequences in the chordates examined in this study that remain to be characterized. If hCOX in actuality represents a basal form of COX-2, then there should also be a COX-1 form in the hagfish. Similarly, if lCOX and sCOX represent basal forms of COX-1 in the lamprey and dogfish, then they should also contain versions of COX-2. These additional forms of COX may

be harder to clone and sequence due to accumulated mutations over evolutionary time, which would render the sequences unidentifiable and possibly non-functional. However, this is not the case in the other vertebrates, as COX-1 and COX-2 seem to each have critical functionality. Using Southern blotting techniques (Southern, 1975) to identify COX DNA sequences in the DNA of hagfish, lamprey, and dogfish could immediately reveal the presence of two forms of COX in these animals. If two forms are present, it could be concluded that they likely represent COX-1 and COX-2 forms, and thus the origin of these forms would coincide with the vertebrate lineage (as predicted in the current phylogenetic analysis). However, if only one form of COX was found the results would suggest a more recent origin of COX-1 and COX-2. This technique could be applied to a wide diversity of chordates to determine how many COX variants they contain.

Additional Taxa

In this study, a comparative evolutionary approach was taken by sampling a wide range of chordates, most of which had no previous record of COX genes. However, there are still many evolutionarily interesting chordates that have not been examined for COX genes. While COX genes are known from multiple mammals and teleosts, other chordate groups have only been minimally sampled. Most notably is the lack of any reported COX genes from any reptile. COX has also only been documented from a single avian species (*Gallus gallus*), a single elasmobranch species (*Squalus acanthias*), and a single marsupial (*Monodelphis domestica*). These groups represent major lineages within the vertebrates and may contain novel forms of COX. The results of this and other recent studies also warrant further investigation within certain chordate groups. For example, evolutionarily ancestral fishes such as the sturgeon, paddlefish, and bowfin could likely contain a single COX-1 and COX-2 gene, while the teleosts studied here have multiple COX-1 or COX-2 genes. Ishikawa and Herschman (2007)

hypothesize that a genome duplication in the teleost lineage and subsequent loss of either a COX-1 (in the zebrafish/trout lineage) or COX-2 gene (in the acanthopterygian lineage) is responsible for this observation. If this is the case, then examining taxa near the base of the teleost lineage may reveal fish with all four variants of COX (COX-1a, COX-1b, COX-2a, and COX-2b). The COX variants from the dogfish shark, lamprey, and hagfish all group weakly with either COX-1 or COX-2. Obtaining COX sequences from other species of hagfish, lampreys, and elasmobranchs may confirm this initial relationship or reveal novel groupings. Finally, although support values for the urochordata and cephalochordata clades are strong regardless of the phylogenetic technique used, only one species from each subphylum was included in phylogenetic analyses. Including more species could give a more complete view of COX evolution in the evolutionarily ancestral chordates. COX sequences from these additional taxa may be readily available in the near future due to the growing regularity of genome sequencing across many taxa. For example, the recently completed *Anolis carolinensis* genome contains COX-1 and COX-2 genes. However, due to the length of time required for whole genome projects, the limited number of genomes available, and the difficulty of searching recently completed, non-annotated genomes, studies that specifically target sequencing COX genes in these taxa are needed.

Function of Cyclooxygenases

As reviewed in Chapter 1, cyclooxygenase plays several roles in chordates because of its role in prostaglandin production. These functions have been examined in some detail in the mammals and to a lesser degree in the fishes. However, no studies have examined the functions of the novel COX forms presented here or any non-vertebrate COX forms in the chordates. Therefore, I have begun preliminary studies investigating the function of these COX forms.

One preliminary method used to infer possible functions for a gene is to determine where its mRNA transcript or protein product is expressed in the organism. For example, Choe *et al*. (2006) concluded that COX-2 may play a role in osmoregulation in the killifish partly due to its high level of expression in the gills and its presence within mitochondrion-rich cells. Following similar procedures, I have found that COX-1b mRNA is also expressed highly in the gills of the killifish (Fig. 3-1a). Similar results were also found for COX-1a and COX-2 in the sculpin (Fig. 3-2a, Fig. 3-2b), hCOX in the hagfish (Fig. 3-3a), and lCOX in the lamprey (Fig. 3-4). To investigate which cells express COX mRNA transcripts, *in situ* hybridization (ISH) was performed using probes designed against the novel COX sequences described here using previous protocols (Choe *et al*., 2006). However, no staining was observed for COX transcripts in the gills of the killifish (Fig. 3-1b) or sculpin (Fig. 3-2b). This is likely due to errors in probe design or hybridization protocol, since COX mRNA was abundant in the gills of both the killifish and sculpin. In the hagfish, probes designed to show hCOX mRNA expression did produce staining patterns in a population of epithelial cells lining gill filaments and lamellae (Fig. 3-3b). This gill structure is similar to those of teleosts but may be functionally different (Evans *et al*., 2005) and since ion transport mechanisms in hagfish may not be directly related to those in teleosts (Choe *et al*., 1999), it is difficult to assign an osmoregulatory function of hCOX based on this staining pattern. Finally, since COX-2 mRNA expression was shown to increase following salinity transfers using qPCR (Choe *et al*., 2006), a similar procedure was used to investigate COX expression after salinity acclimation in the gills of the killifish (Fig. 3-1c) and sculpin (Fig. 3-2c). The only significant result from this experiment was that COX-1b was expressed more abundantly in killifish chronically acclimated to fresh water than seawater. However, the same general trend shown for COX-2 in the killifish (Choe *et al*., 2006) was also

shown for COX-1b in the killifish: an increase in expression 3 hours after salinity transfer (although P = 0.06 in this case) and a return to pre-transfer expression levels by 8 hours after transfer. Taken as a whole, these preliminary results suggest that the different COX forms may be playing various roles in osmoregulation in the killifish, sculpin, lamprey, and hagfish.

Clearly, COX function in the non-mammalian chordates has just begun to be investigated and many questions still remain. Future studies may investigate COX roles in these chordates by examining protein expression patterns using immunohistochemistry, blocking COX function *via* commercially available COX inhibitors, or examining COX expression during reproductive events. Of particular interest are the differential roles of the various COX forms in the teleosts and the possible partitioning of functionality between COX-1a and COX-1b or COX-2a and COX-2b.

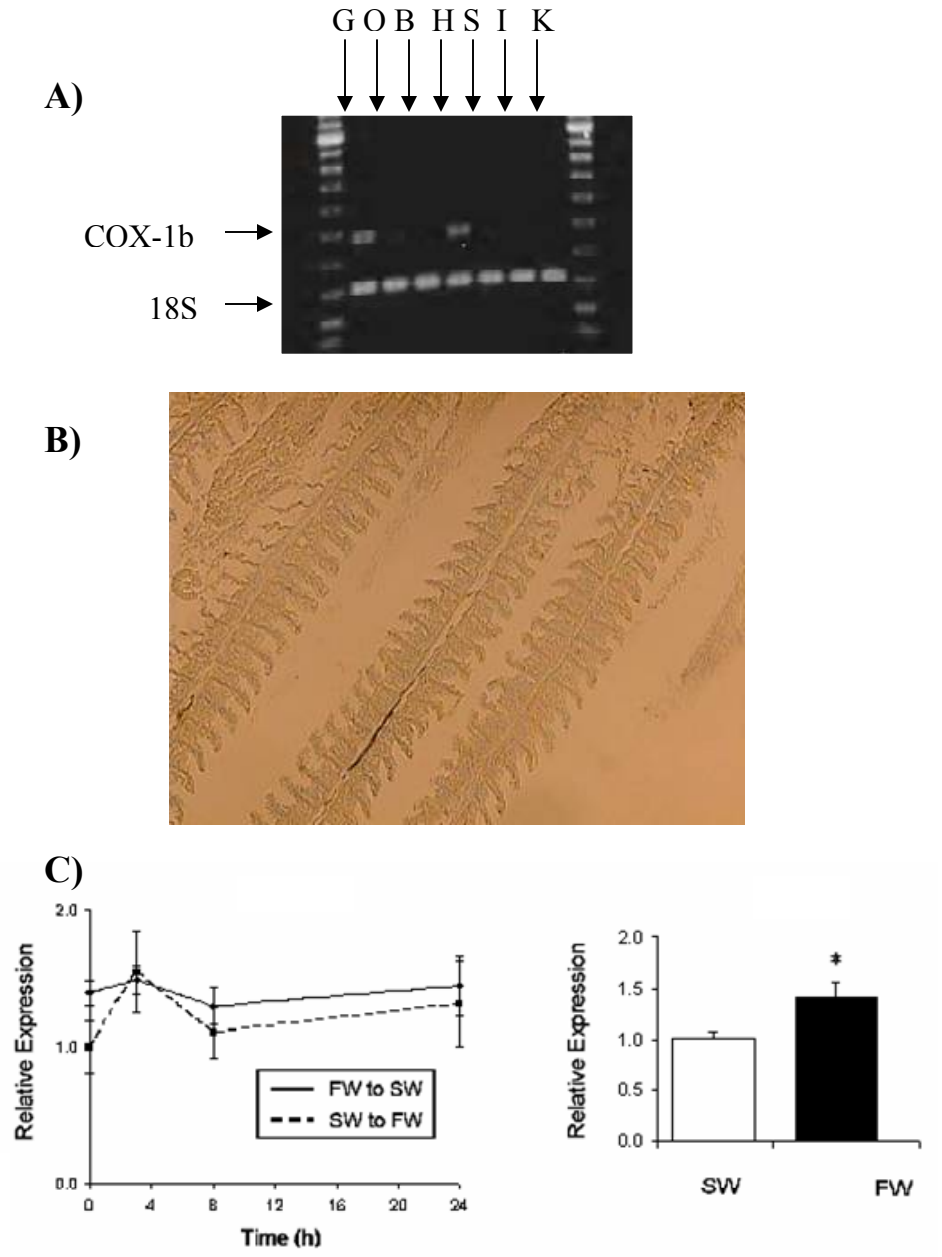

Figure 3-1. Euryhaline killifish (*Fundulus heteroclitus*) COX-1b mRNA expression following procedures outlined in Choe *et al*. (2006). A) COX-1b mRNA expression in various tissues in the killifish. Expression was constant across tissues for the ribosomal protein 18S. COX-1b expression was most abundant in the gills (labeled as G), followed by the heart (H), kidney (K), opercular epithelium (O), stomach (S), intestine (I), and brain (B). B) *In situ* hybridization of COX-1b in the gills of the killifish. C) Expression of COX-1b mRNA in the gills of the killifish following salinity transfers. Expression is normalized to the ribosomal gene L8 and is shown relative to seawater (SW) (FW = fresh water).

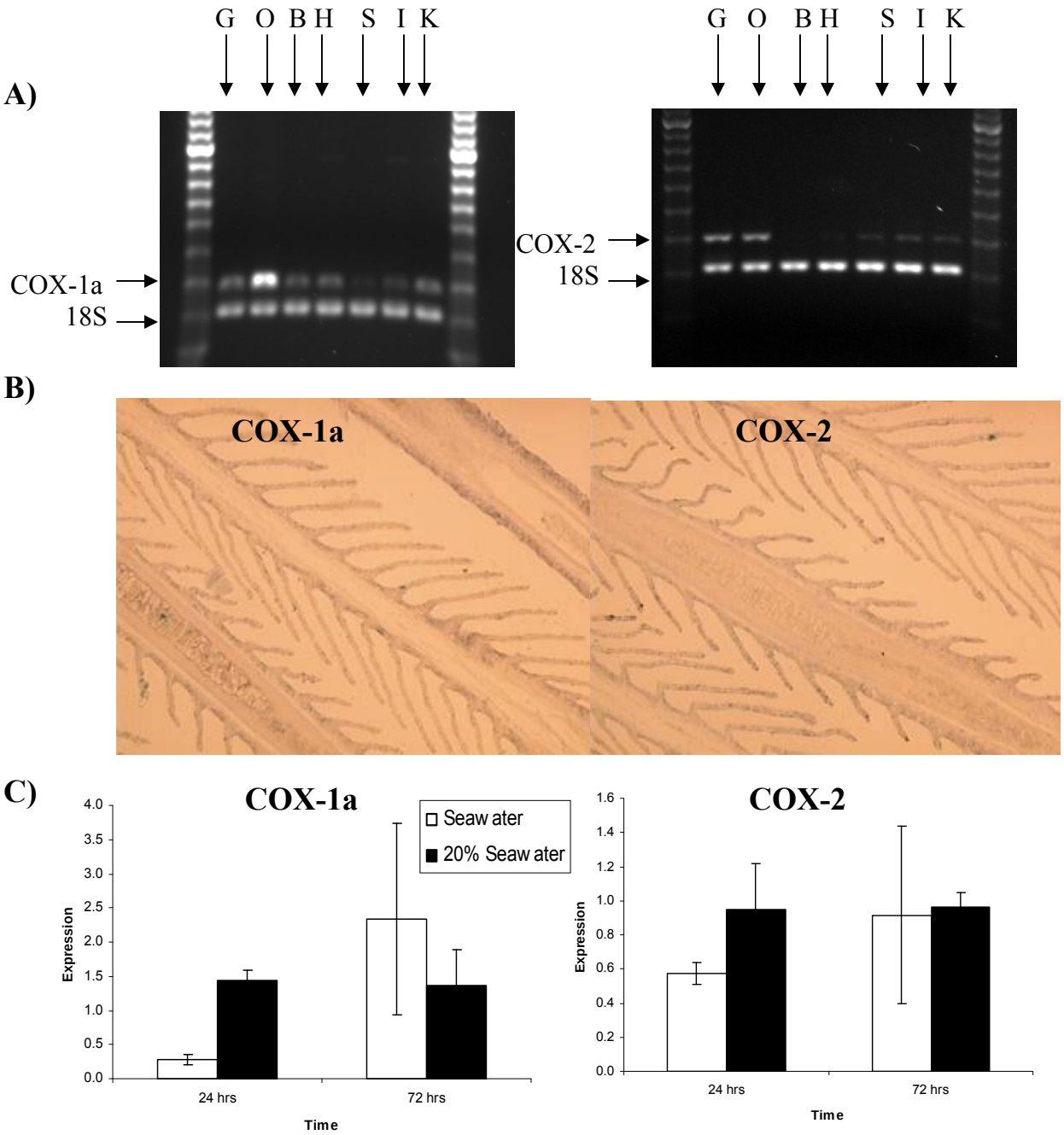

Figure 3-2. Longhorn sculpin (*Myoxocephalus octodecemspinosus*) COX-1a and COX-2 mRNA expression following procedures outlined in Choe *et al*. (2006). A) COX-1a and COX-2 mRNA expression in various tissues in the sculpin. Expression was constant across tissues for the ribosomal protein 18S. COX-1a was most abundant in the opercular epithelium (labeled as O), followed by the gills (G), kidney (K), heart (H), brain (B), intestine (I), and stomach (S). COX-2 was most abundant in the gills, followed by the opercular epithelium, and kidney. B) *In situ* hybridization of COX-1b and COX-2 in the gills of the sculpin. C) Expression of COX-1b and COX-2 mRNA in the gills of the sculpin after 24 and 72 hours following salinity transfers to 100% and 20% seawater. Expression is normalized to the ribosomal gene L8.

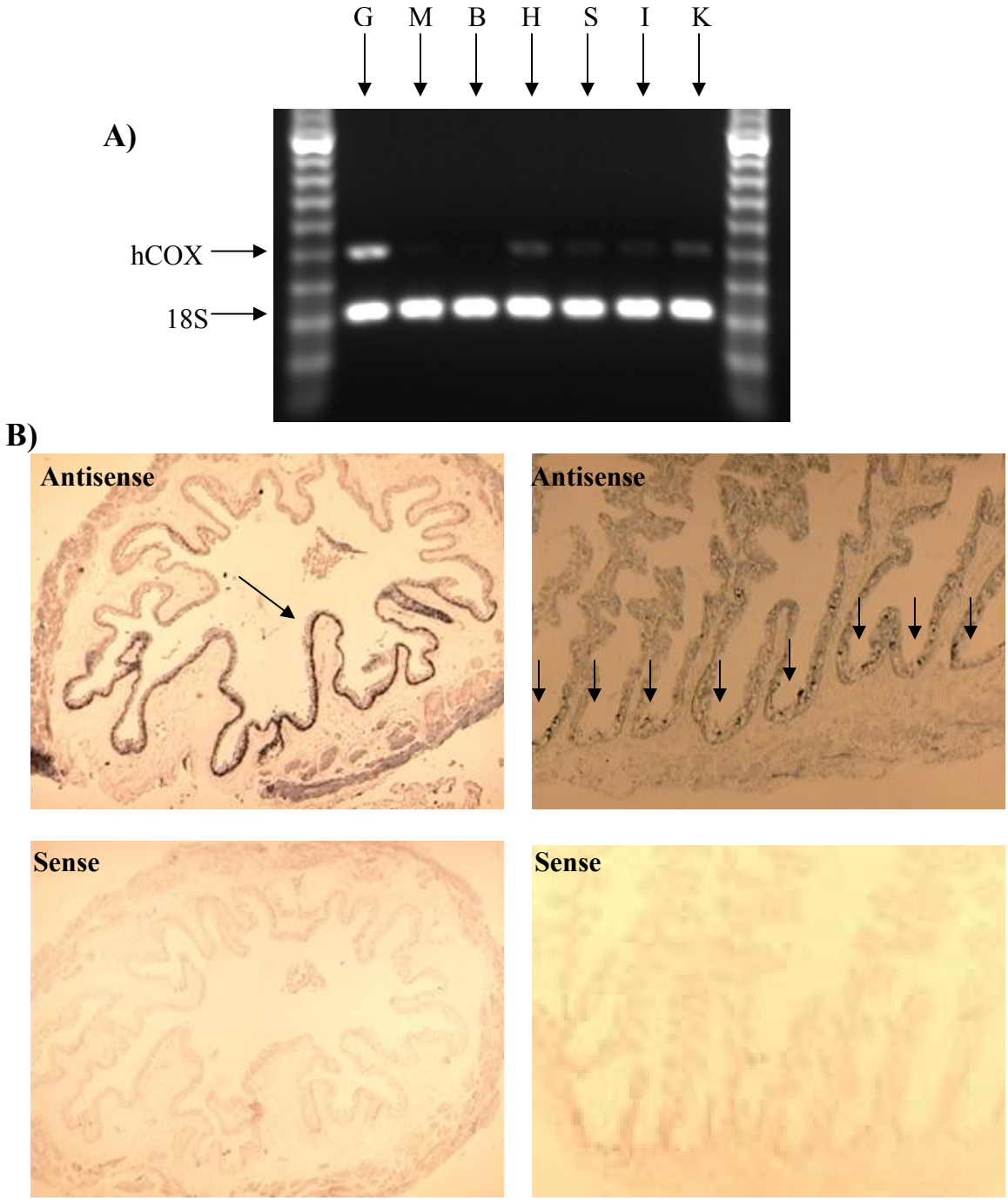

Figure 3-3. Atlantic hagfish (*Myxine glutinosa*) COX (hCOX) mRNA expression following procedures outlined in Choe *et al*. (2006). A) COX mRNA expression in various tissues in the hagfish. Expression was constant across tissues for the ribosomal protein 18S. COX (hCOX) expression was most abundant in the gills (labeled as G), followed by the heart (H), kidney (K), stomach (S), intestine (I), muscle (M), and brain (B). B) *In situ* hybridization of hCOX in the gills of the hagfish from two contrary gill cross-sections. Arrows highlight staining, indicating hCOX mRNA expression. Staining for antisense and sense (control) probes is shown.

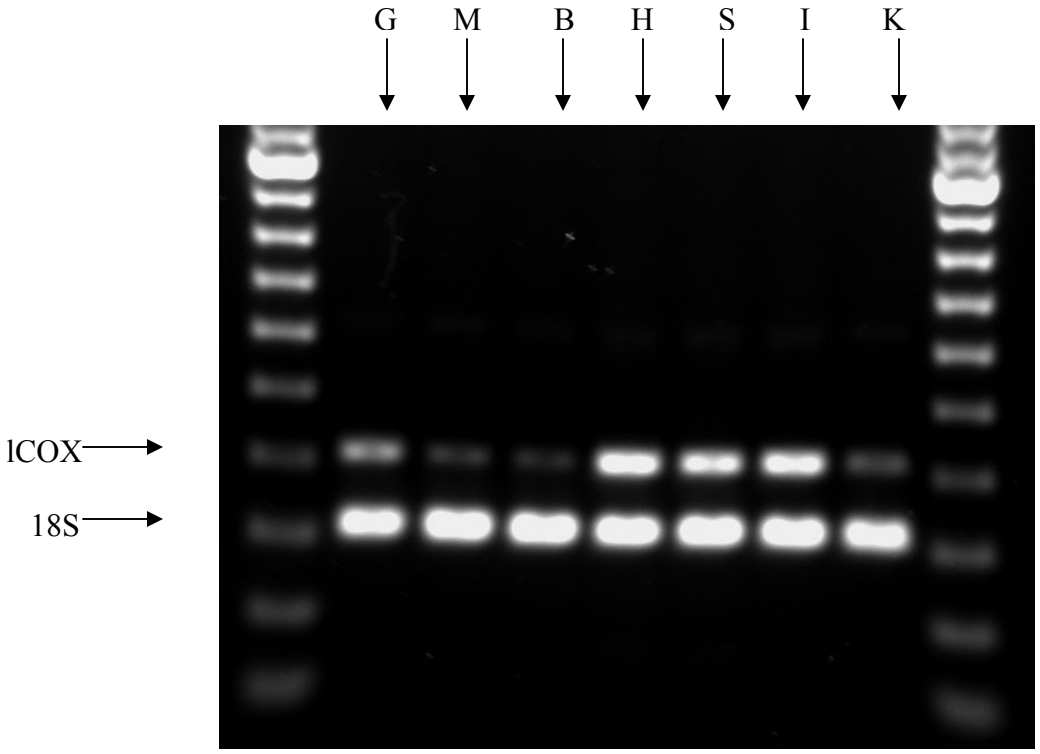

Figure 3-4. Sea lamprey (*Petromyzon marinus*) COX (lCOX) mRNA expression in various tissues following procedures outlined in Choe et al. (2006). Expression was constant across tissues for the ribosomal protein 18S. COX (lCOX) expression was most abundant in the heart (labeled as H), followed by the intestine (I), stomach (S), gills (G), kidney (K), muscle (M), and brain (B)

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BIOGRAPHICAL SKETCH

Justin Chase Havird was born in 1984, in Valdosta, Georgia to Dawn and Kurt Havird. Justin has one younger brother, Joshua. The Havirds soon moved to Lake City, Florida, where Justin spent the majority of his childhood and adolescence. Justin attended Columbia High School in Lake City and graduated with top honors in 2002. Justin then attended the University of Florida in Gainesville where he majored in zoology after an initial interest in aerospace engineering. While at UF, Justin was heavily involved in the university's band program, playing trumpet in the marching, basketball, volleyball, concert, and symphonic bands for several years. As an undergraduate, Justin worked in the laboratory of Dr. David H. Evans for a number of years, investigating ion transporters in the gills of fishes. His senior thesis was titled "Neuronal Nitric Oxide Synthase in the Gill of the Euryhaline Killifish, *Fundulus heteroclitus*." Justin graduated summa cum laude from UF in 2006 with a B.S. in zoology.

In 2006, Justin entered graduate school at the University of Florida in the Department of Zoology. Continuing to work with Dr. Evans, Justin spent three summers at the Mount Desert Island Biological Laboratory in Salisbury Cove, Maine where he collaborated with other researchers investigating fish gill physiology. During his graduate studies, Justin has earned several awards and grants, including a GIAR from Sigma Xi. Justin has also presented his research at scientific meetings and conferences. While earning his M.S., Justin also designed experiments and instructed students in a senior level animal physiology course for 2 years. Justin has also collaborated with other scientists on a wide range of marine life science projects, most notably a revision of the cobitid genus *Lepidocephalichthys* in SE Asia with Dr. Larry Page. After earning his

M.S., Justin plans to continue his academic career by completing a Ph.D. in a related marine science field.