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ASPECTOS MOLECULARES DA DIFERENCIAÇÃO SEXUAL EM TAMBAQUI (*Colossoma macropomum*): identificação de vias não convencionais em uma espécie neotropical

RÔMULO VEIGA PAIXÃO

MANAUS-AM
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Tese apresentada ao Programa de Pós Graduação em Ciência Animal e Recursos Pesqueiros, da Universidade Federal do Amazonas, área de concentração Produção Animal, Biotecnologia Aplicada à Zootecnia e Recursos Pesqueiros, para obtenção do título de Doutor em Ciência Animal e Recursos Pesqueiros.

Orientadora: Dra. Flávia Kelly Siqueira de Souza

Co-orientadora: Dra. Fernanda Loureiro de Almeida O'Sullivan

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Tese apresentada ao Programa de Pós-Graduação em Ciência Animal e Recursos Pesqueiros da Universidade Federal do Amazonas, como requisito para obtenção do título de Doutor em Ciência Animal e Recursos Pesqueiros, área de concentração em Produção Animal.

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BANCA EXAMINADORA

Dra. Fernanda de Almeida O'Sullivan - Presidente
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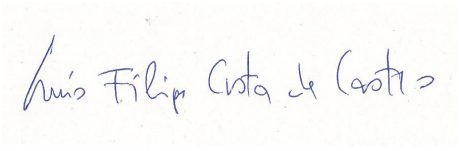

Dr. Luís Filipe C. Castro - Membro
Interdisciplinary Centre of Marine and Environmental Research of the University of Porto (Portugal) - CIIMAR

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RESUMO

Os peixes exibem uma grande variedade de mecanismos de determinação sexual e diferenciação sexual, que são tópicos de grande interesse científico e também relevância para o setor produtivo, quando a exploração de um sexo é mais vantajosa em espécies de importância econômica. O tambaqui *Colossoma macropomum* é uma espécie neotropical de destaque na aquicultura nativa brasileira, e seu mecanismo genético de determinação sexual ainda não foi propriamente identificado e caracterizado, apesar dos esforços utilizando-se de tecnologias ômicas como sequenciamento do genoma de machos e fêmeas, e também transcriptomas de juvenis durante a fase sexualmente indiferenciada. Buscando ampliar os conhecimentos científicos sobre os mecanismos envolvidos no processo de diferenciação sexual do tambaqui, esta tese teve como principal objetivo identificar e avaliar, por análises *in silico* e *in vivo*, os mecanismos moleculares envolvidos neste processo, principalmente os relacionados a hormônios esteróides e seus precursores, bem como os ácidos graxos. **No capítulo 1**, mostramos que a diversidade de peixes não se restringe ao topo da cascata determinante do sexo, mas também nas diferentes vias genicas, hormonais e moleculares necessárias para a desenvolvimento de órgãos reprodutivos e comportamento de machos e fêmeas da espécie. Resultados inesperados acerca da expressão de genes envolvidos na produção e ação do estradiol, como *cyp19a1a* e *cyp19a1b*, e receptores de estradiol, que têm sido recentemente reportados em peixes da ordem Characiformes, indicam que o estradiol possivelmente tenha um papel diferenciado nessa linhagem, se comparado ao classicamente estabelecido na literatura. Nossos resultados **do capítulo 2** sugerem que as variações substanciais na função das aromatases gonadal e cerebral em tambaqui podem ser reflexo das drásticas modificações na região cis e trans-regulatórias provenientes dos rearranjos evidenciados na sequência genômica onde estes genes se econtram localizados, quando comparados com espécies filogeneticamente mais distantes como zebrafish, tilápia, medaka e aruanã. Além disso, as análises de expressão gênica das aromatases *cyp19a1a* e *cyp19a1b* durante a diferenciação sexual e na fase adulta, em conjunto com a inesperada falta de efeito dos disruptores endócrinos na razão sexual do tambaqui, reforçam que o estradiol possa ter um papel menos crítico no desenvolvimento ovariano ou ainda que seja fundamental para o desenvolvimento gonadal e manutenção dos órgãos sexuais em ambos os sexos desta espécie. **No capítulo 3**, a notável expressão diferencial dos genes pertencentes à família elovl, mais expressos nos machos em fase de pré-diferenciação sexual, sugere que o metabolismo dos ácidos graxos tenha um papel importante na diferenciação sexual em tambaqui.

ABSTRACT

Fish exhibit a wide variety of mechanisms of sex determination and sexual differentiation, which are topics of great scientific interest and also relevant to the fish industry, specifically when the exploitation of one sex is more advantageous in species of economic importance. The tambaqui *Colossoma macropomum* is a prominent neotropical species in Brazilian aquaculture. Still, the mechanism of sex determination of tambaqui has not yet been properly identified and characterized, despite efforts using omic technologies such as sequencing the genome of males and females, and also juvenile transcriptomes during the sexually undifferentiated phase. Seeking to expand the scientific knowledge about the mechanisms involved in the tambaqui sexual differentiation process, this thesis had as main objective to identify and evaluate, by *in silico* and *in vivo* analyses, the molecular mechanisms involved in this process, mainly those related to steroid hormones and their precursors as well as fatty acids. In the **Chapter 1**, we show that fish diversity is not restricted to the top of the sex-determining cascade, but also to the different genetic, hormonal and molecular pathways necessary for the development of reproductive organs and the behavior of males and females of the species. Unexpected results about the expression of genes involved in the production of estradiol, such as *cyp19a1a* and *cyp19a1b*, and estradiol receptors, which have been recently reported in fish of the order Characiformes, indicate that estradiol possibly has a different role in this lineage, compared to classically established in the literature. Our results from **chapter 2** suggest that the substantial variations in the function of gonadal and brain aromatase in tambaqui may be a consequence of the drastic changes in the cis and trans-regulatory region resulted from the rearrangements evidenced in the genomic sequence where these genes are located, when compared to species phylogenetically more distant species such as zebrafish, tilapia, medaka and arowana. Furthermore, the gene expression analysis of aromatase *cyp19a1a* and *cyp19a1b* during sexual differentiation and in adulthood, together with the unexpected lack of effect of endocrine disruptors on tambaqui sex ratio, reinforce that estradiol may have a less critical role in the ovarian development or it might be fundamental for the development and maintenance of the gonads in both sexes of this species. In **chapter 3**, the remarkable differential expression of genes belonging to the elovl family, more expressed in males in the pre-differentiation phase, suggests that fatty acid metabolism plays an important role in sex differentiation in tambaqui.

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INTRODUÇÃO GERAL

Os peixes representam o grupo mais diversificado dentre os vertebrados (Nelson *et al*., 2016). Diferentes adaptações morfológicas, fisiológicas e comportamentais destes animais são reflexos de uma alta capacidade adaptativa frente a um amplo espectro de condições ambientais. Além disso, os peixes também mostram diferentes tipos de estratégias reprodutivas, incluindo gonocorismo, hermafroditismo sequencial ou simultâneo e ainda o unissexualismo (Devlin e Nagahama, 2002).

Embora grande parte dessa diversidade seja representada pelos peixes neotropicais (Albert *et al*., 2020), a variedade de mecanismos de determinação e diferenciação sexual em peixes desta região é pouco conhecida. Para um país como o Brasil, conhecido por contemplar uma grande diversidade de espécies de peixes de água doce, direcionar as investigações sobre diferenciação sexual em espécies modelo dos grupos neotropicais mais representativos, como os Characiformes e Siluriformes, seria importante a fim de revelar padrões e tendências conservadas dentro desta biodiversidade (Fernandino e Hattori, 2018).

Os peixes neotropicais incluem várias espécies com características promissoras para a aquicultura e/ou sob o risco de extinção. Entretanto, estudos sobre seus mecanismos de determinação sexual e diferenciação gonadal são fragmentados e limitados a poucas espécies. Aspectos moleculares, celulares, histológicos ou endócrinos envolvidos na diferenciação gonadal e os efeitos dos fatores ambientais são em grande parte desconhecidos. Enquanto o peixe-rei (*Odontesthes bonariensis*) está estabelecido como a espécie modelo de diferenciação sexual ambiental (ESD) em peixes neotropicais (Fernandino *et al*., 2013), a cascata molecular da diferenciação sexual não é conhecida em nenhuma das espécies pertencentes à ordem characiformes, por exemplo, que inclui espécies de grande importância ecológica e econômica como pacu (*Piaractus mesopotamicus*) e tambaqui (*Colossoma macropomum*).

O tambaqui é o peixe mais produzido em cativeiro dentre as espécies nativas brasileiras (Peixe BR, 2021). Trata-se de uma espécie que possui potencial para alcançar altas taxas de crescimento e de qualidade de carne através de programas de melhoramento genético (De Mello et al., 2016; Perazza et al., 2019; Campos et al., 2020). Além disso, o tambaqui exibe um forte dimorfismo sexual em favor das fêmeas, as quais após a maturação sexual são maiores e mais pesadas que os machos da mesma idade (Almeida et al., 2016). Tal descoberta motivou a utilização de tecnologias de nova geração, como o sequenciamento de genomas e transcriptomas, visando a obtenção de marcadores sexuais a serem utilizados como uma

ferramenta para identificar o sexo durante o estágio de crescimento dentro de programas de reprodução seletiva e melhorar a precisão da seleção e os ganhos genéticos.

Recentemente, um mapa de ligação de alta densidade proporcionou a identificação de um QTL fortemente associado ao sexo em tambaqui localizado no cromossomo 26, fornecendo dados que sugerem o sistema de determinação sexual homogametico feminino (XX / XY). No entanto, esta região não é grande o suficiente para indicar heteromorfismo cromossômico (Varela et al., 2021). Diferentemente da maioria dos sistemas de determinação sexual XX descritos em peixes até hoje, nos quais duplicatas de um único gene ou variantes alélicas de genes determinantes do sexo estao presentes no cromossomo sexual (Herpin e Schartl, 2015), em tambaqui os SNPs associados ao sexo não se encontram em regiões codificantes.

O sequenciamento de bibliotecas transcriptômicas (RNAseq) de tambaqui sexualmente indiferenciados proporcionou a identificação de machos e fêmeas com base na expressão diferencial de genes classicamente relacionados à diferenciação sexual (Lobo et al., 2020). No entanto, revelou resultados inesperados a respeito das vias clássicas de sintetização e sinalização do hormônio 17β-Estradiol. A biossíntese de estrógenos é mediada pela enzima esteroidogênica aromatase que converte andrógeno em estrógeno e tem sido hipotetizada como determinante na diferenciação sexual de peixes (Guiguen *et al*., 2010). Em peixes teleósteos, a aromatase é codificada por dois genes, *cyp19a1a* e *cyp19a1b*, os quais emergiram de um evento de duplicação genômica específico dos teleósteos (Böhne *et al*., 2013). c*yp19a1a* é, geralmente, altamente expressa nas gônadas, enquanto que *cyp19a1b* é altamente expresso no cérebro (Guigen *et al*., 2010; Diotel *et al*., 2010). Em tambaqui, a ausência de transcrição da aromatase gonadal e a maior expressão da aromatase cerebral no corpo dos machos (sem presença da cabeça) durante a diferenciação sexual (Lobo et al., 2020), coloca em questão o papel deste esteróide na diferenciação ovariana em tambaqui.

Ademais, a expressão do receptor de estrógeno beta 1 (*esr2a*) que, quando ativado pelo seu ligante 17β-estradiol atua como fator de transcrição de genes essenciais para a diferenciação ovariana, como *foxl*2, *fst*, *rspo1* e *cyp19a1b* (Nicol *et al*., 2013; Vizziano-Cantonnet *et al*., 2008; Zhou *et al*., 2016), exibiu um padrão de expressão baixa em fêmeas de tambaqui comparado aos machos durante a fase sexualmente indiferenciada (Lobo et al., 2020). De fato, a cascata classicamente conhecida dos genes relacionados a diferenciação gonadal de peixes, mais precisamente a via hormonal, tem sido recentemente questionada e notáveis diferenças genéticas têm sido notadas não só na identidade do gene master, como também na

interação dos genes envolvidos, que variam substancialmente em diferentes níveis taxonômicos (Böhne et al., 2013; Herpin et al., 2013; Lau et al., 2016; Nakamoto et al., 2018; Chakraborty et al., 2019; Catanach et al., 2021; Imarazene et al., 2021).

A esteroidogênese é baseada na presença e transporte de colesterol para a mitocôndria, que, em alguns peixes, é dependente da atividade do ácido araquidônico (20: 4n-6; ARA) (Castillo et al., 2006), indicando que os ácidos graxos podem ter efeitos diretos na produção de esteróides sexuais em teleósteos (Norambuena et al., 2013; Xu et al., 2017). Além disso, em mamíferos e no salmão do Atlântico, os lipídios são necessários para o desenvolvimento dos testículos, representando componentes estruturais fundamentais durante o crescimento e maturação testicular (Shi et al., 2018; Oresti et al., 2013; Bogevik et al., 2020). Embora a importância da biossíntese e remodelação de lipídios de membrana durante a diferenciação de células germinativas masculinas, espermatogênese e fertilidade seja bem caracterizada em mamíferos (Oresti et al., 2013), a função dos ácidos graxos no desenvolvimento e maturação dos testículos ainda não é totalmente compreendida em teleósteos, e os estudos até o momento são recentes e restritos ao salmão do Atlântico (Bogevik et al., 2020).

O sequenciamento do genoma de tambaqui proporcionou a identificação de genes importantes na biossíntese de ácidos graxos poliinsaturados (PUFAs) como a *elovl4a* e *elovl4b*, que contribuem para a biossíntese de DHA (Ferraz et al., 2020); *fads2*, *elovl2* e *elovl5* (Ferraz et al., 2019) necessários para biossintetizar ARA, EPA e DHA a partir de ácidos graxos precursores (C18) da dieta. Além disso, apesar das vias responsáveis pelo metabolismo de ácidos graxos, assim como biossíntese e metabolismo de esteróides, terem sido mais enriquecidas em machos de tambaqui sexualmente indiferenciados (Lobo et al., 2020), não houve um aprofundamento a respeito dos genes envolvidos. A hipótese de que mudanças no metabolismo lipídico possam afetar o sexo do tambaqui é interessante do ponto de vista fisiologico e evolutivo, mas tambem importante para o controle sexual da espécie e para o desenvolvimento de novas tecnologias para o cultivo de tambaqui, principalmente se aplicadas a nutrição sexo-especifica durante as fases iniciais do desenvolvimento.

Nesse contexto, o objetivo geral da tese foi investigar os aspectos moleculares da diferenciação sexual do tambaqui, em particular sobre as vias de biossíntese e regulação de estrógenos e biossíntese de ácidos graxos durante esta fase inicial de desenvolvimento gonadal.

A tese é composta por três capítulos em forma de artigos. O **Capítulo I** apresenta uma revisão da literatura científica reunindo as mais recentes descobertas sobre o recrutamento de genes envolvidos na determinação e diferenciação sexual em peixes teleósteos, abordando padrões clássicos e não convencionais já reportados. No **Capítulo II** combinamos análise *in*

silico, tratamentos anti-estradiol, mensuração da expressão gênica e quantificação dos níveis plasmáticos de estradiol durante a diferenciação de tambaqui para caracterizar a função dos parálogos *cypa19a1a* e *cyp19a1b* nesta espécie. No **Capítulo III** identificamos pela primeira vez todos os genes *elovl* (1 a 8), e realizamos uma análise filogenética e de sintenia detalhada que mostra a conservação desses genes quando comparados a outras espécies. Adicionalmente, para abordar a expressão e possível função desta família de genes na diferenciação sexual em tambaqui, analisamos os dados do transcriptoma do tambaqui de machos e fêmeas pré-diferenciados.

OBJETIVOS

Objetivo geral

Caracterizar as vias moleculares de biossíntese e regulação de estrógenos e de biossíntese de ácidos graxos durante a diferenciação sexual de tambaqui.

Objetivos específicos

Realizar um levantamento de literatura sobre as vias moleculares envolvidas no processo de diferenciação sexual de teleósteos;

Realizar análises *in silico* dos genes codantes para aromatase em tambaqui e suas diferenças e semelhanças com outras espécies de teleósteos;

Analisar o envolvimento das *cyp19a1a* e *cyp19a1b* na diferenciação sexual do tambaqui;

Traçar o perfil de expressão dos genes *elovls* em tambaqui, com ênfase na fase de pré-diferenciação sexual.

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CAPÍTULO I

EXPANDING THE KNOWLEDGE OF SEX DETERMINATION SYSTEMS AND SEX DIFFERENTIATION GENE RECRUITMENTS ACROSS TELEOST LINEAGES.

Expanding the knowledge of sex determination systems and sex differentiation gene recruitment across teleost lineages.

Rômulo Veiga Paixão; Fernanda Loureiro de Almeida O'Sullivan

PAIXÃO, R. V. (Rômulo Veiga Paixão)[a]; O'SULLIVAN, F. L. A (Fernanda Loureiro de Almeida O'Sullivan)[b].

[a]Universidade Federal do Amazonas (UFAM), Programa de Pós-graduação em Ciência Animal e Recursos Pesqueiros, Avenida Rodrigo Otávio, 6200 – Coroado, CEP: 69080-900. Manaus, Amazonas, Brazil.

[b]Embrapa Amazônia Ocidental, Rodovia AM-010, Km 29, Caixa Postal 319, CEP: 69010-790

Abstract

Despite the vast literature on the mechanisms of sex determination and differentiation in teleost fishes, some taxonomic groups such as neotropical species have been largely neglected. Advances in sequencing technology and bioinformatics allowed the increase of high-quality genomes from non-model species, revealing new genes involved in the regulation of sex. In this review, we compiled the available data on teleost sex determination and differentiation gene recruitments based on classical cytogenetics as well modern techniques of Next Generation Sequencing. We also described major trends in the evolution among basal teleosts, otophysians and euteleosts, as well as within lineages. The most recent studies show up novel SD mechanisms, unusual MSD genes and evolutionary shifts in gene expression of key players of sex differentiation that confronts classical assumptions regarding the sex steroid pathway towards the gonadal differentiation in fish.

1. Introduction

The recently proposed phylogenetic classification of bony fishes shows major groups such as basal teleosts and successively branching clupeocephalans, otocephala, euteleosts, neoteleosts, and acanthomorphs, leading to the most species-rich clade of modern fishes, the percomorphs (Hughes et al., 2018). Whole-genome duplication (WGD), which occurred on the teleost stem lineage in an ancient common ancestor of all living teleosts, is often proposed as having caused the spectacular evolutionary radiation of teleost fishes (Glasauer and Neuhauss, 2016). The main benefit of WGD acts through gene dosage balance where duplicates are selected for on a network level so as not to disrupt dosage-specific regulation. Gene dosage balance results in the capacity for network evolution to occur, which could be a mechanism that may help to promote sexual plasticity in teleosts (Mank et al., 2006; Edgecombe et al., 2021), reflected by the variety of reproductive strategies such as unisexuality, different types of hermaphroditism and gonochorism; and also by the diversity of SD mechanisms that have classically been divided into two main categories: genetic sex determination (GSD) and environmental sex determination (ESD) or a combination of both (GSD+ESD) (Devlin and Nagahama, 2002; Heule et al., 2014; Li et al., 2018; Fernandino and Hattori, 2018; Stöck et al., 2021).

The classic view of sex development in teleosts established that differentiation of a bipotential gonad is triggered by the interaction between ESD and GSD during a period early in life, where the initial inherited factor causes the first steps of the SD cascade, thus activating further downstream genes of sexual differentiation to form an ovary or testis (Heule et al., 2014). Such heritable genetic factors that differ between sexes are usually located in sex chromosomes, which either may be distinguishable cytologically (heteromorphic) or appear identical (homomorphic). In both cases, female (ZZ/ZW) or male heterogametic (XX/XY) systems, plus polygenic SD or multiple sex chromosomes exist in teleosts and are even found side by side in closely related species (Sember et al., 2021). Also, the phylogenetic distribution of SD mechanisms in teleosts indicates that most if not all of the broad categories of sex determination in teleosts had multiple evolutionary origins, in which sex chromosomes of both XY and ZW type can arise repeatedly and perhaps interconvert quite readily during evolution of lineages, i.e. at genus level (Mank et al., 2006).

Advances in DNA and RNA sequencing technologies allowed the identification of sex determing regions (SDR) that in most of the cases involve a rather simple genetic system with male heterogamety and one major sex determiner (MSD) gene. Most of these genes has gained new function to become the sex-determining gene in different teleost fish, through duplication events (i.e. segmental duplications of a large or small autosomal region containing its precursor gene) that resulted in a novel function as the regulatory mechanism may not have been duplicated with it (Matsuda et al., 2002; Hattori et al., 2012). Other than that, allelic variants have been reported in some teleost systems in which sex chromosomes may even freely recombine, and rarely, the X and Y may differ by just a single nucleotide polymorphism in MSD gene functional domains or cis regulatory region (Kamyia et al., 2012; Myosho et al., 2012).

Up to date, different MSD genes have been identified and characterized across teleosts, which involves a common set of genes that evolved from the conserved downstream regulators of gonadal differentiation (Ortega-Recalde et al., 2020, Stöck et al., 2021). These often include the "usual suspect" genes such as the transcription factors *dmrt1*, *sox3*, and *Tgf-beta* signaling pathway members *Amh, Amhr2, Gsdf* and *Gdf6*, that function as master male sex determinants in several fish species acting at initial stages of gonad development through repression of female pathway gene expression and decrease estrogen level so as to promote testis formation (Li et al., 2018). In contrast, in the female pathway *foxl2* promotes ovarian development by upregulating *cyp19a1a* expression, the key enzyme involved in the synthesis of estrogens, increasing germ cell number and repressing male pathway gene expression (Zhang et al., 2017).

The level of endogenous estrogen is also tightly controlled by epigenetic modification of *cyp19a1a* promoter and it is important for atural sex change which occurs in hermaphrodite teleosts, either protadrous or protogynous (Wu et al., 2016; Wu and Chang, 2017; Li et al., 2018). Epigenetic regulation of *cyp19a1a* was also proposed to be involved in ESD species in response to environmental influences, leading to the gonad fate change (Navarro-Martín et al., 2011; Shao et al., 2014; Li et al., 2018). Moreover, elevated cortisol due to the high temperature may induce masculinization by directly down-regulating the expression of *cyp19a1b* through activation of the *pparaa* signalling pathway during gonadal sex differentiation in medaka (Hara et al., 2020). As PPARα mainly plays roles related to lipid metabolism and is a sensor for free

fatty acids, such study provides the first evidence that lipid metabolism may be involved in environmental sex determination in vertebrates.

However, because studies regarding sex determination and differentiation among fish lineages are unproporcional, unusual master SD genes and unconventional expression patterns of key GD players have been recently reported in fishes from less representative teleosts groups such as basal teleosts and Otocephala, configuring an unexpected outcome compared to what was found mostly in euteleosts. In this context, here we review the current knowledge about sex determination and sex differentation across the teleost phylogeny, providing a sistematic view of the genetic information among lineages.

2. Overview of current knowledge about sex evolution across the teleost phylogeny

Basal teleosts: Osteoglossiformes

The fish superorder Osteoglossomorpha, along with Elopomorpha, occupies the basal position among extant teleostean fishes. Osteoglossiformes currently comprises 244 valid species (restricted to freshwater tropical regions), classified into five families, namely Pantodontidae (2n=46), Notopteridae (2n=38/42/50), Gymnarchidae (2n=34), Mormyridae (2=40-50), and Osteoglossidae (2n=40-56) (Hatanaka et al., 2018; Hilton and Lavoué, 2018). Within Osteoglossidae, genes that has never been assigned as a key factor on sex differentiation cascade of teleosts, such as *id2bbY*, a duplicated copy of the inhibitor of DNA binding 2b (*id2b*) gene, was recently reported as candidate male sex-determining gene in the the Amazonian freshwater fish *Arapaima gigas*. Moreover, the small male-specific region (9656bp) also contains a fragment of *kidins220b* (*kidins220bΔ*) highly divergent from to the homologous, suggesting that this gene fragment is corrupted (Adolfi et al., 2021). Otherwise, the genomic sequence combined with karyotype analysis identified putative sex chromosomes and suggest a ZW/ZZ sex determination system in Asian arowana *Scleropages formosus* (Bian et al., 2016), which belongs to Osteoglossinae subfamily, sister of Arapaiminae. Additionally, among the potential three sex candidate genes detected for *S. formosus*, *fhl3* showed the greatest level of differential expression in ovary versus testis, followed by *dmrt3* and *sf1*.

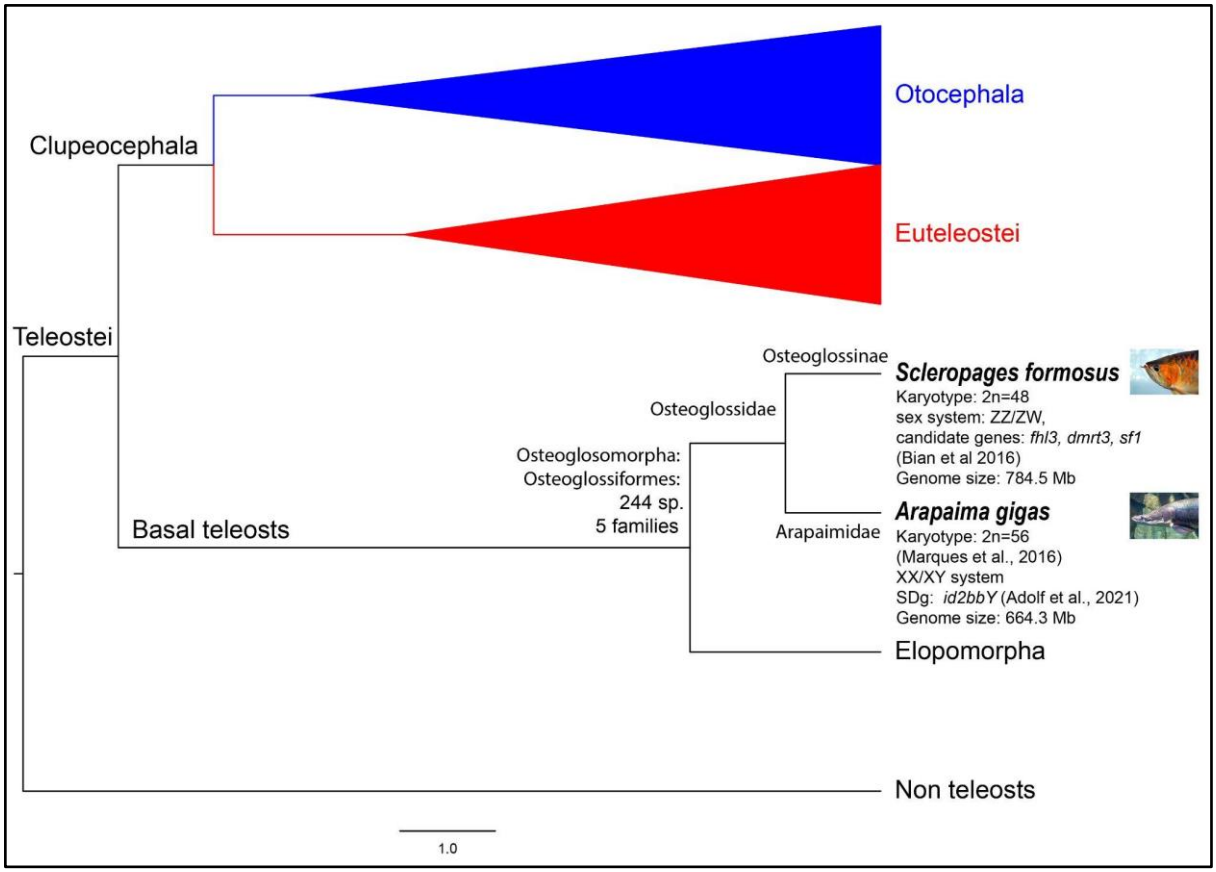


Figure 1: Known sex-determining genes (SDg) and candidate genes in Osteoglossiformes, representative species of basal teleosts, and the phylogeny of the species where they are found. Also, karyotype diploid number (2n), sex system and genome size in megabase (1 Mb = milion bases) informations are shown.

Otocephala: Clupeiformes

Among the remaining teleosts within Clupeocephala, a limited number of studies have attempted to resolve the genetic basis of sex determination and differentiation in Otocephala lineage. Also Otocephala comprise Clupeiformes, Alepocephaliformes and Ostariophysi as main subclades (Straube et al., 2018). Commonly known as herrings, sardines and anchovies, species in the order are cosmopolitan, with 364 species in 84 genera and 5 families (Nelson, 2006, Queiroz et al., 2020), being primarily pelagic marine species inhabiting coastal areas, though some species live in estuaries and freshwaters. The evolution of sex determination within Clupeiformes is characterized by highly divergent sex systems evidenced by *Coilia nasus* karyotype, member of most basal family Engraulidae which exhibit a ZZ/Z0 system (Xu et al., 2014), while in species from family Clupeidae, such as *Clupea harengus* and *Brevoortia pectinata*, sex chromosomes are early in their evolution and still homomorphic with an exception of *Brevoortia aurea*, a species that has heteromorphic sex chromosomes with two X and one Y chromosome (X_1X_2Y) (Brum and Galetti, 1992). Thus, the XX/XY sex

determination system for *C. harengus* was identified through genotypes of the SNPs associated with sex generated by low coverage whole-genome sequencing, but the exact genes for sex determination could not be detected (Kongsstovu et al., 2020). Further, an unusual *Tgf-beta* signalling pathway member named *bmpr1bbY*, which encodes a truncated form of a Bmp1b receptor, was found to be the candidate male-determining gene in *Clupea harengus*, originated by duplication and translocation of a small sex-associated region (~300 kb). The Y region also contains two genes encoding subunits of the sperm-specific Ca2+ channel (*catsperg* and *catsper3Y*) required for male fertility (Rafati et al., 2020).

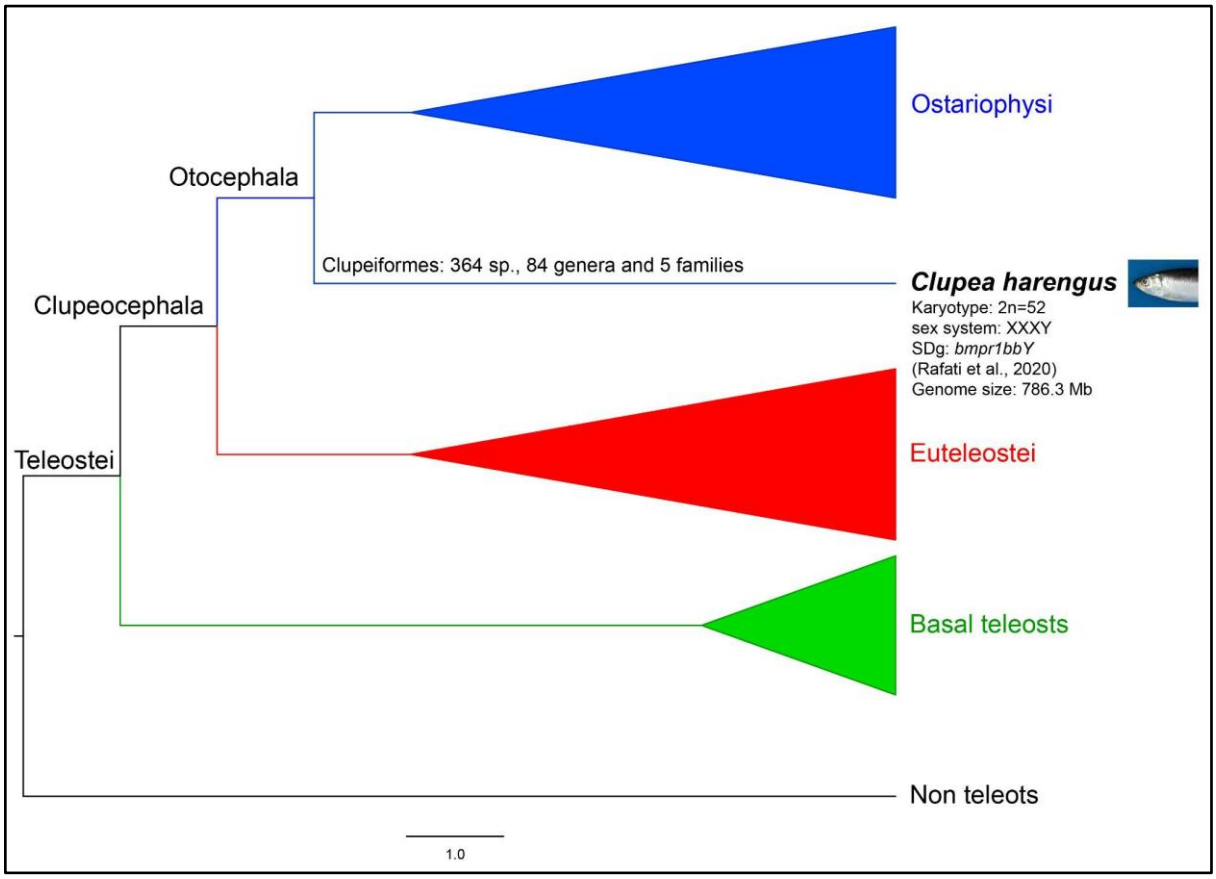


Figure 2: Known sex-determining genes (SDg) in the order Clupeiformes and the phylogeny of the species where they are found. Also, karyotype diploid number (2n), sex system and genome size in megabase (1 Mb = milion bases) informations are shown.

Otocephala: Ostariophysi - Cypriniformes

Within the superorder Ostariophysi, Gonorynchiformes (i.e., Anotophysi; milkfish *Chanos chanos* and relatives, 37 species) are considered monophyletic and the sister group of the Otophysans, by far the largest and most diverse group of primarily freshwater teleosts including 11.508 valid species (Fricke et al., 2020). Moreover, the Neotropical ichthyofauna is

dominated by otophysan fishes, except from the order Cypriniformes (4.776 valid species) occurring throughout North America, Africa, Europe, and Asia freshwater habitats (Nelson 2006). Apart from seven families recognized, the Cyprinidae family includes about 3,000 species in over 360 genera (Nelson et al., 2016; Tao et al., 2019) which contains more polyploid species than any other well-known polyploid group of fishes (Li et al., 2020; Yang et al., 2022).

Cross-species comparisons show that the genome of species from the Cyprinidae subfamily Cyprininae such as common carp *Cyprinus carpio* (n=50) and goldfish *Carassius auratus* (n=50) had one more round of whole genome duplication (WGD) than grass carp *Ctenopharyngodon idellus* (n=24), which in turn had a chromosome fusion in relative to zebrafish (n=25), member of subfamily Danioninae that is distant and equally related to all three carps (Xu et al., 2014, Wang et al., 2015; Kuang et al., 2016). Indeed, bighead carp, silver carp and grass carp shared the same male sex-specific markers and sex-determining DNA sequence, while no similarity were found between it and male-specific marker in common carp, tetraploid fish that might have formed its own sex-determination region or genes in its speciation process (Liu et al., 2018). However, no obvious SD genes could be detected in Y sequences from carps.

Computational identification of Y-linked markers and genes in the grass carp revealed 14 genes never reported in sex differentiation cascade previously, with high similarity to their female homologs in coding sequences but low similarity in intergenic regions, except for two genes with no indication of the biological function (*un-y1* and *un-y2*) and for the SD candidate *ubq-y*, supposed to be an E3 ubiquitin-protein ligase containing a zinc finger domain that shared low sequence similarity to its female homolog (Zhang et al., 2017). In goldfish, while no male-specific SNPs were found in *amh* coding sequence, the only usual suspect master SD in SDR (out of 373 genes), two annotated genes, Stromal cell-derived factor 1 (*sdf1*) and Xaa-Pro aminopeptidase 1-like (*xpnpep1-like*) are located in the SDR with the highest density of sex-specific SNPs, but both are unlikely candidates as potential master sex determining genes (Wen et al., 2020).

Different from carps, zebrafish in nature possess a WZ/ZZ sex-determination mechanism with a major determinant lying near the right telomere of chromosome 4 (*sar4*) while strains providing the zebrafish reference genome lack key components of the natural sex-determination system supporting a polygenic sex determination with genetic elements and environmental perturbations contributing to sex-fate (Wilson et al., 2014; King et al., 2020).

However, although divergent sex determination mechanisms and reproductive strategies can be found in cyprinids, the molecular pathway of gonad development follow the classic view.

In zebrafish, which is termed a juvenile hermaphrodite, all gonads initially develop as an undifferentiated juvenile ovary that later degrade and transform into true ovary or testis (Yang et al., 2017); *cyp19a1a* and forkhead box L2a (*foxl2a)* promote ovary differentiation and maintanence (Yang et al., 2017; Chen et al., 2017); male sex determination is initiated by expression of sex-determining genes that activate downstream factors essential for testis development and spermatogenesis such as anti-Mullerian hormones (*amh*); *dmrt1* and sry-related HMGbox 9 (*sox9*) (Rodríguez-Marí et al. 2005; Schulz et al. 2007; Lin et al., 2017). To date, a dozen of genes involved in sex determination, differentiation and maintenance have been studied through gene knockout in zebrafish (Li and Ge, 2020), including *gnrh3* (Feng et al., 2019), *dio2* (Houbrechts et al., 2019), *wnt4a* (Kossack et al., 2019) that reflects in a male-biased sex ratio; *amh* that lead to a female-biased sex ratio (Lin et al., 2017); *prmt5* (Zhu et al., 2019) *dnd* (Li et al., 2017) *nanos2* (Li and Ge, 2020), *tdrd12* (Dai et al., 2017) in which all developed into infertile males in adults; *nr0b1* (Chen et al., 2016), *fshr* (Zhang et al., 2015), *bmp15* (Dranow et al., 2015), *cyp19a1a* (Lau et al., 2016; Yin et al, 2017), *cyp17a1* (Zhai et al., 2018), *foxl2a/foxl2b* (Yang et al., 2017) and *esr2a/esr2b* (Lu et al., 2017) which lead to complete sex reversal to testis in all mutants that were able to produce normal sperm and fertilize eggs.

In another way, the common carp is a gonochoristic cyprinid in which ovaries and testis directly differentiate from undifferentiated gonads. Moreover, female related genes *cyp19a1a* and *foxl2* and male related genes *amh*, *dmrt1* and *sox9b* increased significantly prior female, and male gonad differentiation, respectively (30–40 dph); Additionally, meiotic marker genes *dmc1* and *sycp3* increased expression significantly in 55 dph female gonads coincide with oocytes in meiosis; while *sycp3* increased expression significantly in 100 dph male gonads coinciding with the first appearance of spermatocytes (Jiang et al., 2020). Recently, an all-male phenotype capable of normal spermatogenesis and sperm was observed in the *cyp17a1*-deficient Common carp, regardless of the individuals' sex-determination genotypes XY or XX (Zhai et al., 2021), demonstrating that the roles of sex steroids in regulating gonad development and differentiation are similar in the family Cyprinidae, regardless of the GSD background.

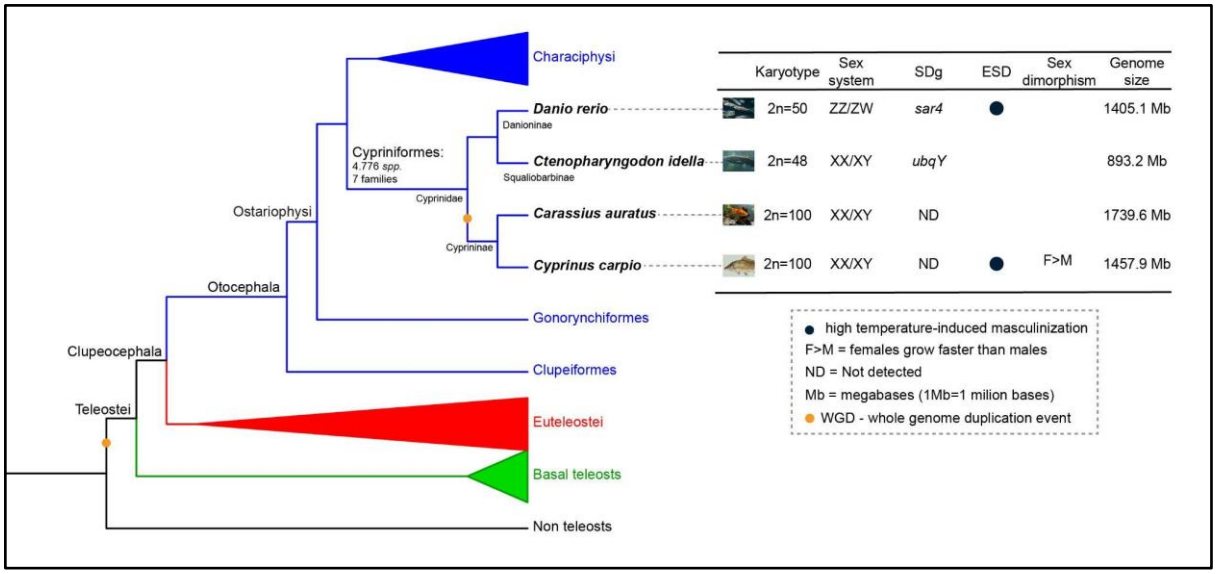


Figure 3: Known sex-determining genes (SDg) in the order Cypriniformes and the phylogeny of the species where they are found. Also, karyotype diploid number (2n), sex system and genome size in megabase (1 Mb = milion bases) informations are shown.

Otocephala: Ostariophysi - Characiphysi - Siluriformes

Siluriformes is one of the largest orders of teleosts popularly known as catfish, subdivided in 38 families with 4.131 valid species, highly diverse and distributed worldwide (Diogo et al., 2004, Eschmeyer and Fong, 2020). Except for the mainly marine Ariidae and Plotosidae families, which also include representatives in brackish and freshwater, the order Siluriformes is composed mainly of freshwater fish with 15 families occurring exclusively in Central and South America: Ariidae (155 species, 58 Neotropical), Aspredinidae (49 species), Astroblepidae (82), Auchenipteridae (127), Callichthyidae (224), Cetopsidae (44), Diplomystidae (7), Doradidae (96), Heptapteridae (231), Lacantuniidae (1), Loricariidae (1024), Nematogenyidae (1), Pimelodidae (116), Pseudopimelodidae (54), Scoloplacidae (6), and Trichomycteridae (365) (Fricke et al., 2020). Despite all this diversity endemic to the Neotropical region, genomic sequence resources are currently available mostly for catfishes from other continents such as North american, Asia and Europe (i.e. *Clarias magur*, *Clarias macrocephalus*, *Clarias batrachus*, *Ameiurus melas*, *Ompok bimaculatus*, *Hemibagrus wyckioides*, *Pangasianodon hypophthalmus*, *Bagarius yarrelli*, *Silurus meridionalis*, *Silurus glanis*, *Tachysurus fulvidraco*, *Ictalurus punctatus*).

Overall, several types of sex systems have been reported particularly for fishes of the order Siluriformes, such as ZZ/ZW (Vissotto et al. 1997), XX/XY (Andreata et al. 1992), XX/X0 (Alves et al. 2006), $X_1 X_1 X_2 X_2/X_1X_2Y$ (Blanco et al. 2014), XX/XY_1Y_2 (Centofante et al.

2006), and $Z_1Z_1Z_2Z_2$ / $Z_1Z_2W_1W_2$ (de Oliveira et al. 2008), $X_1X_1X_2X_2$ /$X_1Y_1X_2Y$ (Ferreira et al., 2016) based on cytogenetic approaches. Molecular methods have made identification of male sex-specific markers indicating an XX/XY sex determination system in Southern catfish *Silurus meridionalis* (Zheng et al., 2019), channel catfish *Ictalurus punctatus* (Ninwichian et al., 2012), yellow catfish *Pelteobagrus fulvidraco* (Dan et al., 2013), African catfish *Clarias gariepinus* (Kovács et al., 2000) and Ussuri catfish *Pseudobagrus ussuriensis* (Pan et al., 2015).

However, the molecular basis of sex determination and sex differentiation in this group remains farly unknown, except for the channel catfish *Ictalurus puntatus* and yelow catfish *Pelteobagrus fulvidraco* in which unusual mechanisms have been recently proposed through modern techniques such as Next Generation Sequecing and CRISPR. Sex determination in channel catfish can be regulated at the RNA level, whereas the Y-linked breast cancer anti-resistance 1 (*bcar1*) isoform inhibits estrogen/estrogen receptor alpha-mediated signal transduction in the undifferentiated gonad driving development toward the testis. The comparison of the channel catfish X and Y chromosome sequences showed that the most significant SNP loci were distributed within a narrower region of 8.9 Mb on Chr. 4, with no sex-specific genes, only 102 bp in the male-specific transcript that is spliced in females contains 3 potential miRNA targeting sites (Bao et al., 2019). More recently, 13 male-specific single nuclear polymorphisms (SNPs) were identified in the coding region of the *zbtb38* gene of channel catfish by targeted next-generation sequencing, where six substitutions resulted in changes in the *zbtb38* amino acid coding on both the X and Y chromosomes. Thus, it is speculated that *zbtb38* on the Y chromosome has an important role in testicular differentiation and is an important candidate gene for sex determination in channel catfish (Pan et al., 2022).

In yellow catfish sex, a chromosome-linked marker Pf62-Y (8 kb) was found located in the intron of a novel PDZ domain-containing gene, which was named *pfpdz1*. The coding sequence of *pfpdz1* in Y chromosome was identical to that in X chromosome except a missense SNP (A/T) that changes an amino acid (Glu/Val) in the N-terminal region. Further, gene editing methods, including transgene and CRISPR/Cas9 mediated gene knockout, showed that the *pfpdz1* gene plays an important role in male gonad development of yellow catfish via regulation of these genes involved in sex determination and differentiation such as *dmrt1* and *sox9a1* (upregulation) and of *cyp19a1*, *foxl2* and *wnt4* (downregulation) leading to partial male-to-female sex reversal when inactivated (Dan et al., 2018). Additionally, eleven significant QTLs were detected on LG3 through high-density genetic linkage map of yellow catfish, where six

sex-related genes were identified from the region of reference genome near these QTLs including *amh*, *gnrhr*, *vasa*, *lnnr1*, *foxl2*, and *bmp15* (Gao et al., 2020).

More recently, it has been showed that the male heterogametic XX/XY sex determination system should co-exist with the ZZ/ZW system in African catfish in which no strictly male- or female-linked loci were detected using high-throughput genome complexity reduction sequencing as Diversity Arrays Technology. Moreover, comparison of moderately sex-linked loci between the two candidate systems showed homology to *dctn4*, *gucdu* and *add3*, uncoventional SD genes in teleosts, and many male-linked SNP/PA loci identical to transposable elements such as *Gypsy*, *Mariner/Tc1*, and *SINE* (Nguyen et al., 2021a). Similar results were obtained for bighead catfish *Clarias macrocephalus* where both XX/XY and ZZ/ZW sex-linked loci were observed occasionally in the same individual, tendind for a male heterogametic sex determination. One male-linked locus shows homology with the *gtsf1l* (gametocyte-specific factor 1-like), which have a testis-enriched expression pattern, whereas two other male-linked loci were partially homologous to transposable elements (Nguyen et al., 2021b).

In the striped catfish *Pangasianodon hypophthalmus*, a male-specific duplication on the Y chromosome of the autosomal anti-Müllerian hormone receptor type gene (*amhr2a*) named *amhr2y* was recently characterized as being sex-linked in males and expressed only in testicular samples (Wen et al., 2022). Additionally, Amhr2by translates as an N-terminal truncated type II receptor, which corresponds to two first exons and part of exon 3 of Amhr2a, lacking its whole extra-cellular domain mediating ligand binding. Sex-linkage studies and genome sequencing of 11 additional Pangasiid species show that *amhr2by* is conserved as a male-specific gene in at least four Pangasiid species, stemming from a single ancient duplication/insertion event at the root of the Siluroidei suborder radiation that is dated around 100 million years ago (Wen et al., 2022).

Regardless the GSD systems, environmental factors such as temperature can also insert major perturbations to sex ratios in catfishes (Patino et al., 1996; Sulis-Costa et al., 2013; Santi et al.,2016; Yu et al., 2021). However, the pattern of response to temperature effect may vary among Siluriformes, with high temperature-induced masculinization in yellow catfish *Tachysurus fulvidraco* (Yu et al., 2021) and in African catfish (Santi et al., 2017), while in channel catfish masculinization was induced by low temperature (Patiño et al., 1996). Moreover, sex dimorphism in growth rate also vary among Siluriformes, with females growing

faster than males in Amur catfish *Silurus asotus*, *Pangasianodon djambal* and African catfish (Legendre et al., 2000; Abanikannda et al., 2019; Shen et al., 2020;), whereas in channel catfish, *Pelteobagrus fulvidraco* and *Pangasianodon bocourti*, males grow faster than females (Davis et al., 2007; Meng-Umphan, 2009; Lu et al., 2015).

Like in most other gonochoric teleost species, ovarian differentiation precedes with the formation of ovary cavity starting from 19 dpf in channel catfish, 25 dpf in *Tachysurus fulvidraco*, 13 days post hatching (dph) in *Pelteobagrus fulvidraco* and 45 dph in African catfish; while the onset of testicular differentiation starts much later, between 90 and 110 dpf in channel catfish, 65 dpf in *T. fulvidraco*, 55 dph in *P. fulvidraco* and 52 dph in African catfish (Patiño et al., 1996; Hong, 2008; Santi et al., 2016; Yu et al., 2021). The genetic pathways underlying sex differentiation and maintenance are well described only in African catfish, where dimorphic expression of various transcription factor (*wt1*, *dmrt1*,*sox9a*, *sox9b*, *sox3*, *ad4bp*/*sf-1*, *foxl2*) and steroidogenic enzyme genes (*3-hsd*, *cyp17*, *17-hsd1*, *cyp19a1*, *cyp11b1*) during gonadal ontogeny were evidenced by quantitative real-time RT-PCR (Raghuveer et al., 2011). Transcriptomic comparison between channel catfish testes and ovaries identified male-preferential genes, such as *gsdf, cxcl12*, as well as other cytokines mediating the development of the gonad into a testis (Zeng et al., 2016).

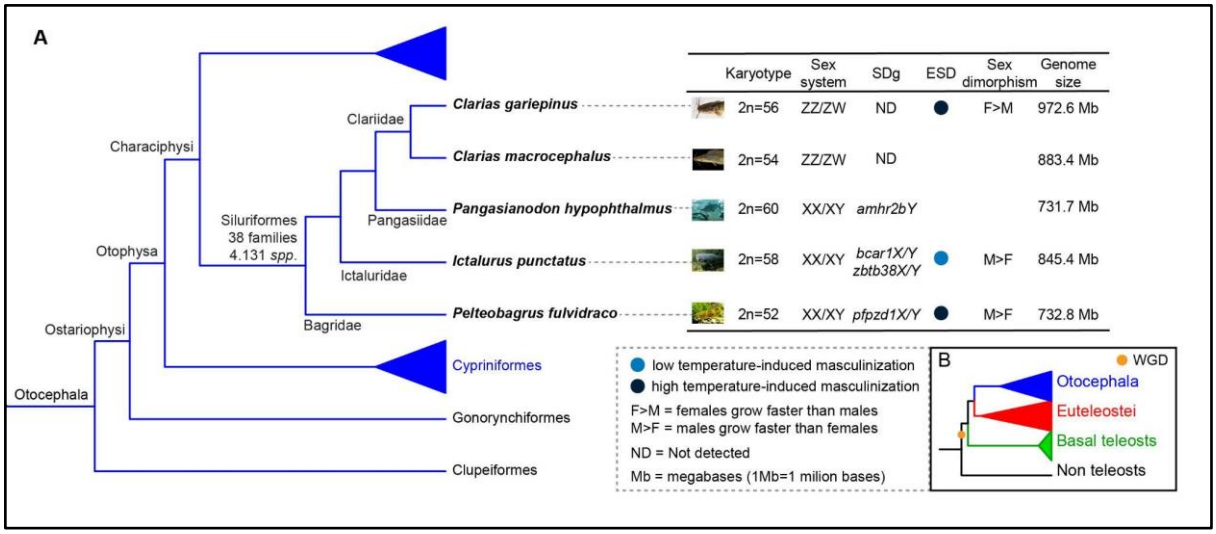


Figure 4: Known sex-determining genes (SDg) in the order Siluriformes and the phylogeny of the species where they are found. Also, informations regarding karyotype diploid number (2n), sex system, occurrence of ESD (Environmental sex determination) and sex dimorphism in growth, genome size in megabase (1 Mb = milion bases) are shown **(A)**. The phylogenetic relationship among Actinopterygii main subdivisions are displayed **(B).**

Otocephala: Ostariophysi - Characiphysi - Characiformes

The order Characiformes or characins is composed by primary freshwater forms that are split into two monophyletic suborders: The african Citharinoidei with about 116 valid species in two families, and the Characoidei with 2215 valid species in 22 families which only two being endemic to Africa and the remaining from Neotropics (Fink and Fink, 1996; Fricke et al., 2020). Across the major characin subseries including Crenuchoidea, Alestioidea, Erythrinoidea, Curimatoidea and Characoidea, an extensive diversity can be found, intra- and interspecifically, including different diploid numbers, hybrid cytotypes and differential intra-genomic spread of repetitive DNAs (Nishiyama et al., 2016; Pucci et al., 2018, Nascimento et al., 2020; Sember et al., 2020).

Several sex systems have been reported for Characiformes species including ZZ/ZW, highly degenerated ZW (Yano et al., 2017) or at initial stages of differentiation (Barros et al., 2018); XX/XY involving heteromorphic or homomorphic sex chromosomes (Freitas et al., 2017); and multiple sex chromossomes $X_1X_1X_2X_2/X_1X_2Y$ (Bertollo et al., 2004; Moraes et al., 2019), $X_1X_1X_2X_2/X_1X_2Y$ (Sember et al., 2017), XX/XY_1Y_2 (Sember et al., 2021) $Z_1Z_1Z_2Z_2/Z_1W_1Z_2W_2$ (Ramirez et al., 2017), ZZ/ZW_1W_2 (Jorge and Moreira-filho, 2000). However, although many species lacking differentiated sex chromosomes, the female heterogamety is more frequent (Galleti et al., 1995; Centofante et al., 2002; Venere et al., 2004;

Vicari et al., 2006; Diniz et al., 2008; Bellafronte et al., 2012; Figliuolo et al., 2020, Sember et al., 2021).

Studies on sex determination and gonad differentiation in Neotropical fish is fragmentary and limited to a very few and distant related species (Fernandino and Hattori, 2018). Despite being an important emerging model species with many resources, including some genome sequences (Di Palma et al., 2007; Hinaux et al., 2013; McGaugh et al., 2014; Herman et al., 2018), *A. mexicanus* (cavefish originating from Pachón cave) sex differentiation has been investigated recently showing unexpected results regarding ovarian differentiation genes (i.e., *foxl2a*, *cyp19a1a*, and *wnt4b*) which displayed an early sexually-dimorphic expression and, surprisingly, exhibited predominant expression in adult testes (Imarazene et al., 2020). More recently, the high-quality genome assembly of *A. mexicanus* B chromosome, male-predominant, provided the characterization of the first B master sex-determining (MSD) in animals with strong functional evidences that *A. mexicanu*s B-sex" chromosome contains duplicated copies of the *gdf6b* gene, that can promote male sex determination in this species (Imarazene et al., 2021).

The presence of B chromosomes has been correlated with the differential expression of major sex-associated genes such as the downregulation of *foxl2* in B chromosome carrier females and with higher levels of *dmrt1* in B chromosome carrier males of *Astyanax scabripinnis*, indicating the adaptive relevance of this chromosome, directly influencing the reproductive cycle dynamic of these animals and expanding the reproduction period, enabling the generation of a greater number of offspring (Castro et al., 2019a, 2019b). In contrast, B chromosomes in *A. scabripinnis* do not seem to share a direct association with hermaphroditism at the level of the analyses that were performed, and hermaphroditism seems to be associated with abiotic factors, possibly functioning as a buffering element in the maintenance of the sex ratio (Cornelio et al., 2017).

For *Astyanax altiparanae*, none of classical genes involved in the female pathway such as *esr*, *rspo*, *foxl2* and *cyp19a1a* exhibited a dimorphic expression in the adult gonads. Additionally, *A. altiparanae* showed a complex gonadal plasticity in terms of genetic expression profiles after E2 treatment (Martinez-Bengochea et al., 2020). To date, no species of the genus *Astyanax* has demonstrated morphologically differentiated sex chromosomes, which could be a facilitating factor for the increase in plasticity regarding sex determination

under the influence of environmental and/or behavioral factors in these fishes (Cornelio et al., 2017).

Among the commercially interesting species from the Serrasalmidae family such as tambaqui (*Colossoma macropomum*) and Pacu (*Piaractus mesopotamicus*), despite the elevated chromosomal diversification within this group, there is no evidence of differentiated sex chromossomes (Adriano et al., 2006; Nakayama et al., 2012; Ribeiro et al., 2014; Favarato et al., 2019). Interestingly, one small B element was recently found in the female genome of *Mettynnis lippincottianus* (Characiformes: Serrasalmidae) highlighting the potential role of intraspecific variation in the karyotype evolution of the genus (Favarato et al., 2019). However, despite the large amount and high diversity of species (101 valid species, distributed in 16 extant genera) (Fricke et al, 2020; Ota et al., 2020) presently only two genomes from serrasalmidae species such as tambaqui (Ferraz et al., 2020; Hilsdorf et al., 2021) and Red-bellied piranha *Pygocentrus nattereri* (Schartl et al., 2019) have been released.

A high density genetic map were constructed recently for tambaqui, male and female, which allowed the identification of a strong sex associated QTL in LG 26, providing data to support an XX/XY female/male heterozygous sex determination (Varela et al., 2021). However, Unlike most XX/XY sex determination systems described in fish to date, in which single gene duplicates or allelic variants of genes known to be related to sex determination and/or differentiation were reported (Herpin and Schartl, 2015), in tambaqui no significant differences were observed between maps and the SNPs associated with sex were not located in coding regions. Furthermore, It has been showed through transcriptome approach that the molecular cascade towards female sex differentiation pathway is represented by the components of the Wnt/β-catenin pathway - with special emphasis on *wnt4* and its downstream genes - and the classic *foxl2*, rather than the classical estrogen signaling components such as estrogen receptors, *esr1*, *esr2a*, *esr2b* and aromatases, *cyp19a1a* and *cyp19a1b* (Lobo et al., 2020).

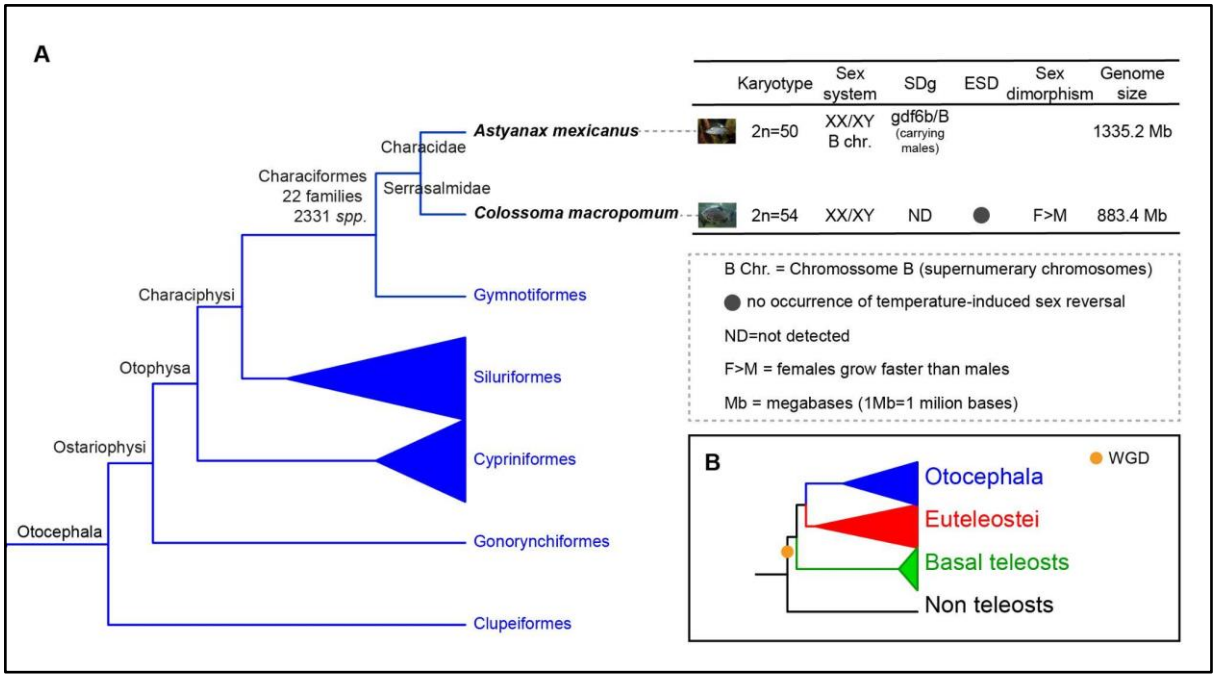


Figure 5: Known sex-determining genes (SDg) in the order Characiformes and the phylogeny of the species where they are found. Also, informations regarding karyotype diploid number (2n), sex system, occurrence of ESD (Environmental sex determination) and sex dimorphism in growth, genome size in megabase (1 Mb = milion bases) are shown **(A)**. The phylogenetic relationship among Actinopterygii main subdivisions are displayed **(B)**.

Euteleostei: Protacanthopterygii (basal Euteleostei)

The Euteleostei lineage comprises four major clades: Lepidogalaxiiformes (1 valid species), Protacanthopterygii (431), Stomiatii (498), and Galaxiiformes (66) plus Neoteleostei (Straube et al., 2018; Fricke et al., 2020). Among basal euteleosts, most literature concerns the sister orders Esociformes and Salmoniformes (Protacanthopterygii) which present variable genome sizes and highly variable karyotypes. Chromosome numbers in species from genus Esox are centered at 2n=50 (Arslan and Alpaslan, 2020), while esociformes outside this genus have varying karyotypes of 2n=22–78, with only *Novumbra hubbsi* exhibiting a diploid chromosome number of 2n=48 (Crossman, 2001). Although no morphologically distinguishable sex chromossomes have reported for this group, genomic resources have recently been generated for norther pike *Esox lucius*, including a whole genome assembly anchored on chromosomes, that permited the identification of an old duplicate of *amh* (*amhby*) as the male sex determination gene in this species (Pan et al., 2019). Given the relatively high level of divergence between *amha* and *amhby* sequences in *E. lucius*, the low level of differentiation between a pair of sex chromosomes harboring an old MSD gene (480 kb) was unexpected.

Esociformes diverged from Salmoniformes prior to the salmonid whole genome duplication (WGD; Ss4R) event which means that it may closely approximate the ancestral 3R genomic state (Berthelot et al., 2014). This phenomenon is evidenced by larger Salmoniformes karyotypes, highly variable across subfamilies Coregoninae (i.e. whitefishes, ciscos, inconn; 2n=58-82), Thymallinae (i.e. graylings; 2n=98-102) and Salmoninae (i.e. trouts, salmons and charrs; 2n=52-86) (Phillips and Ráb, 2001). Several salmonid species have been shown to possess a genetic mechanism of sex determination characterized by an XY chromosomal system, although environmental factors and sex steroids influence the expression of sex phenotype (Davidson et al., 2009). Among identified teleost MSD genes, the *salmonid* MSD gene, named *sdY*, a truncated copy of the *irf9*, is the most intriguing because arose from duplication of an immune-related gene which has not been implicated in sexual development so far (Yano et al., 2012a). The *sdY* sequence is highly conserved in all salmonids being a male-specific Y-chromosome gene in the majority of these species, with the exception of two whitefish species (subfamily Coregoninae), in which *sdY* was found both in males and females suggesting that alternative sex-determination systems may have also evolved in this family (Yano et al., 2013).

The time course of expression in the Atlantic salmon suggests that *sdY*, which appeared to be expressed in males from 58 days postfertilization (dpf), may be involved in the upregulation of *gsdf* and *amh/mis* (at 69, 83, 97, 111 and 124 dpf) and the subsequent repression of *cyp19a* in males (starting at 83 dpf) via the effect of *amh/mis* (Krzysztof et al., 2015). Similarly, in agreement with its male-determining role, *sdY* expression in Rainbow trout was detected only in male gonads, with a peak of expression around 45 dpf, and after this time point *foxl2b1* and *foxl2b2* as well *cyp19a1a* and *nr5a1* are markedly up-regulated in females and down-regulated in males (Betho et al., 2018). Additionally, in vitro and in vivo approaches suggest that in the presence of Foxl2, SdY is translocated to the nucleus where the SdY:Foxl2 complex prevents activation of the aromatase (*cyp19a1a*) promoter in cooperation with Nr5a1 (Sf1) (Betho et al., 2018). Indeed, expression of *cyp19a1* and *fst* in females and *sox9a1* in males were sexually dimorphic between 32 to 35 dpf, and after that the differentiation proceeded with sexually dimorphic profiles for *sox9a2, dmrt1, cyp11b2.1, amh* in males and *foxl2a, foxl2b, hsd3b1, inha* in females (Vizziano et al., 2007). Thus, the overall mechanism of sex differentiation in salmonids can be explained by the blockage of positive loop needed for the synthesis of estrogens in the early differentiating gonad marked by *sdY* expression, that further

disrupts a preset female differentiation pathway and consequently allowing testicular differentiation to proceed.

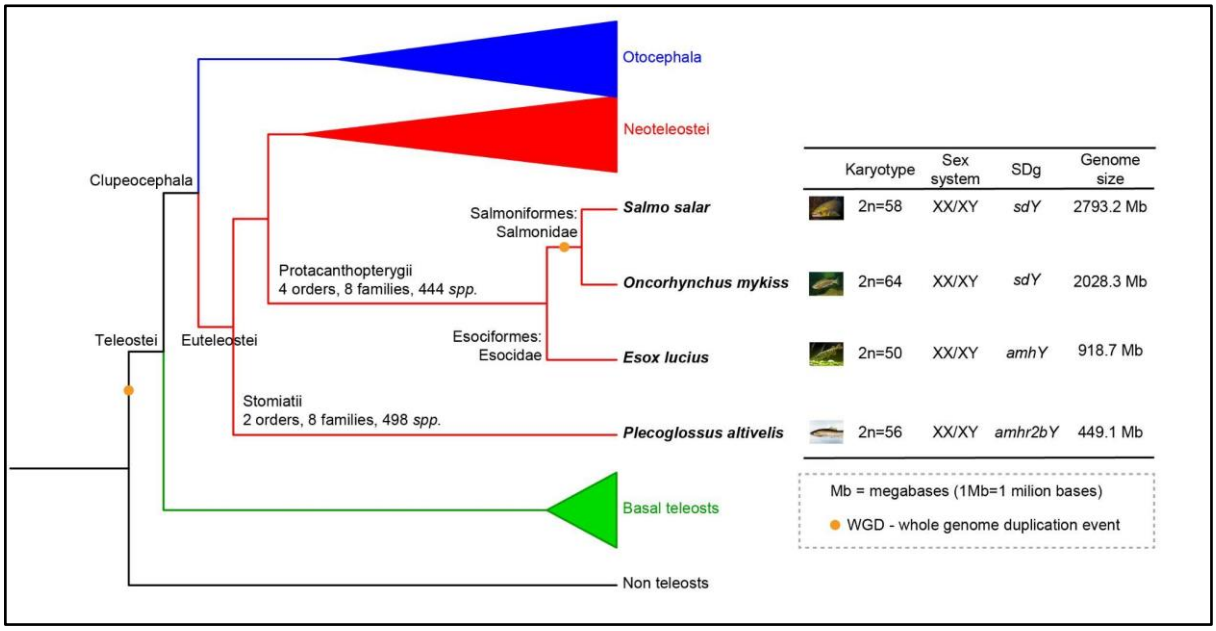


Figure 6: Known sex-determining genes (SDg) in Protacanthopterygii and Stomiati superorders, together with the phylogeny of the species where they are found. Also, informations regarding karyotype diploid number (2n), sex system, genome size in megabase (1 Mb = milion bases) are shown.

Neoteleostei: Acanthomorpha - Gadiformes

Neoteleostei is a large euteleost clade that comprises the orders Ateleopodiformes, Aulopiformes, Myctophiformes, plus Acanthomorpha or spiny-rayed fishes, which represent the largest group of teleost fishes, with estimates of diversity reaching close to 19,000 species (Fricke et al., 2020). The Acanthomorpha includes the following main lineages: the most basal Lampriformes (including open-ocean and deep-sea taxa as oarfish, opahs, and ribbonfishes) and Polymixiiformes (deep-sea beardfishes), that are relative to the other major acanthomorph groups Paracanthopterygii (cods, freshwater trout-perches, cusk-eels, anglerfishes, and toadfishes) and Acanthopterygii (the advanced acanthomorphs) (Chen et al., 2014; Hughes et al., 2018).

Gadiformes (Acanthomorpha: Paracanthopterygii) comprises 621 species distributed in 17 families (Fricke et al., 2020), with diploid chromosome numbers varying from 2*n* = 26 in *Eleginus* species to 2*n* = 46 in European Hake *Merluccius merluccius*, Atlantic cod *Gadus morhua*, and 2n=48 in Poor cod *Trispoterus minutes*, tadpole fish *Raniceps raninus*, and the burbot *Lota lota*, the only freshwater gadiform species (Arai, 2011; Ghigliotti et al., 2012;

García-Solto et al., 2015; Kirtiklis et al., 2016). However, most studies regarding sex determination and differentiation of gadiforms are usually restricted to species important in fisheries such as cods, mainly the Atlantic cod (Johnsen and Andersen, 2012; Johnsen et al., 2013; Nagasawa et al., 2014; Star et al., 2016; Kirubakaran et al., 2019; Kim et al., 2020). In this way, the characterization of the male specific region on LG11 suggest that the *zkY* gene is involved in Atlantic cod *Gadus morhua* sex determination. Cod ZkY and the autosomal proteins Zk1 and Zk2 possess an identical zinc knuckle structure, but only the Y-specific gene *zkY* was expressed at high levels in the developing larvae before the onset of sex differentiation (kirubakaran et al., 2019). This study also documents the presence of X- and Y-zinc knuckle protein sequences in other gadoids suggesting that this arrangement predates the split of Arctic cod from Atlantic more than 7.5 MYA and Lotinae (*Lota lota*)– Gadinae (*Gadus morhua*) split 45 MYA. Although the *zkY* MSD gene has not been recruited from the conserved genes within the sex differentiation cascade, Zinc knuckle proteins are members of the large family of zinc finger proteins possessing a versatility of tetrahedral Cys- and His-containing motifs that bind to DNA and RNA target sites, in a way similar to other transcription factors such as DMRT (Michalek et al., 2011).

Atlantic cod is a differentiated gonochoristic species in which the first molecular signs of sex differentiation commenced at around 12 mm total body length (TL), resulting in a sex-dimorphic expression pattern of *cyp19a1a* before ovarian cavity formation (18 to 20 mm TL); and in a male-specific increase of *amh* from 20 mm TL prior to testis development which occured later and was morphologically evident after 30 mm TL (Haugen et al., 2012).

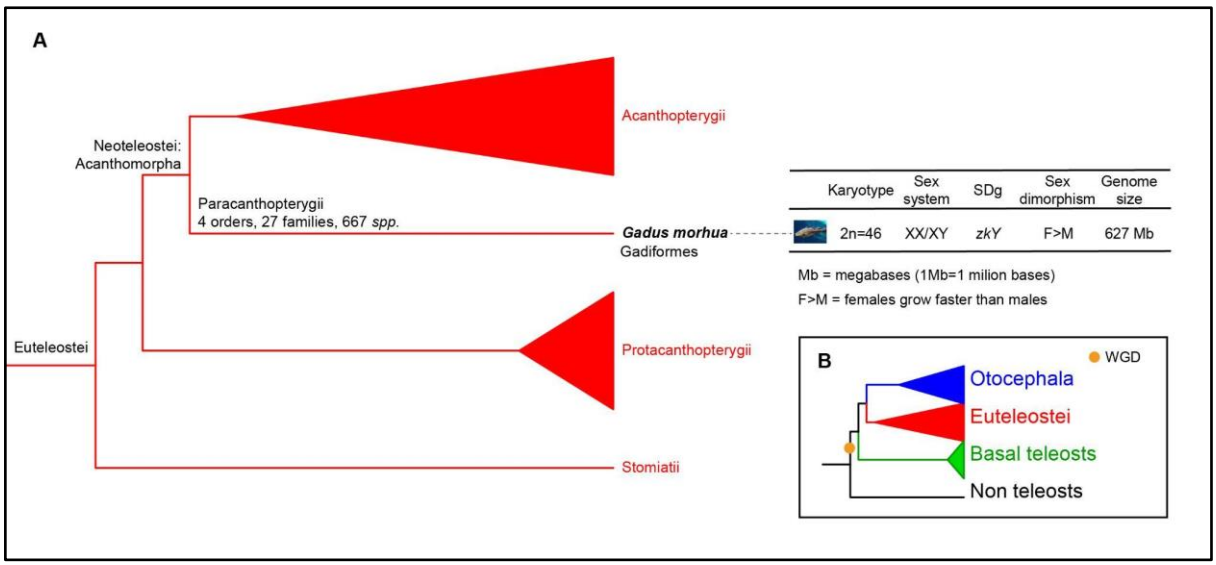


Figure 7: Known sex-determining genes (SDg) in the order Gadiformes and the phylogeny of the species where they are found. Also, informations regarding karyotype diploid number (2n), sex system, occurrence of sex dimorphism in growth, genome size in megabase (1 Mb = milion bases) are shown **(A)**. The phylogenetic relationship among Actinopterygii main subdivisions are displayed **(B).**

Neoteleostei: Acanthomorpha - Acanthopterygii - Percomorpha

The superorder Acanthopterygii comprises the orders Beryciformes, Trachichthyiformes and Holocentriformes plus the Percomorpha lineage, the most species-rich clade of modern fishes, which in turn is subdivided into six series: Pelagiaria (tunas), Syngnatharia (seahorses, pipefishes), Carangaria (harboring extremely asymmetric benthic flatfishes, dolphinfish and jacks), Anabantaria (containing many air-breathingand predominantly freshwater fishes), Ovalentaria (composed of cichlids, atherinomorphs, blennies, clingfishes) and Eupercaria (basses, groupers, sticklebacks, and sculpins) (Hughes et al., 2018).

Notable karyotype similarities have been highlighted across Percomorpha lineages (Molina et al., 2013; Costa et al., 2016) that exhibit small or modest reductions (2n=20-46), expansions (2n=50), and also polyploid deviations from the probable ancestral state of 2n=48, the most frequent number of chromossomes (Mank and Avise, 2006; Nirchio et al., 2014; Motta-Neto et al., 2019) with the rare presence of diferentiated sex chromosomes, except for the species from the order Gasterosteiformes in which degenerated Y chromosome is frequent (Sember et al., 2021). However, the vast genomic sequence resources currently available for percomorph species reflects the largest number of master SD characterized in this group.

Percomorpha: Ovalentaria - Beloniformes

The order Beloniformes comprises 293 valid species distributed in six families and 35 genera (Fricke et al., 2020). Medaka fishes or ricefishes belong to Oryzias genus within Adrianichthyidae family, which live in fresh-, brackish-, and seawater of East and South Asia and the Indo-Australian archipelago (Parenti, 2008). Oryzias fishes have been intensively investigated for their mechanisms of sex-determination, where *dmrt1b*/*dmy* has been reported as MSD gene for the first time in *Oryzias latipes* and *O. curvinotus* (Matsuda et al., 2002; Nanda et al., 2002), which arose through a segmental duplication of a small autosomal region containing its precursor gene *dmrt1* and some neighboring genes, followed by the insertion of the duplicated region on the proto-Y chromosome, directly creating a region of suppressed

recombination (258 kb). The *dmy* was expressed only in somatic cells of XY gonads at the beginning of sexual differentiation (5 days after hatching) and mutation of *dmy* caused XY medakas to develop into females (Matsuda et al., 2002). The molecular mechanism of *Dmy* was characterized by RNAi in which *Dmy* knockdown suppressed male pathway (*gsdf, sox9a2*, etc.) and favoured female cascade (*rspo1*, etc.) in embryonic XY gonads, resulting in a fertile male-to-female sex-reversal (Chakraborty et al., 2016). Furthermore, *wt1a* stimulated the promoter activity of *Dmy*, suggesting *wt1a* as a regulator of *Dmy* (Chakraborty et al., 2016). Therefore, although the sex of medaka is genetically determined, that germ cells are also important for feminization of gonads (Kurokawa et al., 2007; Nishimura et al., 2018). High temperatures also decrease the number of germ cells, which often accompanies female-to-male sex reversal (Selim et al., 2009; Hayashi et al., 2010). The influence of estrogen in early germ cell development and its consequences on sexual identity in medaka *O. latipes* was evidenced by transgenerational *esr2b*-knockdown (ERβ2-KD) line and *esr2b*-null mutants (Chackraborty et al., 2019). Although fetal estrogenic concentration and actions are instrumental in gonadal sex differentiation in medaka, null mutation of *cyp19a1a* does not affect the early gonadal development, suggesting.that traces of remaining estrogen (i.e. maternal estrogen from yolk stores) or other estrogen by-products might be enough to drive the normal gonadal development (Nakamoto et al., 2018). Indeed, zygotic estrogen receptor beta 2 (*esr2b*) and brain type aromatase (*cyp19a1b*) transcription onset, at 2 days after fertilization, precedes the gonadal type aromatase (*cyp19a1a*) expression at 5-10 days after hatching (Chakraborty et al., 2011; Okubo et al., 2011), and *esr2b* knockdown reduces the *cyp19a1b* expression in both gonad and brain (Nakamoto et al., 2018). Moreover, elevated cortisol due to the high temperature may induce masculinization by down-regulating directly the expression of *cyp19a1b* through activation of the peroxisome proliferator-activated receptor (*pparaa*) signalling pathway during gonadal sex differentiation in medaka (Hara et al., 2020).

Different sex chromosomes have been observed in other *Oryzias* species, and their their sex-determining loci are located on different chromosomes, suggesting that these mechanisms are regulated by different genes and have evolved rapidly. $sox3^{Y}$ evolved as the sex-determining gene in *O. dancena* by activating a downstream *gsdf* gene and regulated by Y chromosome-specific distant *cis*-regulatory sequence at the sex-determining locus that dominantly affects distant *Sox3* expression (Takehana et al., 2014). Additionally, *gsdf* functions as the male-determining gene in *O. luzonensis*, where the gene has a Y- and X-chromosome-specific allele, called $gsdf^{Y}$ and $gsdf^{X}$, varying by 12 silent nucleotide substitutions, with male

expression caused by a mutation in a *cis*-regulatory region of $gsdf^{Y}$ in a putative binding site for steroidogenic factor 1 (Sf1), causing a loss of the binding site in $gsdf^{Y}$ but not in $gsdf^{X}$ (Myosho et al., 2012).

Percomorpha: Ovalentaria - Atheriniformes

The order Atheriniformes, commonly known as silversides, rainbowfishes, and blue eyes, includes about 385 fish species distributed in 11 families (Fricke et al., 2020), occuring in a wide range of environments from freshwater lakes, lagoons and rivers, to estuaries and coastal marine waters, and are globally distributed in tropical and temperate regions.temperate regions (Campanella et al., 2015). A duplicate copy of *amh* (*anti-Müllerian hormone*) has been found on the Y chromosome in species from Atherinopsidae family such as *Odontesthes bonariensis* (Yamamoto et al., 2014), *Odontesthes hatcheri* (Hattori et al., 2012) and Cobaltcarp silverside *Hypoatherina tsurugae* (Bej et al., 2017). Although they share the same MSD gene, *O. hatcheri* exhibit the weakest sex ratio response to water temperature in contrast to *O. bonariensis*, in which sex ratios reach 100% female or 100% male at environmentally relevant temperatures of 17°C (female producing temperature) and 29°C (male producing temperature), respectively (Strüssmann et al., 1997; Yamamoto et al., 2014; Fernandino and Hattori, 2019). In *O. hatcheri*, *amhy* is expressed during gonadal formation starting at 6 days after fertilization, whereas *amha* is expressed later (12 weeks after fertilization) and Knockdown of *amhy* in XY embryos led to an up-regulation of female factors and, consequently, to the development of ovaries. Similarly, expression analyses in *O. bonariensis* revealed that *amhy* transcription began during embryo stage and decreased by the end of sex determination period, while the expression of *amha*, although not as early as that of *amhy*, coincided temporally with the period when the pejerrey gonads are still sexually labile (Yamamoto et al., 2014).

Percomorpha: Ovalentaria - Cyprinodontiformes

The order Cyprinodontiformes contains 1435 valid species distributed in 14 families, found primarily in Africa and the Americas (Fricke et al., 2020). Viviparity and Annualism are the most remarkable life history strategies found in some Cyprinodontiformes, and both traits have the potential to affect diversification in different ways (Helmstetter et al., 2016). The turquoise killifish *Nothobranchius furzeri* is an annual fish with fast lifecycle and short lifespan that can reach sexual maturity in < 3 weeks while first signs of sexual dimorphism are apparent

at 2 weeks after hatching. This can be reflected in the unusual *gdf6Y* as the master sex-determining gene in *N. furzeri* as well intra-species Y chromosome polymorphism found across strains from Zimbabwe-GRZ, Mozambique-MZM, and borderland between Mozambique and Zimbabwe-MZZW (Reichwald et al., 2015).

Percomorpha: Ovalentaria - Cichliformes

In the order Cichliformes, that comprises a total estimated number of over 3000 species with rapid organismal diversification (Salzburger, 2018; Ronco et al., 2020), it has been reported by the integration of male and female whole genome sequencing (244 taxa) and transcriptome (66 taxa) from several cichlid lineages that Tanganyikan cichlids have the highest rates of sex chromosome turnover and heterogamety transitions (XY to ZW) known to date (Taher et al., 2021). *amh/amhy* have been reported in other species from *Oreochromis* genus (Li et al., 2015; Curzon et al., 2020), however, functional evidence for a master SD gene in cichlids was reported only for tilapia *Oreochromis niloticus* in which a male-specific duplication of *amh*, denoted *amhy* was identified, characterized and mapped to the QTL region on LG23 for SD (Eshel et al., 2014). Furthermore, this study also reported the discovery of sexually differentially expressed genes and microRNAs at a very early stage of tilapia embryonic development (2 dpf), but only amh was differentially expressed in male embryos and testis among the fifty-one genes that were positional candidates in the 1.47 Mbp critical region of the SD in tilapia (Eshel et al., 2014). Moreover, prior the first signs of gonadal sex differentiation in tilapia (23-26 dah), which can be the formation of the ovarian cavity in the XX gonad or the efferent duct in the XY gonad, the sex-specific expression of *foxl2* and *cyp19a1a* in XX gonads and *dmrt1* in XY gonads during early gonadal differentiation (5–6 dah) is critical for undifferentiated gonads to differentiate into either the ovary or testis in the Nile tilapia (Ijiri et al., 2008). In addition, aromatase plays a key role during temperature-induced masculinization of tilapia (D'Cotta et al., 2001). The critical role of estradiol-17beta in tilapia ovarian differentiation has been proved by *foxl2* and *cyp19a1a* mutant lines, where $Foxl2^{-/-}$ and $cyp19a1a^{-/-}$ XX fish displayed female-to-male sex reversal, demonstrating that *foxl2* promotes ovarian development by upregulating *cyp19a1a* expression, increasing germ cell number, and repressing male pathway gene expression such as *sf1*, *dmrt1*, *gsdf*, *cyp11b2* and *star1* (Li et al., 2013; Zhang et al., 2017).

Apart from that, within the adaptive radiation of East African cichlids, sex determination in *Astatotilapia burtoni* involve the presence of multiple, novel sex

determination alleles, and thus the presence of polygenic sex determination (Roberts et al., 2016). Moreover, recruitment of sex differentiation genes into roles other than its usual role have been reported in East African cichlids, where the well-known female-associated aromatase genes (*cyp19a1a* and *cyp19a1b*) have shown a new role in testis differentiation (Böhne et al., 2013). This unconventional pattern was further supported by aromatase inhibitor treatments that failed in transforming ovaries into functional testis (Göppert et al., 2016), contrasting with the complete functional sex reversal obtained for model species, even long after the original sex differentiation time window in adult females, such as Nile tilapia (*Oreochromis niloticus*) which belongs to the same order (Paul-Prasanth et al., 2013; Sun et al., 2014).

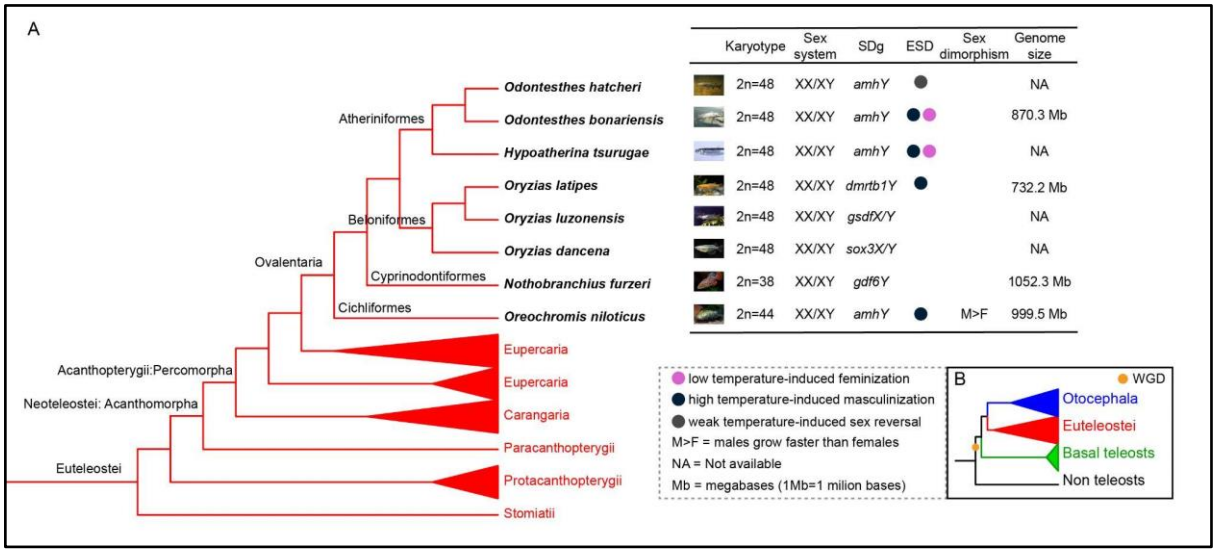


Figure 8: Known sex-determining genes (SDg) in the Ovalentaria subseries and the phylogeny of the species where they are found. Also, informations regarding karyotype diploid number (2n), sex system, occurrence of ESD (Environmental sex determination) and sex dimorphism in growth, genome size in megabase (1 Mb = milion bases) are shown **(A)**. The phylogenetic relationship among Actinopterygii main subdivisions are displayed **(B).**

Percomorpha: Eupercaria

Also within Eupercaria series, a single-nucleotide polymorphism (SNP) within *amh receptor* namely *amhr2* have also been reported to be responsible for SD in tiger pufferfish *Takifugu rubripes* from the order Tetraodontiformes (Kamiya et al., 2012); *dmrt1* was identified to be robustly associated with sex in *Larimichthys crocea*, from the order Acanthuriformes, by GWAS (Lin et al., 2021) and comparative genomics (Lin et al. 2017). Furthermore, 21 significant sex-associated single nucleotide polymorphisms (SNPs) on chromosome 22 were identified near 14 candidate genes related to sex differentiation (*dmrt1*,

dmrt2 and *dmrt3*) spermatogenesis (*spef2*, *strbp*, *odf2*, *piwil2*, *cfap157*, and *golga3*), sex hormone metabolic process, estrogen response, and cholesterol biosynthetic process (*hsd17b4*, *znf366*, *fam102a*, and sec14l), in *L. crocea* (Lin et al., 2021).

Among the order Perciformes, the largest group within Eupercaria with 4.113 valid species distributed in 65 families (Fricke et al., 2020), usual suspects MSD genes are recurrent. In the spotted scat *Scatophagus argus* (Scatophagidae) a truncated *Dmrt1* (*Dmrt1b*) was found to exist in both males and females, whereas a 3.2 kb fragment between exon 2 and exon 3 of a normal *Dmrt1* could be amplified only in males implying that *Dmrt1* is located on the Y chromosome (Mustapha et al., 2018). Similarly, in the yellow drum *Nibea albiflora* (Scianidae), a male-specific 45 bp deletion was found located in the first intron of the *Dmrt1* gene through comparative analysis between male and female (Sun et al., 2018). The characterization of the yellow perch *Perca flavescens* sex determination locus, based on a chromosome-scale genome assembly of male and female, revealed a male-specific duplicate of the anti-Mullerian hormone type II receptor gene (*amhr2by*) inserted at the proximal end of the Y chromosome (chromosome 9) (Feron et al., 2020). *amh/amhy* have been reported as MSD gene in rockfish *Sebastes schlegelii* (Scorpaenidae) (Fowler and Buonaccorsi, 2016; Song et al., 2021), in three species from the genus Siniperca (Sinipercidae) such as the mandarin fish (*Siniperca chuatsi*), the spotted mandarin fish (*Siniperca scherzeri*), and the big eye mandarin fish (*Siniperca kneri*); and in threespine stickleback *Gasterosteus aculeatus* (Gasterosteidae) (Peichel et al., 2020). Otherwise, in the Rock Bream *Oplegnathus fasciatus* (Oplegnathidae), which exhibit a $X_1X_1X_2X_2/X_1X_2Y$ multiple SD systems based on conventional cytogenetic (Xu et al. 2013), tightly linked sex-specific SNPs were enriched in two regions (Chr2 and Chr6), which was coincident with the SD regions that harbor three candidate sex-determining genes (*CCDC63*, *ITR*, *WNT4*) which were furtherly detected in transcriptome data of testes and ovaries (Gong et al., 2022). In *Anoplopoma fimbria* (Scorpaenidae) the characterization of sex locus on the Y chromosome resulted in identification of *gsdf* as the main candidate for fulfilling the master sex determining (MSD) function, which arose by allelic diversification (different X and Y Chromosome copies of this gene) initiated by insertion within the $gsdf^{Y}$ promoter region, bringing *cis*-regulatory modules that led to transcriptional rewiring and thus creation of a new MSD gene (Herpin et al., 2021).

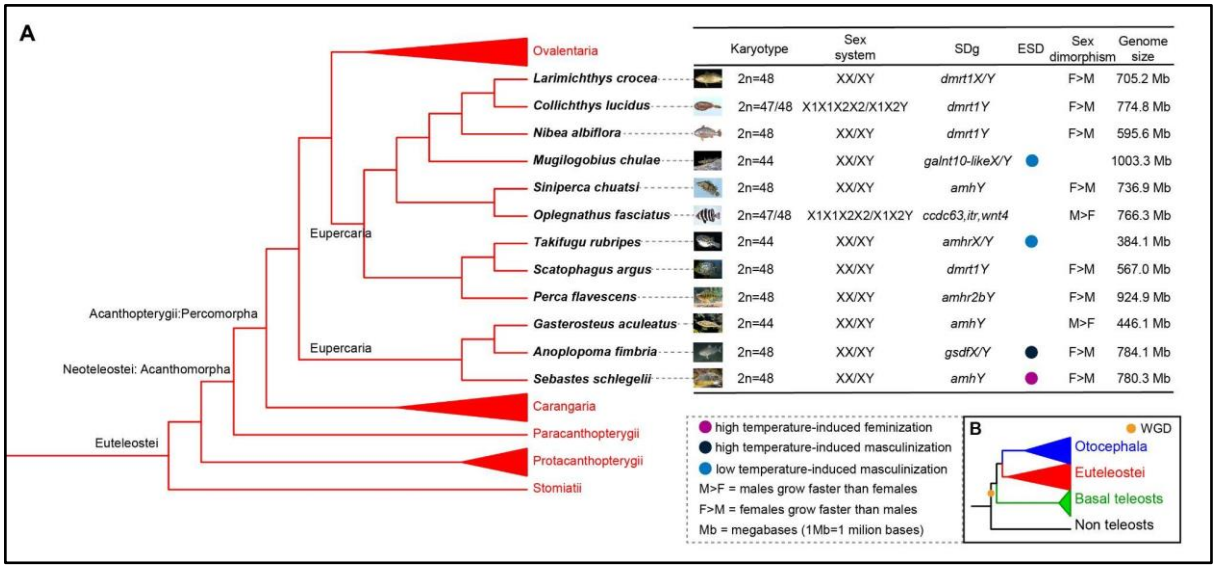


Figure 9: Known sex-determining genes (SDg) in the Eupercaria subseries and the phylogeny of the species where they are found. Also, informations regarding karyotype diploid number (2n), sex system, occurrence of ESD (Environmental sex determination) and sex dimorphism in growth, genome size in megabase (1 Mb = milion bases) are shown **(A)**. The phylogenetic relationship among Actinopterygii main subdivisions are displayed **(B).**

Percomorpha: Carangaria

Among Carangaria series, usual MSD gene suspects can be found in species with ZW system such as the Chinese half-smooth tongue sole *Cynoglossus semilaevis* (Percomorpha: Pleuronectiformes), in which *dmrt1* is located on the Z chromosome while a corrupted version of *dmrt1* is present on the W (Chen et al., 2014). Further, gene expression and knockout studies supported *dmrt1* function as a master SD gene in *C. semilaevis* (Chen et al., 2014; Cui et al., 2017). Novel and unexpected SD genes have been recently reported in other Carangaria species such as *sox2* in *Scophthalmus maximus* from the order Pleuronectiformes, in which the only diagnostic variant detected between Z and W chromosomes can be located on the regulatory element of *sox2* leading to differential expression through a complex regulation of this gene involving a lncRNA (Martínez et al., 2019). Similarly, a missense SNP in the third exon of the gene encoding the steroidogenic enzyme 17β-hydroxysteroid dehydrogenase 1 (*Hsd17b1*) was perfectly associated with ZZ/ZW sex determination in yellowtail amberjack *Seriola lalandi*, from the order Carangiformes, whereas *Z* alleles (containing the less enzymatically effective Glu144 or Lys144) cause silencing of the steroid conversion during sex differentiation, leading to a shortage of E2 and establishement of testis (Koyama et al., 2019). In *Trachinotus ovatus*, Z-linked allele in the sex defining SNP (Chr16) was assumed to result in the intron retention (64 bp), the reading frame shift and the introduction of a premature termination codon of *hsd17b1* (Guo et al., 2021).

More recently, the presence of sex-associated *cyp19a1a*-like gene in Y chromosome from trevally *Pseudocaranx georgianus* (Percomorpha: Carangiformes), in addition to autosomal paralogues of *cyp19a1a* and *cyp19a1b*, revealed the first evidence of c*yp19a1a* duplication and up-recruitment to assume the role of the sex determining gene in teleost, suggesting that it might play a role in the masculinisation of genetically male fish in this species. (Catanach et al., 2021). In this context, the usage of *cyp19a1a* as part of the cascade of sex determination, differentiation, and reproduction across teleosts may differ substantially at different taxonomic levels.

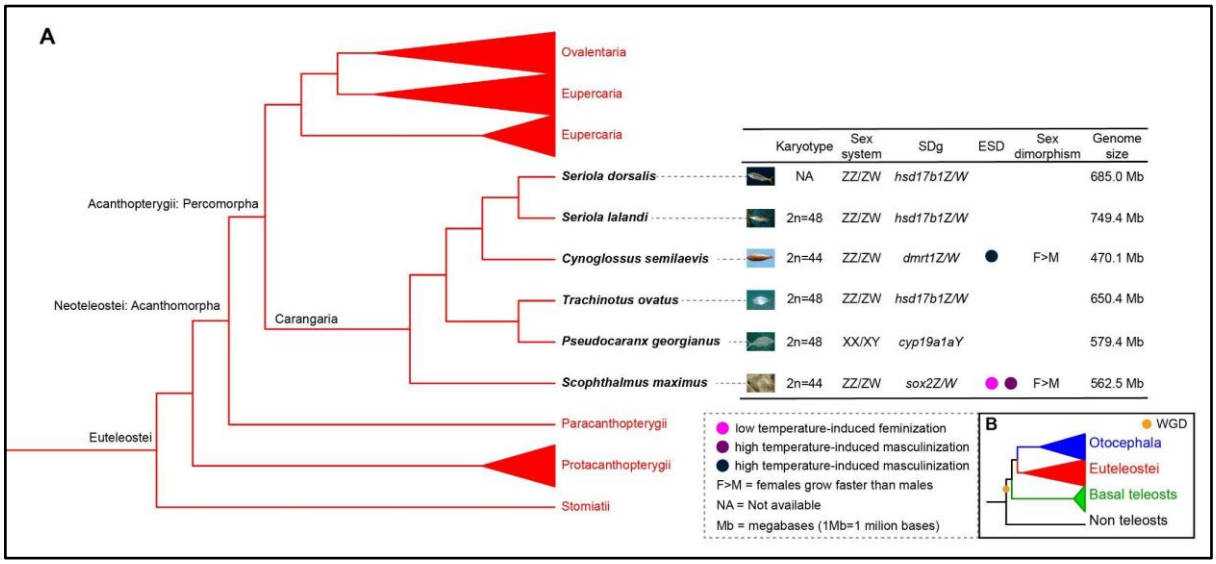


Figure 10: Known sex-determining genes (SDg) in the Carangaria subseries and the phylogeny of the species where they are found. Also, informations regarding karyotype diploid number (2n), sex system, occurrence of ESD (Environmental sex determination) and sex dimorphism in growth, genome size in megabase (1 Mb = milion bases) are shown **(A)**. The phylogenetic relationship among Actinopterygii main subdivisions are displayed **(B).**

3. Concluding remarks

In this review, we attempted to summarize the most recent findings in teleost concerning sex determination and sex differentiation gene recruitments that have been obtained through Next Generation Sequecing in the last years. Looking across fish families and genera, the genetic basis of SD can be profoundly different (Figure 11; Supplementary table 1). Novel SD master genes from TGF-beta signaling pathway were also described in the most basal teleosts such as *A. gigas* (Osteoglossomorpha) and *C. harengus* (a basal order of Otocephala), although never been assigned to an important role in sex determination and dierentiation of teleosts before. Further, more complex and unknown SD mechanisms involving sex-linked

SNPs in non coding regions (i.e. introns, intergenic regions, UTR 5' and 3') of unusual master SD gene suspects (i.e. *bcar1* and *ubq-y*) were found in non-neotropical Otophysians.

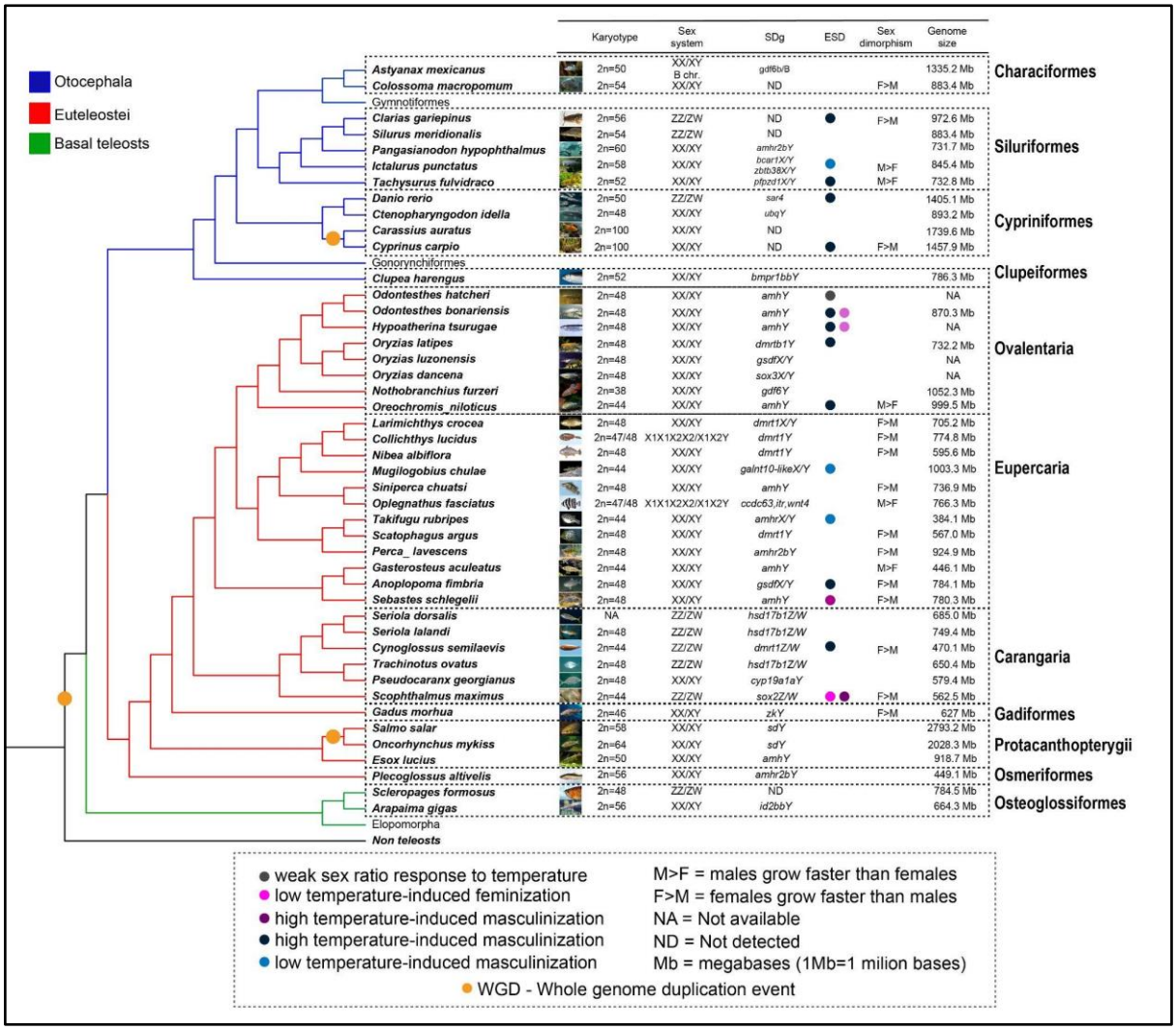


Figure 11: Phylogenetic view of known sex-determining genes (SDg) in teleost lineages and the phylogeny of the species where they are found. Also, informations regarding karyotype diploid number (2n), sex system, occurrence of ESD (Environmental sex determination) and sex dimorphism in growth, genome size in megabase (1 Mb = milion bases) are shown.

Interestingly, the first B master sex-determining was described for the first time in the Characiformes model species *A. mexicanus*, which also exhibited an unexpected expression profile of genes classically involved in female sex differentiation, similar to was found in other Characidae representatives from South America and in *Colossoma macropomum*, member of distant related Serrasalmidae family within the order. Given the huge biodiversity and species richness that are currently vastly unexplored compared to other teleost subdivisions, the discovery of novel SD genes and systems can be expected from Characiphysi orders.

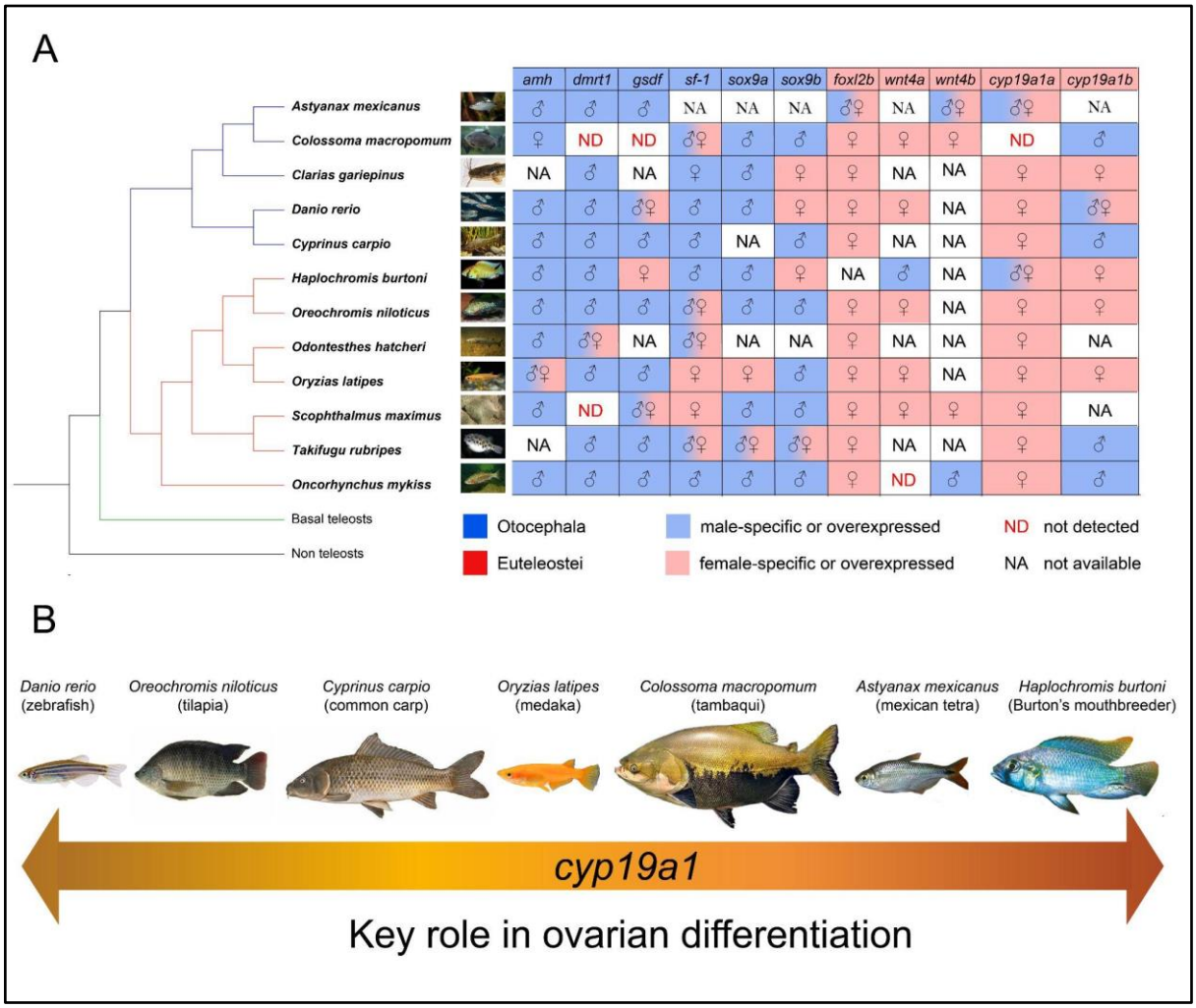
amh is the recurrent master SD gene found only in euteleosts: Different from Otocephala, *amh* emerged as SD master gene at different times in the Euteleost evolution, being recruted to the top of sex determination cascade in the early euteleost *Esox lucius*, as well in the modern Percomorpha clade species from the order Atheriniformes (i.e. *H. tsurugae*, *O. hatcheri*) and Cichliforme (i.e *Oreochromis niloticus*) corresponding to Ovalentaria subseries; and Gasterosteiformes (i.e. *G. aculeatus*), corresponding to Eupercaria subseries.

Usual master SD gene suspects are found mostly in Percomorpha clade with XX/XY GSD system: Other than *amh*, up-recruitment of key sex differentiation genes have been found also in species from orders Belontiformes (i.e. *sox3*, *dmrt1b* and *gsdf* in medakas) and Cyprinodontiformes (i.e. *gdf6* in *N. fuzeri)*, within Ovalentaria, and in Tetraodontiformes (i.e. *amhr2* in *T. rubripes*), within Eupercaria. Interestingly, in species from the order Pleuronectiformes *dmrt1* and *sox2* arose as MSD in a ZZ/ZW system.

The most recent outcomes suggest the up recruitment of estradiol-synthesizing genes to assume the role of the sex determination in carangid species (Carangaria series within Percomorths), such as *cyp19a1a* in *Pseudocaranx georgianus* and *hsd17b1* in *Seriola dumerili* and *Seriola dorsalis*. This findings bring out new well-known members of the sexual development regulatory network to the hall of master SD genes found across Percomorphs, in addition to Dmrt1, Sox3 and Tgf-beta signalling pathway members. Exceptions to this rule were found out of this clade, in which unusual suspects master SD genes were found with no obvious function in sex differentiation molecular cascade, i.e. *zkY* in *Gadus morhua* (Paracanthopterygii: Gadiformes) and sdY in salmonids (Procanthopterygii: Samoniformes).

Evolutionary shifts in *cyp19a1a* gene recruitment found in two distant related species-rich clades: A limited number of studies point out to the plasticity of *cyp19a1* genes, indicating that diversity in teleosts not only affects the core of sex-determinating cascade but also includes sexual differentiation downstream gene-regulatory network (Figure 12; Supplementary table 2). Estrogen-independent ovarian differentiation has been already suggested in *O. latipes* (Euteleostei: Percomorpha), based on the disruption of *cyp19a1* genes, and most recently in species from the order Characiformes (Otocephala: Characiphysi), such as *A. mexicanus* and *C. macropomum* based on unexpected expression profiles of *cyp19a1a* during sex differentiation. Moreover, the recruitment of *cyp19a1a* into roles other than its usual role (aromatisation of androgens into estrogens within the ovaries), as has been reported in Cichlids and Characids, suggest novel testis function in this lineages evidenced by its overexpression in

adult male gonads. This emerging hypothesis is reinforced by the existence of a *cyp19a1aY* copy in *Pseudocaranx georgianus* (Euteleostei: Percomorpha), which might play a role in the masculinisation of genetically male fish through an unknown mechanism.



F**igure 12:** Comparative phylogenetic view of the main sex differentiation gene expression profiles across teleost species, adapted from Heule et al., 2014 **(A)**. A limited number of studies point to the plasticity of *cyp19a1* genes **(B)**, indicating that diversity in teleosts not only affects the core of the sex-determining cascade, but also includes the regulatory network of genes downstream of sexual differentiation.

By the way, despite the diversity of sex determination system (GSD, ESD, GSD+ESD) the critical role of estrogens in fish sex differentiation and sexual plasticity has been proved by transcriptome, epigenetics, chemical treatment and especially by gene knockout analyses (Li et al., 2019). Indeed, knockout of *cyp19a1a*, but not *cyp19a1b*, leads to female-to-male sex reversal in zebrafish and tilapia (Yin et al., 2017, Zhang et al., 2017) whereas medaka XX aromatase loss of function mutant ovaries developed normally only

through the early developmental stages but undergo ovary degeneration and partial female-to-male sex reversal after puberty (Nakamoto et al., 2018).

The evolutionary lability of sex-determining modes in fishes through a phylogenetic point of view has been encouraged primary by Mank et al. (2006). Further, critical rethinking of the current models for the evolution of sex-determining genes with a stronger focus on the interactions further downstream in the network has been suggested previously by Bohne et al. (2013) and Heule et al. (2014). More recently, with the hope to pursue a *sexomics* integrative research initiative across vertebrates, Stöck et al. (2021) reviewed the sex evolution across major clades of vertebrates with information on SD, sexual development and reproductive modes, offereing an up-to-date review of divergence times, species diversity, genomic resources, genome size, occurrence and nature of polyploids, SD systems, sex chromosomes, SD genes, dosage compensation and sex-biased gene expression. Altogether, this review provided a foundation for future mapping of sex determination genes and their interactions through comparative studies across teleost fishes with a closer inspection at family-level and genus-level phylogenies

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Supplementary material

Supplementary table 1 - Infograph displaying an updated list of teleost with known genetic sex determining systems, including the corresponding taxonomic groups, heterozigosity type at order level, ocurrence of environmental influences at order level, genome size, chromosome karyotype, SD genes and allelic vaiation at species level.

Taxa					Heterogamety		Environmental influence	Species ID	GSD system	Assembled genome size (Mb)	Karyotype	SD gene candidate
					Male (XY)	Female (ZW)						
Osteoglossomorpha		Osteoglossiformes			X	X		*Arapaima gigas*	XX/XY	664.3	2n=56	*id2bbY*
Clupeocephala	Otocephala	Clupeiformes			X	X		*Clupea harengus*	XX/XY	790.0	2n=52	*bmpr1bbY*
		Ostariophysi: Otophysa	Cypriniformes		X	X	X	*Cyprinus carpio*	XX/XY	1457.9	2n=100	?
								Carassius auratus	XX/XY	1739.6	2n=100	?
								Ctenopharyngodon idellus	XX/XY	893.2	2n=48	*ubqY*
								Danio rerio	ZZ/ZW	1405.1	2n=50	*sar4*
			Characiphysi	Siluriformes	X	X	X	*Ictalurus punctatus*	XY	845.4	2n=58	*bcar1X/Y, zbtb38X/Y*
								Pelteobagrus fulvidraco	XY	732.8	2n=52	*pfpdz1X/pfpdz1Y*
								Clarias gariepinus	ZW/XY	-	2n=56	?
								Clarias macrocephalus	ZW/XY	883.4	2n=54	?
								Pangasianodon hypophthalmus	XY	731.7	2n=60	*amhr2bY*
				Characiformes	X	X		*Astyanax mexicanus*	XX/XY, B chromosome	1335.2	2n=50	*gdf6b/B-carrying males*
								Colossoma macropomum	XX/XY	1767.9	2n=54	?
	Euteleostei	Stomiati		Osmeriformes	X			*Plecoglossus altivelis*	XX/XY	449.1	2n=56	*amhr2bY*
		Protacanthopterygii		Esociformes	X			*Esox lucius*	XX/XY	918.7	2n=50	*amhY*
				Salmoniformes	X		X	*Salmo salar*	XX/XY	2793.2	2n=58	*sdY*
								Oncorhynchus mykiss	XX/XY	2028.3	2n=64	*sdY*
		Neoteleostei: Acanthomorpha	Paracanthopterygii	Gadiformes	X			*Gadus morhua*	XX/XY	627	2n=46	*zkY*
			Eupercaria	Tetraodontiformes	X			*Takifugu rubripes*	XX/XY	384.1	2n=44	*amhr2X/amhr2Y*
				Acanthuriformes	X			*Larimichthys crocea*	XX/XY	705.2	2n=48	*dmrt1X/dmrt1Y*
				Centrarchiformes	X			*Siniperca chuatsi*	XX/XY	736.9	2n=48	*amhY*
				Perciformes	X	X		*Nibea albiflora*	XX/XY	595.6	2n=48	*dmrt1Y*
								Scatophagus argus	XX/XY	567.0	2n=48	*dmrt1Y*
								Collichthys lucidus	X1X1X2X2/X1X2Y	774.8	2n=47/48	*dmrt1Y*
								Gasterosteus aculeatus	XX/XY	446.1	2n=44	*amhY*
								Sebastes schlegelii	XX/XY	780.3	2n=48	*amhY*
								Perca flavescens	XX/XY	924.9	2n=48	*amhr2bY*
								Anoplopoma fimbria	XX/XY	784.1	2n=48	*gsdfX/gsdfY*
								Oplegnathus fasciatus	X1X1X2X2/X1X2Y	766.3	2n = 47/48	*ccdc63, ltr, wnt4*
			Gobiaria	Gobiiformes	X	X		*Mugilogobius chulae*	XX/XY	1003.3	2n=44	*galnt10-likeX/galnt10-likeY*
			Carangaria	Pleuronectiformes	X	X	X	*Cynoglossus semilaevis*	ZZ/ZW	470.1	2n=44	*dmrt1Z/dmrt1W*
								Scophthalmus maximus	ZZ/ZW	562.5	2n=44	*sox2Z/sox2W*
				Carangiformes	X	X		*Seriola dorsalis*	ZZ/ZW	685	NA	*hsd17b1Z/hsd17b1W*
								Seriola lalandi	ZZ/ZW	749.4	2n=48	*hsd17b1Z/hsd17b1W*
								Trachinotus ovatus	ZZ/ZW	650.4	2n=48	*hsd17b1Z/hsd17b1W*
								Pseudocaranx georgianus	XX/XY	579.4	2n=48	*cyp19a1aY*
			Ovalentaria	Cichliformes	X	X	X	*Oreochromis niloticus*	XX/XY	999.5	2n=44	*amhY*
				Atheriniformes	X		X	*Hypoatherina tsurugae*	XX/XY	-	NA	*amhY*
								Odontesthes bonariensis	XX/XY	870.3	2n=48	*amhY*
								Odontesthes hatcheri	XX/XY	-	2n=48	*amhY*
				Cyprinodontiformes	X	X	X	*Nothobranchius furzeri*	XX/XY	1052.3	2n=38	*gdf6Y*
				Beloniformes	X	X	X	*Oryzias dancena*	XX/XY	-	2n=48	*sox3X/sox3Y*
								Oryzias latipes	XX/XY	732.2	2n=48	*dmrt1bY*
								Oryzias luzonensis	XX/XY	-	2n=48	*gsdfX/gsdfY*

Supplementary table 2 - Comparative expression profiles of the main sex differentiation genes across teleost species, adapted from Heule et al., 2014, including the corresponding taxonomic groups. ♂, male specific or overexpressed; ♀, female specific or overexpressed; NA, not tested; ND-Not detected.

Gene	Otocephala: Otophysa					Protacanthopterygii	Euteleostei					
							Neoteleostei: Acanthomorpha					
	Cypriniformes		Characiphysi				Ovalentaria				Eupercaria	
			Siluriformes	Characiformes			Beloniformes	Atheriniformes	Cichliformes		Tetraodontiformes	Pleuronectiformes
	Zebrafish (D. rerio)	Common carp (C. carpio)	African catfish (C. gariepinus)	Pachon cavefish (A. mexicanus)	Tambaqui (C. macropomum)	Rainbow trout (O. mykiss)	Medaka (O. latipes)	Patagonian pejerrey (O. hatcheri)	Nile tilapia (O. niloticus)	Burton's haplo (A. burtoni)	Fugu (T. rubripes)	Turbot (S.maximus)
amh	♂	♂	NA	♂	♀	♂	♀/♂	♂ (first amhy, then amha)	♂	♂	NA	♂
dmrt1	♂	♂	♂	♂	ND	♂	♂	♀/♂	♂	♂	♂	ND
gsdf	♂/♀	♂	NA	♂	ND	♂	♂	NA	♂	♀	♂	♂/♀
sf-1	♂	♂	♀	NA	♂/♀	♂	♀	♂/♀	first ♀, then ♂	♂	♂/♀	♀
sox9A	♂	NA	♂	NA	♂	♂	♀	NA	♂	♂	♂/♀	♂
sox9B	♀	♂	♀	NA	♂	♂	♂	NA	♂	♀	♂/♀	♂
foxl2	♀	♀	♀	♀/♂	♀	♀	♀	♀	♀	NA	♀	♀
wnt4b	♀	NA	NA	♀/♂	♀	ND	♀	NA	♀	♂	NA	♀
wnt4a	NA	NA	NA	NA	♀	♂	NA	NA	NA	NA	NA	♀
cyp19a1A	♀	♀	♀	♀/♂	ND	♀	♀	♀	♀	♀/♂	♀	♀
cyp19a1B	♂/♀	♂	♀	NA	♂	♂	♀	NA	♀	♂	♂	NA

CAPÍTULO II

PHYLOGENOMIC AND EXPRESSION ANALYSIS OF *Colossoma macropomum cyp19a1a* AND *cyp19a1b* AND THEIR NON-CLASSICAL ROLE IN TAMBAQUI SEX DIFFERENTIATION

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Phylogenomic and expression analysis of *Colossoma macropomum cyp19a1a* and *cyp19a1b* and their non-classical role in tambaqui sex differentiation

R.V. Paixão [a], G.F. Silva [b], A.R. Caetano [c], L.C. Cintra [d], E.S. Varela [e], F.L.A. O'Sullivan [b,*]

[a] Universidade Federal do Amazonas (UFAM), Programa de Pós-graduação em Ciência Animal e Recursos Pesqueiros, Avenida Rodrigo Otávio, CEP: 69080-900, 6200 Manaus, AM, Brazil
[b] Embrapa Amazônia Ocidental, Rodovia AM-010, Km 29, Caixa Postal 319, CEP: 69010-790, Brazil
[c] Embrapa Recursos Genéticos e Biotecnologia, Final Av. W/5 Norte, C.P. 02372, CEP 70770-917, Brasília, DF, Brazil
[d] Embrapa Agricultura Digital, Avenida André Tosselo, 209, Cidade Universitária, CEP: 13083-886, Campinas, SP, Brazil
[e] Embrapa Pesca e Aquicultura, Av. NS 10, cruzamento com a Av. LO 18 Sentido Norte Loteamento - Água Fria, Palmas, TO 77008-900, Brazil

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ABSTRACT

The genes coding for Cytochrome P450 aromatase (*cyp19a1a* and *cyp19a1b)* and estrogen (E_2) receptors (*esr1*, *esr2a* and *esr2b*) play a conserved role in ovarian differentiation and development among teleosts. Classically, the "gonad form" of aromatase, coded by the *cyp19a1a*, is responsible for the ovarian differentiation in genetic females via ligation and activation of the Esr, which mediates the endocrine and exocrine signaling to allow or block the establishment of the feminine phenotype. However, in neotropical species, studies on the molecular and endocrine processes involved in gonad differentiation as well as on the effects of sex modulators are recent and scarce. In this study, we combined *in silico* analysis, real-time quantitative PCR (qPCR) assay and quantification of E_2 plasma levels of differentiating tambaqui (*Colossoma macropomum)* to unveil the roles of the paralogs *cypa19a1a* and *cyp19a1b* during sex differentiation. Although the synteny of each gene is very conserved among characids, the genomic environment displays striking differences in comparison to model teleost species, with many rearrangements in *cyp19a1a* and *cyp19a1b* adjacencies and transposable element traces in both regulatory regions. The high dissimilarity (DI) of SF-1 binding motifs in *cyp19a1a* (DI = 10.06 to 14.90 %) and *cyp19a1b* (DI = 8.41 to 13.50 %) regulatory region, respectively, may reflect in an alternative pathway in tambaqui. Indeed, while low transcription of *cyp19a1a* was detected prior to sex differentiation, the expression of *cyp19a1b* and *esr2a* presented a large variation at this phase, which could be associated with sex-specific differential expression. Histological analysis revealed that anti-estradiol treatments did not affect gonadal sex ratios, although Fadrozole (50 mg kg^{-1} of food) reduced E_2 plasma levels ($p < 0{,}005$) as well *cyp19a1a* transcription; and tamoxifen (200 mg kg^{-1} of food) down regulated both *cyp19a1a* and *cyp19a1b* but did not influence E_2 levels. Altogether, our results bring into light new insights about the evolutionary fate of *cyp19a1* paralogs in neotropical fish, which may have generated uncommon roles for the gonadal and brain forms of *cyp19a1* genes and the unexpected lack of effect of endocrine disruptors on tambaqui sexual differentiation.

Abbreviations: aa, amino acid(s); AhR, Aryl hydrocarbon receptor; AIs, Aromatase inhibitors; AR, Androgen receptor; bp, base pair(s); cDNA, DNA complementary to RNA; CDS, Coding DNA sequence; CRE, cAMP response element; CRX, Cone-rod homeobox protein; dNTP, deoxyribonucleoside triphosphat; DBP, D site-binding protein binding site; EDCs, Endocrine Disrupting Chemicals; ELISA, enzyme-linked immunosorbent assay; ERE, Estrogen-response element; ERs, Estrogen receptors; ETOH, ethyl alcohol; FRA-1, Fos related antigen-1; Foxl2, forkhead box L2; GATA-1, GATA binding factor 1; hERE, ERE half sites; LTR, long terminal repeat(s); IRF-2, Interferon regulatory factor 2; Myr, million years; NF-Y, Nuclear transcription factor Y; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; ORF, open reading frame; P53, Cellular tumor antigen p53; PR, Progesterone receptor; SERMs, Selective Estrogen Receptor Modulators; SF-1, Steroidogenic factor 1; SMAD3, Mothers against decapentaplegic homolog 3; SOX9, SRY-Box Transcription Factor 9; TBP, Transcription binding protein; TCF-4E, Transcription factor 4; TEs, Transposable Elements; TFBSs, transcription factor binding sites; TIR, Terminal inverted repeats; UTR, untranslated region(s); WT1-KS, Wilms tumor protein.

* Corresponding author at: Rodovia AM-010, Km 29, Caixa Postal 319, CEP: 69010-970, Manaus, Amazonas, Brazil.
E-mail address: fernanda.almeida@embrapa.br (F.L.A. O'Sullivan).

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1. Introduction

In teleosts, steroids are well known for playing key roles in sex differentiation and reproduction. A general and classic feature of ovarian development in teleost fish is the sex-biased synthesis of estradiol during the critical period of ovary differentiation, as a reflection of the sex-specific differential expression of the *cyp19a1*. The *cyp19a1* encodes for Cytochrome P450 aromatase, the catalytic enzyme that aromatizes androgens into estrogens (Devlin & Nagahama, 2002; Guiguen et al., 2010; Rajakumar & Senthilkumaran, 2020). Furthermore, estrogens, mainly 17β-estradiol, act as primarily transcriptional regulators of target genes mediated by the nuclear estrogen receptors (ERs), which recognize and activate gene transcription through binding to the genomic element called the estrogen-response element (ERE) (Amenyogbe et al., 2020). The administration of endocrine disruptor chemicals (EDCs) such as aromatase inhibitors (AIs) and Selective Estrogen Receptor Modulators (SERMs), which block this E_2-ER path, induces sex reversal in fish, resulting in genetic females that are functional males (Dang & Kienzler, 2019).

Due to a second, fish-specific, whole genome duplication (3R) event that occurred during the evolution of teleosts, the *cyp19a1* gene was duplicated, giving rise to the paralogs *cyp19a1a*, mostly expressed in the gonads, and *cyp19a1b*, which is mostly expressed in the brain (Guiguen et al., 2010; Diotel et al., 2010). Additionally, although both Cyp19a1 proteins have similar enzymatic activity, some divergences in their gene promoter sequences have supported their differential tissue expression and regulation in different fish species (Lin et al., 2020). Recent studies on teleosts using different gene editing methodologies (TALEN and CRISPR) showed that the knockout of *cyp19a1a* results in all-male offspring in zebrafish (Lau et al. 2016) and tilapia (Zhang et al., 2017). However, *cyp19a1a* deficiency in XX medaka mutants did not affect the ovarian development, but caused a partial ovary degeneration and partial sexual inversion after puberty due to a failure in yolk accumulation (Nakamoto et al., 2018). This establishes that not in all cases the initial expression of *cyp19a1a* is important for the gonadal fate, but it is important for the normal ovary development. In contrast, *cyp19a1b*-null mutants showed a normal sex ratio in zebrafish and medaka, providing evidence that *cyp19a1b,* is not crucial for ovarian fate determination in these species (Yin et al., 2017, Nakamoto et al., 2018).

While the process of sex differentiation and the role of estrogen and AIs in the gonad final phenotype are well characterized in model and some economically important fish species, the elucidation of sex determination mechanisms and associated molecular pathways for sex differentiation in neotropical fishes remain key areas of research (Fernandino & Hattori, 2019). The Characiformes order is the most speciose and diverse group of fish in the neotropics, where they inhabit a large variety of freshwater habitats. Moreover, neotropical fish are the basis of native finfish production in Brazil, which recently increased steadily along with the continuous growth of the aquaculture industry in South America. Among different farmed neotropical species, the tambaqui (*Colossoma macropomum*), a member of the family Serrasalmidae, outstands as the major native species in fish farming in Brazil and in neighboring countries (Peixe, 2022). Juvenile female tambaqui apparently proceeds throughout ovarian cavity formation, which marks the ovary differentiation, in the absence of *cyp19a1a.* Moreover, RNA seq data of undifferentiated tambaqui showed that the expression of *cyp19a1b* and *esr2a* were higher in the trunks of putative males (Lobo et al., 2020). To better explore this unconventional pattern, we performed a phylogenomic analysis of both *cyp19a1* paralogs and of their coded aromatases, comparing with other teleost's and evaluated the expression of *cyp19a1a*, *cyp19a1b* and *esr2a* in different stages of tambaqui development, under normal growing conditions and treated with AI compounds before and during sex differentiation.

2. Materials and methods

2.1. In silico analysis

2.1.1. Identification of cyp19a1a and cyp19a1b sequences in tambaqui and other teleost

The identification of tambaqui *cyp19a1a* and *cyp19a1b* was done by blast searches, using *Pygocentrus nattereri* CDS sequences as query, against juvenile trunks, ovary and testis RNAseq data, and on a local assembly of the *C. macropomun* genome (*unpublished*) in addition to the *C. macropomum* genome assembly at National Center for Biotechnology Information (NCBI; Bioproject PRJEB40318). The introns prediction, splicing junctions and protein deduction from the genomic databases were performed with Augustus or manually by BLASTX tool with identification of nucleotides consensus for introns 5′(GT) and 3′(AG). The retrieved sequences were manually curated using the Unipro UGENE v. 35.1 software to construct the predicted CDS, which were subsequently used to design species-specific primers for real-time quantitative PCR (qPCR) and to deduce the amino acid sequences.

We further examined the *cyp19a1* sequences from several animal species with available sequenced genomes at NCBI and Ensembl databases, using the TBLASTN algorithm. Teleost representatives include the superorder Elopomorpha (*Megalops cyprinoides*, *Anguilla anguilla*), Osteoglossomorpha (*Sclerophages formosus*, *Arapaima gigas*, *Heterotis niloticus*, *Gymnarchus niloticus*, *Mormyrus iriodes*, *Paramormyrops kingsleyae*, *Brevimyrus niger*, *Mormyrus lacerda*), Otocephala (*Denticeps clupeoides*, *Coilia nasus*, *Limnothrissa miodon*, *Alosa alosa*, *Clupea harengus*, *Chanos chanos*, *Danio rerio*, *Carassius auratus*, *Cyprinus carpio*, *Electrophorus electricus*, *Ictalurus punctatus*, *Tachysurus fulvidraco*, *Pangasianodon hypophthalmus*, *Ameiurus melas*, *Hepsetus odoe*, *Pygocentrus nattereri*, *Astyanax mexicanus*) and Eutelostei (*Gasterosteus aculeatus*, *Takifugu rubripes*, *Dicentrarchus labrax*, *Monopterus albus*, *Esox lucius*, *Oreochromis niloticus*, *Oryzias latipes*, *Gadus morhua*, *Salmo salar*, *Onchorhynchus mykiss*). We also included non-teleost actinopterygian such as *Lepisosteus oculatus*, *Acipenser ruthenus* and *Erpetoichthys calabaricus*, some tetrapods (Sarcopterygii; *Latimeria chalumnae*, *Homo sapiens*, *Gallus gallus*, *Xenopus tropicalis*, *Anolis cariolinensis*), Chondrichthyes (*Callorhinchus milii*, *Amblyraja radiata*, *Rinchodon typus*) and one Cyclostomata representative (*Eptatretus burgeri*). Cyp19 sequences found in *Branchiostoma floridae* (Cephalochordata) were used as outgroup. Annotations were added to sequences from species (9) with non-annotated databases.

2.1.2. Promoter analysis of tambaqui cyp19a1a and cyp19a1b

The comparative intergenic analysis of both *cyp19a1* genes was carried out on the entire region from the 5′ flanking region to their respective adjacent gene neighbors. We used the fishTEDB and NCBI as databases to search for transposable elements through homology search by BLASTn and BLASTx tools, respectively. We further searched for putative transcription factor-binding sites in the promoter by applying the PROMO web tool (https://alggen.lsi. upc.es/home.html), using previous reports that have been validated TFBS from aromatase promoter region (Tanaka et al., 1995, Tchoudakova et al., 2001, Chang et al., 2005, Sridevi et al., 2012, Lin et al., 2020).

2.1.3. Phylogenetic analysis of Cyp19a1a and Cyp19a1b

Multiple sequence alignments were performed with MUSCLE, included in MEGA7. Phylogenetic relationships were estimated using maximum likelihood and Bayesian approaches. The best-fitting model for amino acid substitution matrix (JTT + I + G4) was selected based on the proposed model tool from IQ-Tree 2.0, which was also used to perform the Maximum likelihood analysis to obtain the best tree. Node support was assessed with 10,000 bootstrap pseudo replicates using the ultrafast routine. Bayesian searches were conducted in MrBayes v.3.1.248, with two independent runs of six simultaneous chains for 1,000,000 generations, and every 1000 generations were sampled using

default priors.

2.1.4. Synteny analysis of cyp19a1a and cyp19a1b loci among teleosts

The Genomicus vertebrate server (https://www.dyogen.ens.fr/genomicus), which is synchronized with Ensembl releases, was used for chromosomal localization of aromatase genes and neighboring genes that appear in conserved position between the phylogenetic representative fish species for synteny analysis. The genomic region (44 neighboring genes) of *cyp19a1a* and *cyp19a1b* from *C. macropomum* were identified and manually compared using the NCBI scaffold annotation from other representative species.

2.2. In vivo experiments

2.2.1. Ethics statement

The authors assert that all procedures of this work comply with the Ethical Principles of Animal Experimentation, adopted by the Brazilian College of Animal Experimentation (COBEA), and were approved by the local Ethics Committee on Animal Use (CEUA) – Embrapa Amazônia Ocidental (n° 05/2018, SEI 21158.003669/2018-77). The project has an Authorization of Access to Genetic Heritage under register A5784B5.

2.2.2. Animals and rearing conditions

Recently-hatched tambaqui larvae were obtained from a commercial farm (from Presidente Figueiredo, Brazil) and transported to the Embrapa Amazônia Ocidental facilities, in Manaus. Fish were maintained in acclimatization for 7 days, fed crumbled fish pellets with 45 % crude protein (CP), *ad libitum*, three times/day. After this period, juveniles were randomly distributed into nine polyethylene tanks (310 L), in a density of 200 juveniles/tank for receiving the treatments in triplicates. The temperature, oxygen, and pH of the water were measured daily. Treatments lasted 6 weeks during which the feed (experimental diets) was provided *ad libitum* four times a day.

2.2.3. Experimental diets

Fadrozole at dose of 50 mg kg^{-1} and tamoxifen at dose of 200 mg kg^{-1} were dissolved in 70 % ethanol and incorporated into fish pellets (according to Hines & Watts, 1995) for feeding sexually undifferentiated tambaqui (2 cm of length) for 40 days, which covered the period when the first morphological signs of sex differentiation appears (Lobo et al., 2020). The control diet was treated with ethanol 70 %, in the same volume: pellet weight as treatment diets. Hormone dosages were established based on different studies showing effects of tamoxifen and fadrozole on fish sex ratio (Afonso et al., 2001; Navarro-Martín et al., 2009; Singh et al., 2012). All feed required for the entire treatment was prepared in one batch. After the treatment, each fish group was transferred to and maintained in net cages up to 12 months of age, with the exception of one replicate of the tamoxifen treatment, which was lost due to an accident. All cages were placed in the same earth pond. During this phase, the animals were fed fish pellets of 32 % CP at a rate of 5 % of the tank biomass, twice a day.

2.2.4. Samplings

The first sampling was done just before the start of the treatment (only in the control tanks; T_0, n = 5/tank), 20 and 40 days after the beginning of treatment (at the middle and end of the treatment, respectively; n = 9/tank). Fish were desensitized with benzocaine and sacrificed by medullary sectioning. The trunks were snap frozen in liquid nitrogen, and stored at −80 °C. At the last sampling, blood was collected from the caudal vein of each fish prior to killing. The plasma was obtained by centrifugation, frozen and stored at −80 °C until analysis. The gonads were dissected, fixed in the Bouin solution for 24 hrs, and kept in 70 % ETOH for histological processing.

To characterize *cyp19a1a*, *cyp19a1b* and *esr* expression levels in non-treated tambaqui, 5 to 15 fish were collected at hatching, and in the sizes of 1.5, 2, 3, 4 and 6 cm of total length, covering the period of pre-differentiation, and morphological differentiation of the gonads (Lobo et al., 2020). Additionally, we collected gonads and brains of 6 sexually immature adult tambaqui (3 males and 3 females), and 8 gonads from sexually maturing tambaqui (5 females and 3 males) for RNA isolation and qPCR analysis (Supplementary Fig. 1C to F).

2.2.5. Gene expression assays

Total RNA from juveniles' trunks, brains and extracted gonads was extracted using mortar and pestle for tissue maceration with TRIzol reagent (Life Technologies; Carsbald, USA) in a proportion of 1 ml per 100 mg of tissue. Total RNA samples were treated with RNAse-free DNA (RQ1 RNAse-free; Promega, Madison, USA) to remove any possible genomic DNA residues. The concentration and integrity of the RNA were assessed by spectrophotometry (Nanodrop 1000; Thermo Scientific) and in agarose gel electrophoresis (1.5 %), respectively. Only samples with a 260/280 ratio between 1.8 and 2.1 were used for cDNA synthesis, which was performed using High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific, Waltham, MA, USA), according to the manufacturer's protocol. The cDNAs were used as a template to amplify and quantify the transcript levels of *cyp19a1a, cyp19a1b* and *esr2a*. All qPCR primers (Supplementary Table 1) were designed using the Integrated DNA Technologies (IDT) tools (https://www.idtdna.com), based on tambaqui genomic nucleotide sequences. Amplification efficiency for each primer set was calculated from a 1:4 serial dilution of a pool of tambaqui ovaries and testes cDNA. Melt curves were inspected to ensure amplification of single amplicons, which were sequenced afterwards to confirm the primers specificity of each assay. The qPCR reactions were performed in the 7500 Fast Real-Time PCR System v2.3 (Applied Biosystems), in duplicates, using 2 μL of cDNA (120 ng), 1 μL of each primer (200 nM), 12,5 μL Fast SYBR Green PCR Master Mix (Applied Biosystems), and nuclease-free water to a final volume of 25 μL. qPCR data was analyzed using the $2^{-(\Delta\Delta Ct)}$ method where the expression of the target gene was first normalized to the *β-actin* (Nascimento et al., 2016) and then calibrated to the mean of the pool of larvae (first sampling) for the non-treated fish (i.e. differentiating juveniles and gonads of adult tambaqui) and of the control group in the treated fish (experimental groups).

2.2.6. Quantification of E_2 plasmatic concentration and histology

The circulating concentration of 17βestradiol was estimated by an enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen, Camarillo, USA) and the samples were analyzed in duplicates. Absorbance was measured using a microplate reader (Multiskan FC Thermo Scientific®, Leicestershire, UK). Analyses were carried out following the manufacturer's instructions, and a standard curve was run for each ELISA plate. No dilution was needed in any assay.

For sex identification, the fixed gonads were dehydrated in increasing concentrations of ETOH, cleared in xylene and embedded in paraffin. The sections were cut in 3 μm and stained with haematoxylin-eosin following conventional histological procedures. Histological slides were used to identify the phenotypic sex of each fish, based on gonadal structures, such as ovarian lamellae and/or the presence of oogonial nests in females and the presence of spermatogonia and/or spermatogenic cysts in males (Supplementary Fig. 1 A and B).

2.2.7. Statistical analysis of the data

To calculate the variation in sex ratios between treatment groups vs control, Chi-square (X^2) tests were performed. Based on ΔΔCt values, the differences of gene expression among different time points of tambaqui early development as well as the differences between control and treatments in some time points were tested by Kruskal-wallis test followed by post hoc Dunn's multiple comparison test, since that did not confirm one or all of the assumptions for parametric test (normal distribution, homogeneity of variances and homoscedasticity). One-way ANOVA was used to identify significant differences of gene expression and E_2 levels between control and treated fish. All statistical analyses

were performed in Graph Pad Prism v. 8.4.3.

3. Results

3.1. Tambaqui cyp19a genes

In the tambaqui genome, the *cyp19a1a* (LOC118804009) is located in the scaffold NW_023496020.1, while *cyp19a1b* (LOC118811907) is

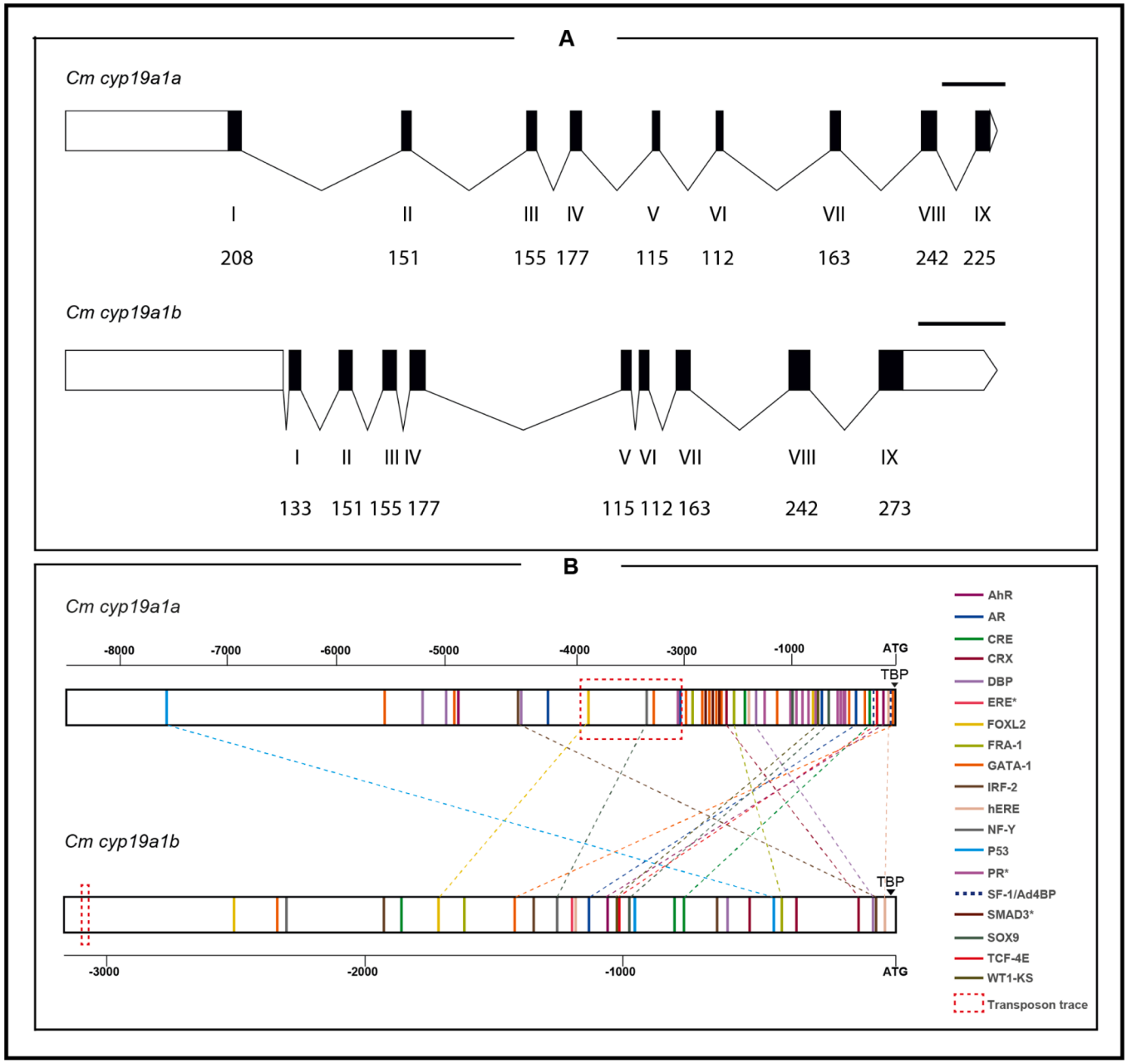

Fig. 1. Characterization of the genomic sequences of *Colossoma macropomum cyp19a1* paralogues. A) Illustrative scheme of relative positions of introns and exons of *C. macropomum cyp19a1a* and *cyp19a1b*. The exons are boxed and the introns are depicted by lines. Position and length of exons are indicated by roman numerals and cardinal numbers, respectively. The thin line shown above the graphic is a scale bar with 1000 base pairs; B) cis-regulatory elements distribution in the promoter region of *C. macropomum cyp19a1a* and *cyp19a1b*. TATA-box are indicated by arrowheads. Binding sites that were predicted to be<5 % dissimilar to the matrix (DI < 5 %) are represented by solid colored lines, with the exception of the SF-1 site which is represented by dotted line (DI > 10 %). Elements that are shared by both *cyp19a1* copies are linked by dotted lines with its respective colour, connecting the closest elements from the ATG (start codon). Asterisks indicate elements that are exclusively present in one of the duplicates. Transposon traces are indicated by red dotted squares. AhR: Aryl hydrocarbon receptor responsive element; AR, androgen receptor binding site; CRE, cAMP response element; CRX, Cone-rod homeobox protein binding site; DBP, D site-binding protein binding site; ERE, estrogen response element; Foxl2, forkhead box L2 binding site; FRA-1, Fos related antigen-1 binding site; GATA-1, GATA binding factor 1 responsive element; IRF-2, Interferon regulatory factor 2 binding site; NF-Y, Nuclear transcription factor Y binding site; SF-1/Ad4BP, steroidogenic factor 1/Ad4-binding protein; hERE, ERE half sites; P53, Cellular tumor antigen p53 binding site; PR, Progesterone receptor binding site; SMAD3, Mothers against decapentaplegic homolog 3 binding site; SOX9, SRY-Box Transcription Factor 9 binding site; TCF-4E, Transcription factor 4 binding site; WT1-KS, Wilms tumor protein binding site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

located in the scaffold NW_023494792.1. Both genes are organized into nine exons, with 1548 and 1521 bp of CDS sequence, respectively (Id shared = 72 %), resulting in a predicted protein of 515 and 506 amino acids, respectively (Id shared = 66,7%) (Fig. 1a). Cyp19a1a predicted aa sequence shares, respectively, 94, 80 and 75 % of identity with *Pygocentrus nattereri*, *Astyanax mexicanus* and *Danio rerio*, whereas Cyp19a1b have the highest amino acid identity with *P. nattereri* (Id = 95 %), followed by *D. rerio* (Id = 77,5%) and then by *A. mexicanus* (Id = 72,8%).

3.2. Intergenic and promoter analysis of tambaqui cyp19a1 genes

Tambaqui *cyp19a1a* and *cyp19a1b* display striking differences in 5′intergenic regions, with 8358 bp and 3248 bp, respectively. The tambaqui *cyp19a1a* promoter region from −3004 to −3993 bp had high similarity (79.33 %) to the PM_Contig_11 sequence of the *Periophthalmus magnuspinnatus,* classified as class II transposon, terminal inverted repeats (TIR) order and TC1-Mariner superfamily in FishTEDB database, and 41.08 % of identity with TCB1 transposase from *Polypterus senegalus* in the NCBI database. Similarly, tambaqui *cyp19a1b* 5′ intergenic region also contains a small trace from −3155 to −3194, which is 99 % similar to the TC1-Mariner from *Anguilla japonica* (Supplementary Fig. 2).

Sequence analysis upstream of the ATG start codon of tambaqui *cyp19a1a* and *cyp19a1b* revealed a TBP motif as well as several potential regulatory elements (Fig. 1b; Supplementary Fig. 3). We found putative binding sites for nuclear transcription factor Y (NF-Y), GATA-1, Nkx2-1, WT1-KS, cAMP response element-binding protein (CRE), SOX9, Forkhead box L2 (FOXL2), aryl hydrocarbon receptor/nuclear transfer responsive element (AhR/Arnt), androgen receptor (AR) and estrogen receptor recognition half sites (hERE), in both *cyp19a1a* and *cyp19a1b* 5′ flanking region. Most of these predicted sites displayed next to 0 % of dissimilarity index (DI) to the reference matrix, except for AhR and NF-Y binding sites which displayed up to 1 % and 2 % of DI, respectively. Moreover, these binding sites have been described in the promoter regions of both *cyp19a1a* and *cyp19a1b* from other teleost species (Tanaka et al., 1995, Tchoudakova et al., 2001, Chang et al., 2005, Sridevi et al., 2012, Lin et al., 2020).

Tambaqui *cyp19a1a* and *cyp19a1b* promoter regions share many putative transcription factor binding sites, but there are differences in position and number of copies that may reflect the particularities in their individual regulation. Among several GATA-1 putative binding sites predicted in tambaqui *cyp19a1a* 5′ flanking region (n = 29), four are located proximal to the transcription start site (nt −54, −285, −500, −1112) in contrast *cyp19a1b* that exhibited only five putative GATA-1 binding sites (nt −1428, −2103, −2355, −2363, −2918). Additionally, we detected four putative AR binding sites in the tambaqui *cyp19a1a* promoter region (nt −458, −747, −2062, −3308) and only one in *cyp19a1b* (nt −1138).

A full ERE binding site (GGGTCAGCCTGACCT, nt −1204) was manually identified in the tambaqui *cyp19a1b* but not in the *cyp19a1a* promoter region, which is consistent with previous studies in fish (Tanaka et al., 1995, Tchoudakova et al., 2001, Chang et al., 2005, Sridevi et al., 2012, Lin et al., 2020). Moreover, no SF-1 response element was predicted in the tambaqui *cyp19a1a* promoter region with less than 10 % of the DI. Otherwise, two SF-1 responsive elements were predicted in the tambaqui *cyp19a1b* promoter region with 10.36 % (nt −777) and 8.41 % (nt −3209) of DI.

Among the less discussed transcription factors in fish, we predicted 8 putative binding sites for progesterone receptor A (PR-A), 2 for progesterone receptor B (PR-B) and 3 for SMAD3, all exclusively present in tambaqui *cyp19a1a* promoter region with 0 % of DI. Moreover, the tambaqui *cyp19a1b* promoter region contains putative binding sites for IRF-2 (nt −79, −700, −1355, −1940, −2262), DBP (nt −91, −653), CRX (nt −147,-389, −572), FRA-1 (nt −447, −1626) and P53 (nt −477, −959), while same binding sites were predicted in tambaqui *cyp19a1a* promoter region, but in a position more distant from transcription start site (up to 1 kb).

3.3. Phylogenetic analysis of teleost's Cyp19a1a and Cyp19a1b

A total of 86 aromatase protein sequences from 52 genomes (Supplementary Fig. 4) were retrieved to assess the phylogenetic relationship and to predict ancestral gene duplications and losses among vertebrates. The reconstructed aromatase phylogenetic tree clustered the sequences in three major groups of vertebrates, namely chondrichthyan, sarcopterygian and actinopterygian (the last comprehending non-teleosts, basal teleosts and clupeocephala) (Fig. 2a). In both Cyp19a1a and Cyp19a1b subclades, *C. macropomum*, together with *P. nattereri, Hepsetus odoe* and *A. mexicanus* sequences represented the Characiformes clade which, together with the Siluriformes and Gymnotiformes, represents the unexplored Characiphysi group.

The single *CYP19A1* of non-teleost actinopterygians, reedfish (*Erpetoichthys calabaricus*), spotted gar (*Lepisosteus oculatus*) and the sterlet sturgeon (*Acipenser ruthenus*), branched basis to the well supported actinopterygian teleost clade (bootstrap probabilities in ML/posterior probability in Bayesian tree inference: 100/1.0), in agreement with the phylogenetic relationships within this major group. The single Cyp19a1 of basal teleosts, including European eel (*Anguila anguila*), Asian arowana (*Scleropages formosus*) and the Amazonian pirarucu (*Arapaima gigas*) grouped in a well-supported cluster at the base of the clade composed by duplicated Cyp19a1 sequences from the remaining teleosts (Clupeocephala).

In teleosts, the duplicated aromatase clade most likely resulted from a specific third round of whole genome duplication event (TWGD) that occurred in the lineage. Additional paralogs that were found in teleosts, such as salmonids and carps, that independently underwent another round (4R) of WGD, and in the non-teleost *A. ruthenus,* which lineage experienced the independently 3R after their divergence from the other ray-finned fish, are indicated. The absence of *cyp19a1a* sequence in the black bullhead catfish (*Amerius melas*) and in The Lake Tanganyika sardine (*Limnothrissa miodon*; Fig. 2b), as well as *cyp19a1b* additional paralogs in the common carp (*Cyprinus carpio)* and Atlantic salmon (*Salmo salar*) databases suggest gene loss in these lineages.

3.4. Synteny analysis

The order of 38 (out of 44) genes is perfectly conserved between tambaqui's scaffold NW_023496020.1 carrying *cyp19a1a* and the red-bellied piranha's chromosome 7 (Fig. 3a). We also found that *cyp19a1a* from Mexican tetra surface fish (*A. mexicanus*) is located at an unplaced scaffold (NW_019171364.1), even though the assembly is at chromosome level, sharing 22 genes with the congeneric representative species (11 in complete synteny). However, small rearrangements are evident as part of tambaqui and red-bellied piranha's *cyp19a1a* 3′ neighboring genes are in synteny with Mexican tetra chromosome 18.

Compared to distant related species, the degree of rearrangements in *cyp19a1a* adjacencies are more evident. In zebrafish (*D. rerio*) chromosome 8, sixteen genes out of 44 were found to be conserved around the *cyp19a1a*, whereas thirteen neighboring genes are in 3′ adjacencies and only three were found in 5′. In contrast, from 25 *cyp19a1a* conservative neighboring genes shared between japanese medaka (*O. latipes*) chromosome 3 and red-bellied piranha chromosome 7, eighteen are in 5′ and only seven are in 3′ adjacencies. Moreover, the reversed order of *usp8* from the twenty-second gene upstream to *cyp19a1a* in red-bellied piranha to the eighth gene downstream japanese medaka *cyp19a1a*, indicates local rearrangement in the *cyp19a1a* gene environment. Despite these particularities, the comparison of all ortholog regions showed that the syntenic organization of the gene group *gldn-cyp19a1a-tnfaip8l3* is highly conserved among the species analyzed.

In regard to *cyp19a1b* neighboring genes, there is a strong synteny conservation among red-bellied piranha chromosome 11, tambaqui scaffold NW_023494792.1 and mexican tetra chromosome 8, despite the duplication of some genes such as *vw5a*-like that varied in the number of copies in each species (Fig. 3b). In the zebrafish chromosome 25,

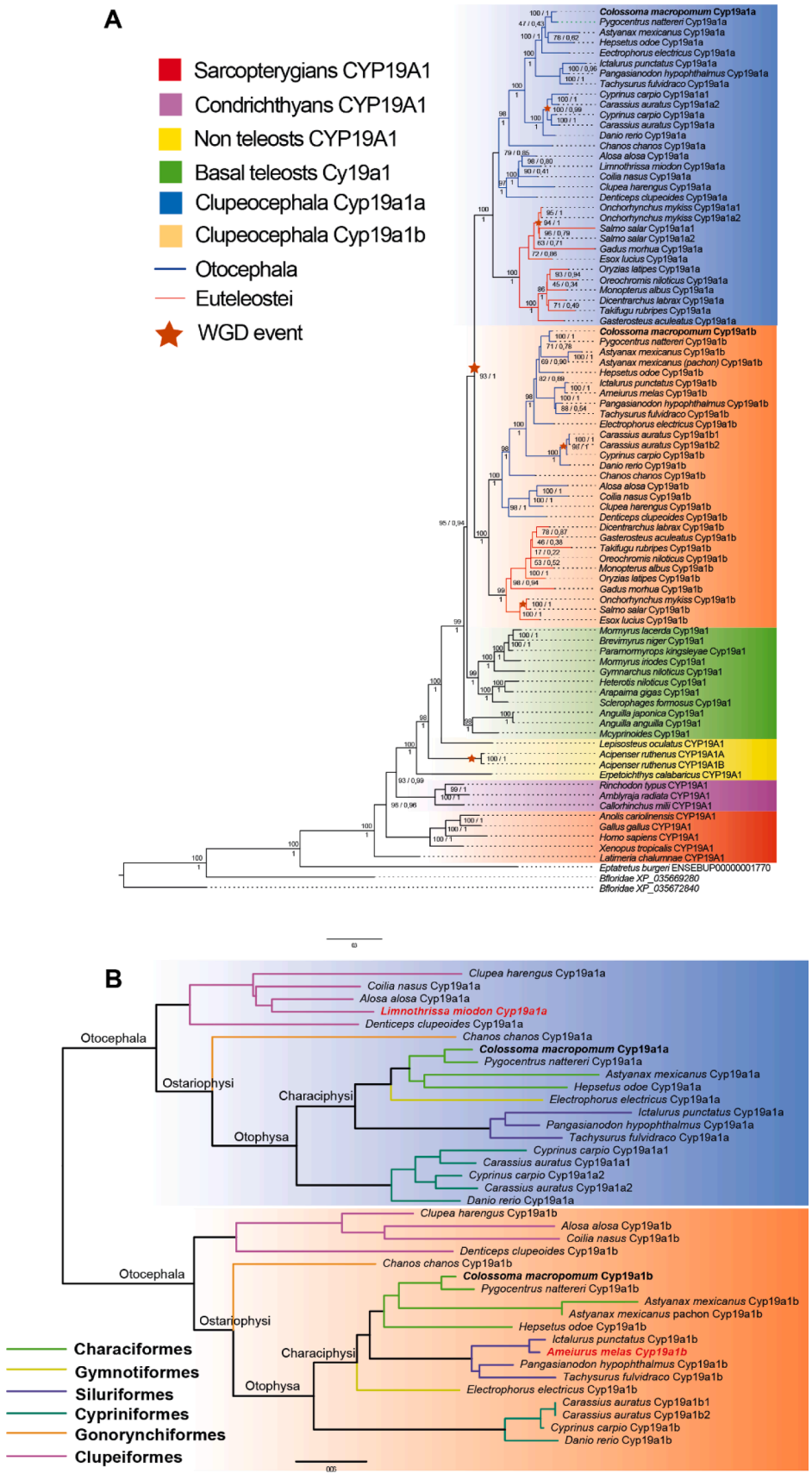

Fig. 2. Phylogenetic tree of vertebrate aromatase amino acid sequences. A) Phylogenetic tree built using Maximum Likelihood and Bayesian analysis on the basis of 86 aromatase sequences from 52 species representative of Sarcopterygians, Chondrichthyans and Actinopterygians. The Cephalochordata *Branchiostoma floridae* CYP19 tandem duplicated protein sequences and the Cyclostomata *Eptatretus burgeri* CYP19 were used as the outgroup. Values on the nodes are relative to the Bootstrap of ML (%; upper of branch or left of slash) and posterior probability of BI (lower of branch or right of slash) respectively. The red stars show the position of the teleost-specific (Ts3R), the salmonid-specific (Ss4R), the carp-specific (Cs4R) and sterlet (Ars3R) whole-genome duplications (WGD) after the 1R/2R events. The scale bar indicates the average substitutions per site; the dotted lines associate the taxon names with the branch ends. B) Phylogenetic tree of aromatase sequences from representative species of Otocephala group evidencing single aromatase Cyp19a1a in *Limnothrissa miodon* and single aromatase Cyp19a1b in *Ameiurus melas*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

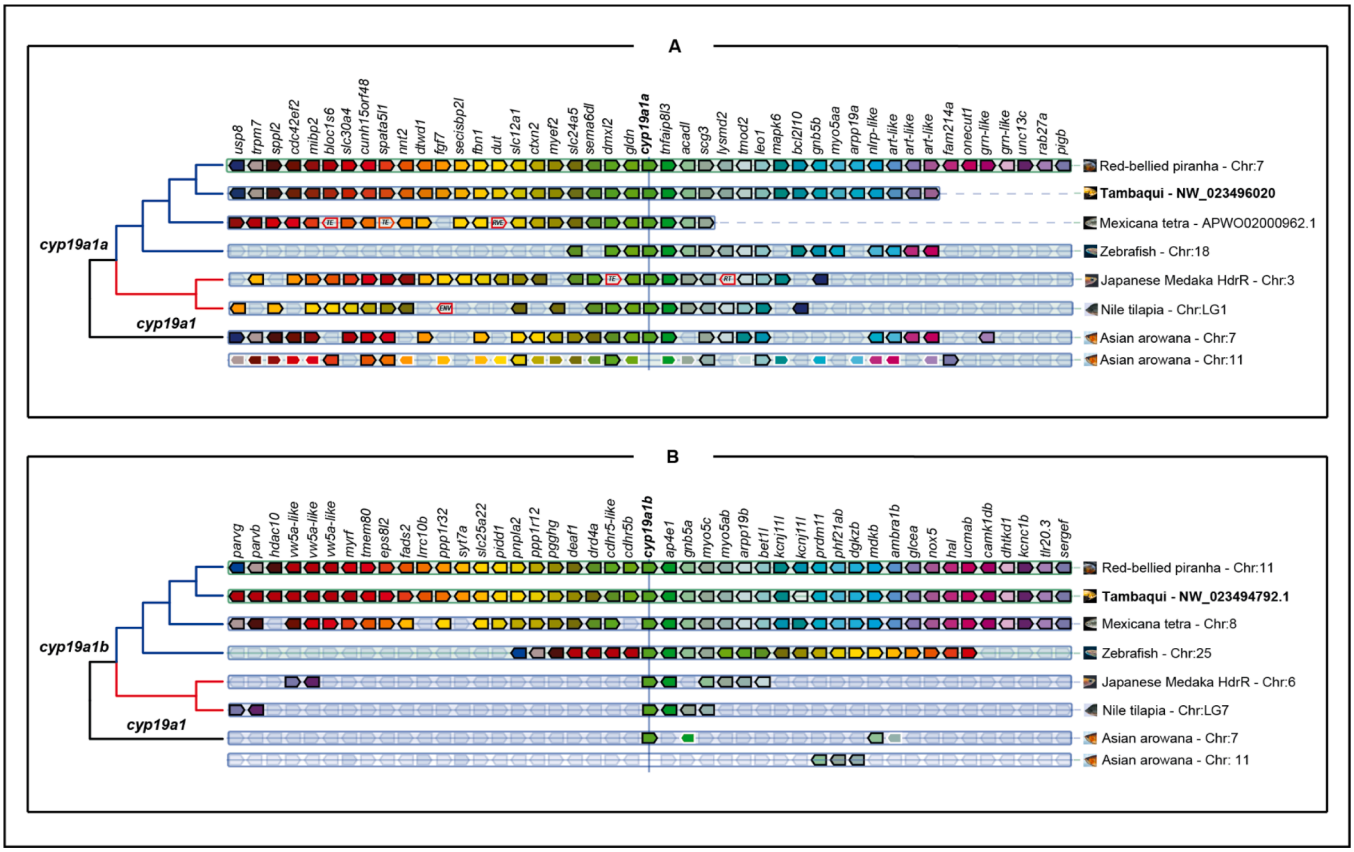

Fig. 3. Synteny analysis of A) *cyp19a1a* and B) *cyp19a1b* neighbouring genes in *C. macropomum* and in different species that share the same ancestral species. Orthologs of these genes in other species are shown in matching colours. Genes outlined in white are paralogs of the genes in the same colour but outlined in black. Shaded genes correspond to genes that are not orthologous to any genes from the species used in the query (reference). The tree on the left of the display is the phylogenetic tree of the gene shown in the middle that intersects the vertical line.

however, the *vw5a*-like tandem duplications lie upstream and adjacent to *cyp19a1b*, while these genes are distant located upstream of *cyp19a1b* in the characiform representatives. Additionally, the zebrafish region features the reversed order and orientation of a block containing fourteen genes, including the *cdhr5b* which is the conserved *cyp19a1b* upstream adjacent gene among red-bellied piranha, tambaqui and mexican tetra.

On the other hand, the japanese medaka chromosome 6 segment shares only seven genes with red-bellied piranha's *cyp19a1b* neighboring genes. The *kcnc1b* and *sergef* genes are in the reverse order and the remaining five genes form the syntenic gene group *cyp19a1b, ap4e1, gnb5a-myo5c, myo5ab and arpp19b* are positionally conserved in all species analyzed. Moreover, a second *gldn* paralog is present and is adjacent to japanese medaka *cyp19a1b* upstream region, which is a conserved feature previously described by Lin et al (2020) in the single *cyp19a1* from basal and non-teleost fishes, as well as in *cyp19a1a* from Clupeocephala. Such results indicates that critical rearangements occured in the *cyp19a1b* cis regulatory region across teleost lineages.

3.5. Transcript level profiling of estrogen signaling cyp19a1a, cyp19a1b and esr2a in tambaqui juvenile, gonads and brain.

Transcripts of *cyp19a1a*, *cyp19a1b* and *esr2a* could be detected in the pool of larvae (12 h post-hatching) samples and it was used as the calibrator for the expression in pre- and differentiating fish (decapitated juveniles); ovaries, testes and brain of differentiated tambaqui (Fig. 4). The expression of *cyp19a1a* was marked by a slight, but significant, increase in 2 cm juvenile trunks. The values remained then unchanged until they reached 6 cm total length. However, in pre-differentiating juveniles of 3 and 4 cm long, the expression of *cyp19a1a* tended to split into two groups. After the end of ovary differentiation (6 cm long) the values gathered again. Maturing ovaries and testes presented the highest expression of *cyp19a1a*.

On the contrary, the expression of *cyp19a1b* increased 10 fold from hatched larvae to juveniles with 1.5 cm and presented a large variation of values in fish of 3 cm, i. e. prior to morphological differentiation. After this phase, the values decreased, but two groups could be identified at 4 and 6 cm length. The expression of *cyp19a1b* in trunks of pre- and differentiating tambaqui was very similar to those found in adult gonads and male brains, and lower than in female brains.

Esr2a was overexpressed in 2 cm (pre-differentiating) fish ($p < 0.005$). At this stage, the expression average increased over 1200 fold, and the individual values could be distinguished in two groups, one whose expression was around 300 times over the hatched larvaes and other with values above thousand times.

In the differentiated gonads, *cyp19a1a* transcription was highest in the testis. Among females, *cyp19a1a* transcripts were more abundant in maturing ovaries while *esr2a* was more expressed in immature ovaries. Furthermore, in sexually immature males the expression of *cyp19a1b* and *esr2a* transcripts were higher in testis than in the brain, while in immature females *cyp19a1b* was more expressed in the brain than in the ovaries. However, both *cyp19a1a* and *cyp19a1b* transcripts were more abundant in the brain of immature females compared to immature males.

3.6. Effect of EDCs fadrozole and tamoxifen on tambaqui sex development

There was no mortality during the experiment, nor any visual effects on the body or behavior of the fish during or after the treatments with

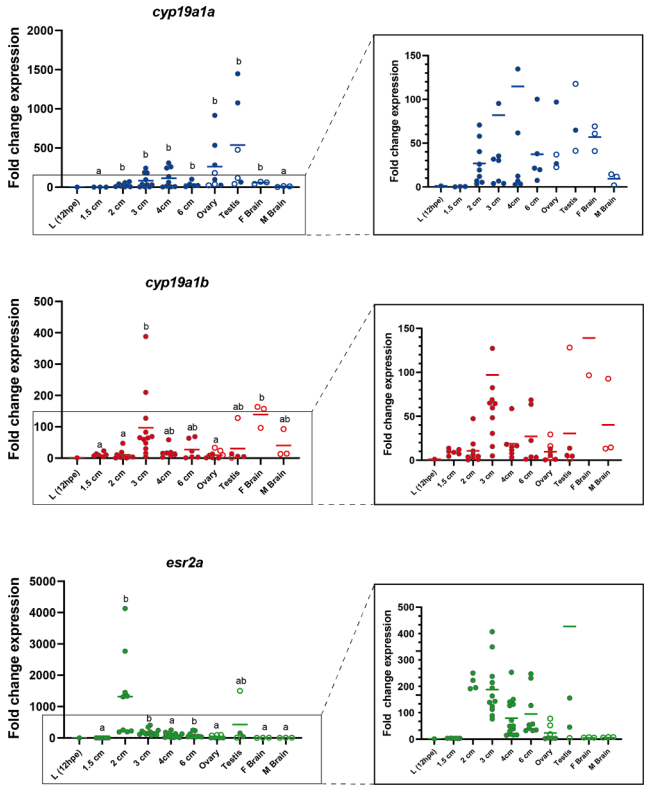

Fig. 4. Expression of *cyp19a1a*, *cyp19a1b* and *esr2a* from different stages of juvenile development and gonads of *C. macropomum*. Results are presented as scatterplots of individual expression values of relative expression to the values of the pool of 12 h post-hatched larvae (L) and the averages as lines. Gonad and brain samples from immature fish are represented by unfilled circles and from maturing fish by filled circles. Different letters mean different averages between the groups. $P < 0.005$.

fadrozole (Fz) or tamoxifen (Tx). There was no masculinization effect in neither treatment (n = 74 to 97 fish evaluated/treatment; Supplementary Table 2). Exposure to Fz reduced the E2 plasma concentration at the end of the treatment (fortieth day), but after 20 days the values were similar to the controls (Fig. 5). Exposure to Tx did not affect E2 plasma values. In both treatments, twenty days after the beginning of EDCs

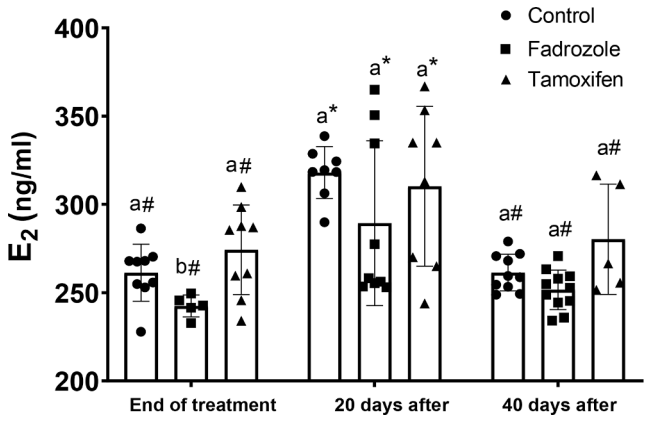

Fig. 5. Concentration of 17β estradiol (E_2) in *C. macropomum* treated with fadrozole (50 mg kg^{-1} of food) or tamoxifen (200 mg kg^{-1}) during 40 days. Samples were collected at the end of treatment, 20 and 40 days after the end of the treatment. Each bar represents the mean ± SD. Different letters denote statistical differences between treatments at the same point and different symbols show statistical differences between the collection points in the same treatment ($p < 0.05$).

administration, *cyp19a1a* transcription was down-regulated in comparison to controls. Otherwise, *cyp19a1b* transcription at day 20 was only down-regulated by Tx and neither Tx nor Fz altered the expression of *esr2a*. No further differences in gene expression were found in EDC-treated tambaqui compared to the control group at 40 days (end) of treatment (Fig. 6). Since no sex changes were observed, it is expected that after some time the transcriptional levels return to normal.

4. Discussion

The role of aromatase on the sex differentiation of vertebrates has been frequently addressed at the gonad level where estradiol, acting via estradiol receptors, is a pivotal factor. There is a single aromatase gene (*CYP19A1*) in Chondrichthyes, Sarcopterygii and non-teleost Actinopterygii, but some mammals as pigs, peccaries and other suiformes have two or more genes resulting from the duplication of a common ancestor (Conley et al., 2009). However, many teleosts exhibit two functionally conserved aromatase genes, namely *cyp19a1a* and *cyp19a1b*, as a result of the third round of whole-genome duplication (TWGD) specific to the teleost lineage. The most recent phylogenomic study revealed a single 3R-aromatase paralog *cyp19a1* present in basal teleosts including eels and Asian arowana, that most likely exhibits conserved properties of the ancestral single *CYP19A1*, such as the conservation of the brain-and gonad-type responsive elements in the *cyp19a1* promoter, in

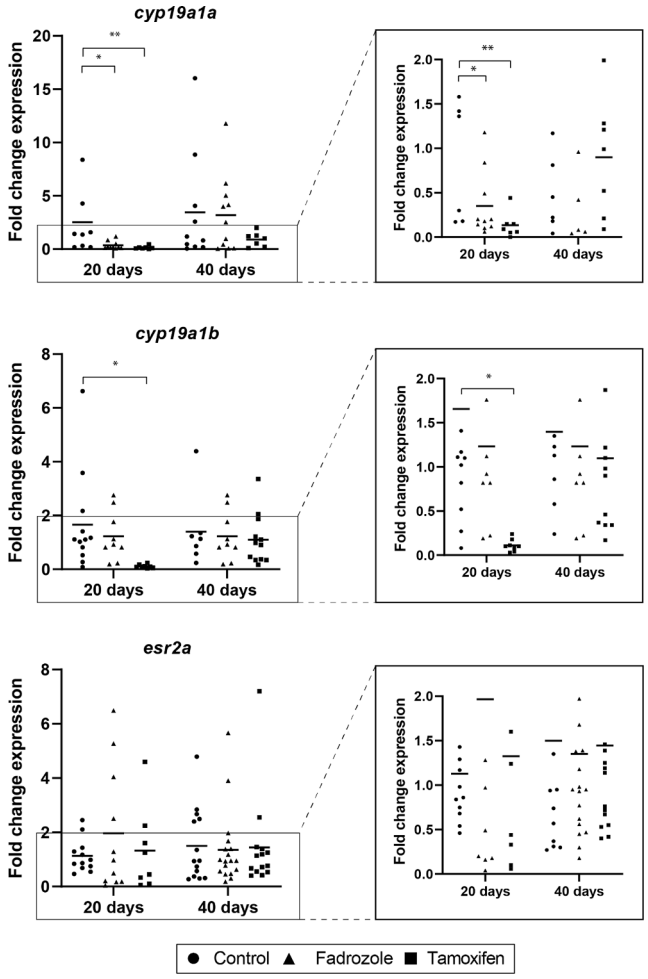

Fig. 6. Transcript abundance of *cyp19a1a*, *cyp19a1b* and *esr2a* in undifferentiated tambaqui trunks during treatment with fadrozole (50 mg kg^{-1} of food) or tamoxifen (200 mg kg^{-1}). Results are presented as scatterplots with individual expression values. Asterisks denote significant differences between control and treatment group ($p < 0.05$).

agreement with the main expression and role of the single *cyp19a1* in both brain and gonad (Lin et al., 2020). In the present study, more aromatase sequences from the Otocephala representatives were included in the phylogenetic analysis which displayed a tree topology similar to previous studies. We also discovered the existence of a single aromatase in the black bullhead fish (*Ameiurus melas*; Siluriformes) and *Limnothrissa miodon* (Clupeiformes) genomes, which correspond to the Cyp19a1b and Cyp19a1a clade, respectively.

A deep phylogenomic view into the Otocephala clade clearly shows additional possibilities regarding the function of duplicated aromatases. Despite the great conservation of *cyp19a1a* and *cyp19a1b* neighboring genes across Characiformes, the variations in their organization compared to the commonly studied zebrafish (*Danio rerio*) and japanese medaka (*Oryzias latipes*) draw our attention. The zebrafish genome notably seems to have experienced many interchromosomal rearrangements during evolution by extensive translocations, while the medaka genome has preserved its Clupeocephala ancestral genomic structure without undergoing major interchromosomal rearrangements (Kasahara et al., 2007). Thus, the variations detected right upstream the *cyp19a1b* genomic location among these species suggest that lineage-specific rearrangement events happened in this locus after the split of Clupeocephala into Otocephala and Euteleostei which may have somehow impacted the regulatory area in different levels.

Changes in the genomic neighborhood after the split from the common ancestor are linked to divergences in gene expression levels in different tissues, potentially leading to phenotypic divergences (De et al., 2009). Although the adjacent genes of *cyp19a1a* among all species compared are conserved, the presence of transposable element (TE) traces such as transposase at this location served as a variation source in the gonadal aromatase genomic environment. Additionally, TEs can be the source of developmental innovations through their recruitment as new coding sequences and new ncRNAs, and by acting as regulatory sequences (Etchegaray et al., 2021). Indeed, an alternate 5′ regulatory region of the human and mouse *CYP19A1* includes a promoter derived from LTR retrotransposon that drives expression in the placenta, as well as an associated upstream enhancer (Kamat et al., 2005). Therefore, TE traces found within *gldn-cyp19a1a* intergenic region in tambaqui, covering the promoter region of both genes, can be associated with notable differences in bp size compared to *cdhr5b-cyp19a1b* intergenic region and with the alterations in *cis*-regulatory elements detected among the groups analyzed.

In this context, the tambaqui *cyp19a1a* promoter sequence provided the most controversial insight due to the absence of steroidogenic factor 1 (SF-1) responsive element, conversely found in the 5′-flanking regions of all Clupeocephala *cyp19a1a*, basal teleost *cyp19a1* and non-teleost actinopterygian *CYP19A1* sequences (Lin et al., 2020). The modulation of *cyp19a1a* expression by SF-1 nuclear receptor, also referred to as AD4BP, NR5A1 or FTZ-F1, has already been demonstrated in Nile tilapia (Yoshiura et al., 2003), medaka (Watanabe et al., 1999), zebrafish (von Hofsten and Olsson, 2005), human ovary (Michael et al., 1995) and rat granulosa cells (Shapiro et al., 1996). Moreover, even though SF-1 binding sites were found in other Characiformes, such as red-bellied piranha and mexican tetra *cyp19a1a* promoter sequences, the highest dissimilarity (DI) values compared to the consensus sequence predicted in all other teleost investigated suggest that remarkably differences in *cyp19a1a* regulation may occur in this clade, even between phylogenetically close species.

Regarding the *cyp19a1b* promoter region, no SF-1 response elements were predicted in tambaqui, similar to other Clupeocephala species such as zebrafish and tilapia, but with the exception of medaka (Lin et al., 2020). This particularity can be associated with the retention of *gldn* paralog linked to *cyp19a1b* in Japanese medaka. Such diversity in aromatase cis regulatory region may result in disparities in gene expression, affecting the plasticity of the gene regulation underlying gonadal morphogenesis (Nakamoto et al., 2018; Li & Ge, 2020; Imarazene et al., 2021). Overall, although both tambaqui *cyp19a1a* and *cyp19a1b* promoter regions share many putative transcription factor binding sites, the differences in position and number of copies may reflect the particularities in their individual regulation and expression.

Although the main molecular pathway towards tambaqui ovary and testis differentiation could be characterized by RNA seq of the trunks of undifferentiated tambaqui, only the *cyp19a1b* (significantly more expressed in the future males than females), but not *cyp19a1a,* could be detected by this approach (Lobo et al., 2020). In the present study, the expression of both genes were identified by real-time quantitative PCR (qPCR) during the early stages of sex development. As expected, based on the previous RNA seq data, *cyp19a1b* transcripts in tambaqui larvae trunks increased after hatching (while *cyp19a1a* decreased) and peaked at very high values just prior to differentiation. On the other hand, the increase in *cyp19a1a* expression occurs later (fish from 2 cm) and the pattern of two distinct groups could be observed in fish of 3 and 4 cm length. Although we couldn't link the transcription profile to the corresponding sex of the individuals, in *A. mexicanus,* a non-dimorphic expression profiles of *cyp19a1a* were detected in males and females during early development (Imarazene et al., 2021).

Given the presence of a well-conserved estrogen-responsive element (ERE) in the proximal promoter region of *cyp19a1b* (Fig. 1b), and the positive auto-regulatory loop of estradiol via ERs in fish brains (Diotel et al., 2010), we traced a parallel between *esr2a* and *cyp19a1b* transcription profiles during sex development of tambaqui. From hatching, the expression of estrogen receptor beta 1 (*esr2a)* increased in pre-differentiating tambaqui up to 1000-fold, which are values far above the *esr2a* expression in differentiated gonads (in immature and mature juveniles). Moreover, at this undifferentiated phase, *esr2a* expression in fish trunks split into two distant groups and in fish bigger are back to similar (and low) transcription values. Soon after the split of *esr2a*, the expression of *cyp19a1b* rises, reaching levels above the recorded in brain and gonads of adult fish. *cyp19a1b* transcripts then decrease during morphological differentiation (4–6 cm), at the same time as *cyp19a1a* expression splits into two groups. Therefore, we suspect that *cyp19a1b* and *esr2a* are involved in the gene pathway that drives tambaqui sex differentiation, while *cyp19a1a* seems to be a consequence of the already triggered E_2-ER system activation and might be responsible for the further maintenance of the developing ovary. The involvement of *cyp19a1b* in sex differentiation has been suggested in pejerrey *Odontesthes bonariensis,* however with high expression in brain, and not in the trunk, preceding the expression of *cyp19a1a* in the trunk before the first signs of gonadal differentiation (Karube et al., 2007; Strobl-Mazzulla et al., 2008).

Fradozole reduced the E_2 plasma levels at the end of treatment, and down regulated *cyp19a1a* (significantly) and *cyp19a1b* at the beginning of the treatment, and tamoxifen reduced the expression of both aromatase coding genes, but without effect on the levels of estradiol. These observations are in agreement with the physiological effects of EDCs in fish in which SERMs blocks estrogen function by binding to ERs and suppressing *cyp19a1a* or *cyp19a1b* expression, while AIs decrease E_2 levels by acting directly on aromatase production (Cheshenko et al., 2008; Scholz and Klüver, 2009). However, none of the treatments changed the expression of *esr2a* and nor were able to distress the sex ratio in tambaqui. Such data is contradictory to the masculinization effect of aromatase inhibitors widely reported in a variety of fish species (Babiak et al., 2012, Shen et al., 2015, Evliyaoğlu et al., 2019, Qin et al., 2020, Ayobahan et al., 2020), even at very low concentrations (Muth-Köhne et al., 2016, Joshi et al., 2019). And also incompatible to the tamoxifen effect that, acting as either agonist or antagonist, stimulating or inhibiting gonadal differentiation, leads to abnormal sexual development and biased sex ratio (Singh et al, 2012, Navarro-Martín et al., 2009, Singh et al., 2015, García-Hernández et al., 2016, Pandit et al., 2017). Such complexity in EDC responses has been reported in *Astyanax altiparane* as a result of a mechanism of resilience, in which males resisted the E_2-induced feminization by enhancing the expression of genes related to testicular differentiation (Martinez-Bengochea et al.,

2020). Likewise, zebrafish females are able to resist heat-induced masculinization, maintaining ovarian morphology despite changing their gonadal transcriptome to a testis-like one (Ribas et al., 2017) and *A. burtoni* ovaries cannot be transformed into functional testis by AI, but rapid changes towards a male-like phenotype happens, such as body coloration, hormone levels and behavior (Göppert et al., 2016).

The higher expression of *cyp19a1a* in testis than in ovaries and the upregulation of *esr2a* in all pre-differentiation fish suggest that the estrogen pathway is necessary for tambaqui gonad development and maintenance in both sexes. Nonetheless, the reproductive state may influence the level of *cyp19a1a*, and more reproductive states should be analyzed in the future. Estrogen production via aromatase in fish testis has been described to be involved in spermatogenesis, where estrogen is necessary for the renewal of spermatogonial stem cells and possibly differentiation (Schulz et al., 2010; Kobayashi et al., 2011). Similar *cyp19a1a* transcription sex bias towards males were observed in *A. mexicanus*, with high values in testes and low basal levels in ovaries during gametogenesis (Imarazene et al., 2021). In another Characid, *A. altiparanae, cyp19a1a* are expressed equally in adult testes and ovaries (Martinez-Bengochea et al., 2020).

The expression pattern of *cyp19a1a* and *cyp19a1b* was more abundant in female brains when compared to even some individual gonads. This observation also confronts the classical assumption of the tissue-specificity of aromatase expression (Guiguen et al., 2010). In most fishes studied so far, the *cyp19a1b* is more expressed in the brain of males than of the females and it is responsible for the male sexual behaviour such as aggression and court strategies (Goto-kazeto et al., 2004; Black et al., 2005; Strobl-Mazzulla et al., 2008; Gonçalves et al., 2008; Renn et al., 2008; Colman et al., 2009; Diotel et al., 2010; Huffman et al., 2013; Göppert et al., 2016, Silva de Assis et al., 2018). However, it was recently demonstrated through the *cyp19a1b* expression on the female brain of the African cichlid fish, *Astatotilapia burtoni*, that the estrogenic signaling in female brain might have important roles in the regulation of reproductive cycling and social behaviors (Maruska et al., 2020). These results on the *cyp19a* expression in gonads and brains of male and female tambaqui are uncommon in fish, and therefore require proper and specific investigation.

Taken together, the present study reveals another level of flexibility on the sex-differentiation pathway of teleosts, contributing to the recent discussion on the unexpected expression of genes related to ovarian differentiation, especially in characids. Such an unconventional mechanism can be explained by the modifications on the *cyp19a1a* promoter region as a consequence of transposable elements' activity. Indeed, the chromosomal evolution of Serrasalmidae involved different rearrangements (fissions, translocations and pericentric inversions) due to TEs, which have crucial roles in the differentiation and evolution of sex chromosomes. During tambaqui sex differentiation, besides the bimodal expression of *cyp19a1b* first, followed by *cyp19a1a*, the outstanding bimodal gene expression of *esr2a* from hatching to differentiation, suggests that estrogen receptor is crucial for the sex identity in *C. macropomum*. Likewise, although the endocrine disruptors caused some changes in *cyp19* genes transcription, the *esr2a* expression and sex ratio were not altered by those compounds. In conclusion, our data on the complete structure, synteny, phylogenetic and expression characterization of tambaqui *cyp19a1a* and *cyp19a1b* provided new information concerning the evolution and evolutionary impact of TEs in the Neotropical freshwater fish genomes, which can be considered groundbreaking in the classical knowledge regarding estrogens and estrogen receptors duties in fish sex differentiation.

CRediT authorship contribution statement

R.V. Paixão: Methodology, Investigation, Validation, Formal analysis, Writing – original draft. **G.F. Silva:** Conceptualization, Supervision, Investigation, Writing – review & editing. **A.R. Caetano:** Resources, Data curation, Writing – review & editing. **L.C. Cintra:** Resources, Data curation. **E.S. Varela:** Resources, Data curation. **F.L.A. O'Sullivan:** Conceptualization, Supervision, Funding acquisition, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2022.146795.

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Supplementary material

Supplementary table 1. Primer sequences used for quantitative realtime PCR.

ID	Primer sequence	Primer length	Amplicon
cmesr2a forward	TGACCCTTCACTGCTCAC	18 bp	107 bp
cmesr2a reverse	CCCTATTGGTTTGCTGTTCTC	21 bp	
cmcyp19a1b forward	CTTCCTCAGAATCCCTCTCAACG	23 bp	177 bp
cmcyp19a1b reverse	TGCTCCACAAGACTTCCCATC	21 bp	
cmcyp19a1a forward	ACCTCCTCCACAAACTCA	23 bp	81 bp
cmcyp19a1a reverse	GAGAAGATTCAGGACATCCAC	21 bp	

Supplementary table 2. Effect of EDCs on tambaqui sex ratios using Chi-square (X^2) test.

Treatment	Male (%)	Female (%)	Total (N)
Control	54,43	45,56	79
Fadrozole	42,27	57,73	97
Tamoxifen	51,36	48,46	74

Figure S1. Histology of tambaqui gonads. A and B) ovary and testes of juvenile tambaqui at early identification of the phenotypic sex; C and D) ovary and testis of immature fish (body weight of XX ± xx gr); E and F) ovary and testis of maturing tambaqui (body weight of XX ± xx gr). C, D, E and F represent the maturation status of the fish used for gonad and brain qPCR. Asterisks point previtellogenic oocyte, arrows show spermatogonia, ST- somatic tissue, MO- mature oocyte, SC- spermatocyte, SZ- spermatozoa.

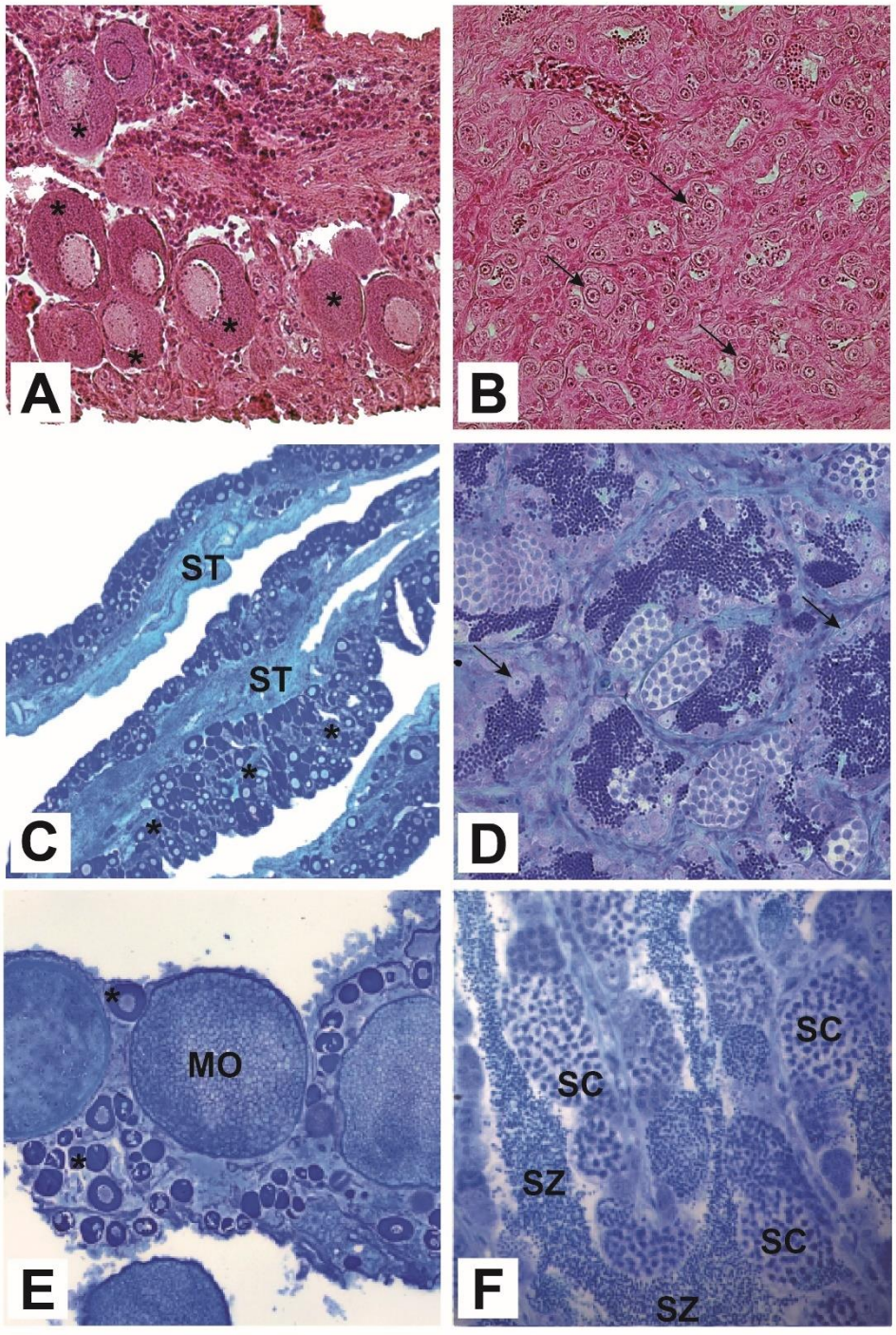
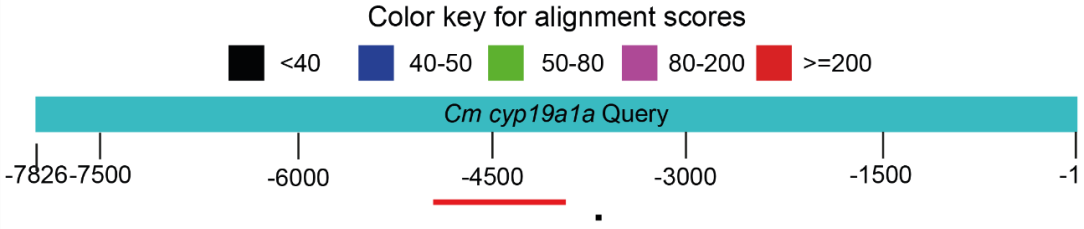


Figure S2: Sequences producing significant alignments against *Colossoma macropomum cyp19a1a* (A) and cyp19a1b (B) 5' intergenic regions in FishTEDB genomic database; and the most significant alignment between the translated TEs found similar to C. macropomum *cyp19a1a* (C) and *cyp19a1b* (D) 5' intergenic regions segment in NCBI protein database.

Fig. S2.A

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PM_Contig_11#DNA/TcMar
Species: Periophthalmus magnuspinnatus
TE class II, TIR order, Superfamily TC1-Mariner
Length=1610
Score = 556.962 bits (301), Expect = 1.89641E-156
Identities = 794/1029(1%), Gaps = 45/1029(0%)
Strand=Minus/Plus

Query        ------CTGCATTAAAAACAGATATGATTCTCTAGTGGAAATCACTGCATGGGCTCAGAAACACTTCTGA
Subject      ACAACCCCAATTCCAATGAAGTTGGGATGCT---GTGTAAA----------ACTTAAAAAAA------A
                   *    *  **   ** *  *** **   *** ***           ** * *** *      *
Query        AATCCACTGCGTGTGAACACAACTCATCACTGCATCCACAAATGCAAGTCAAAACTCTAAAACACAAAGA
Subject      AATACA--------GAATACAATG---------ATTTGCAAATCCTTTTCAA---CCTATATTGAACTGA
             *** **        *** ****           **   ***** *   ****    *** *    *  **
Query        AGAAACCGTACATAAACAGGATCCAGAAACGCTGCCACCTTCTTTGGGCCCGAGCTCATATAAAATGGAC
Subject      ATAAAC--TACAAAGACATGATA-----------------TTTAATGTTCAAACT--GATAAACTTTAT
             * ****  **** * *** ***                    **   *  * * **   ***** *  *
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             **     * *     ** * * *  *  *    * * *  *  * ** **  * **     *** *  **
Query        ACAAAG---TGT---CCTCTGGGCTGAAGACCACTCAGCTTGTTATCAAGGTTCAAAAGCCAGTATTCAT
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             ***  *   ***   ** *** * *  * * ***    * * *** ***   **** *   **  **
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               *** *   * ** ** ** *          * *      ****       * *** *       ****
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             *  ********* * *  * **  *                   *  **   * ***  ***  * ** *
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                 * ***  ****  * **     *** ** ** *      *** * ** * *   * ** ** ***
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Subject      ------TTACTACGAAGCCACGCTGTTGTAACACGTGCAGAATG------TGGCTTGGCATTGTCTTGCT
                   **** **       * ***** *** **   * * ***      **** ** *  ****  * *
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                     ***    *** *     ** ** **       ****    ****          **  *  *
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             **        *   ** ** *  ***  ** **** ***  * *                 **  *
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Subject      CCATACCATCACAGATGCTGGCTTTTGAACTTTGCGCTGATAACAATCCGGATGGTCCTTTTCCTCTTTG
              ** ** *  *****           *** ** * **   ** * * * *      *  *  *** *
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Subject      GCCCGCAGGACACGACGTCCATGATTTCCAAAAACAATTTGAAAT---GTGGACTCGTCAGACCACAGCA
             *  * **    ***    *   *  **   **** **  *****    ****  *   *** ** * *
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              *  ******             ** ** ***    **   *  ** ** *         ****  *
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               * *****  ** **     ***** * * *  ** *       * ***   ** *         **  *
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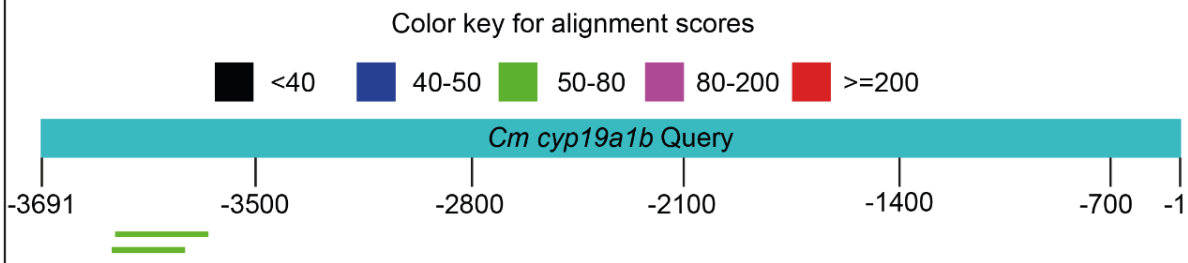
Subject       GATGTAGCGACGAACTGTGTTAACTGACAATGGTTTCCTGAAGTGTTCCTGAGCCCATGTGGTAATATCC
                    *    *  **   ** *  *** **   *** ***           ** * *** *      *
Query         TGCACAGCATCCCAACTTTTTTGGAAATGGGGTTGT
Subject       TTTACACATTCATGTCTGTTTTTAATACAG------
              *  ***   **    ** ****  * *  *
```

```
seq ID: N.bri_rnd-1_family-314#DNA/TcMar-Tc1
species: Neolamprologus brichardi
TE class II, TIR Order, superfamily TC1-Mariner
Length=108
Score = 65.753 bits (35), Expect = 1.40126E-8
Identities = 39/41(1%), Gaps = 0/41(0%)
Strand=Minus/Plus

Query         TTTTATACTCTATCATGTTACTGACCTGTTGCCAATTAACC
Subject       TTTTATACCCAATCATGTTACTGACCTGTTGCCAATTAACC
              ******** * ******************************
```

Fig. S2.B

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japan_eel_rnd-5_family-3443#DNA/TcMar-Tc1
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Score = 143.312 bits (77), Expect = 2.95754E-32
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Strand=Minus/Plus

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Subject       AGTACTGTGCAAAAGTCAGAGACCACCCTTTCTTTTATTTACTTTCCAGTGAAAACAGCCATTAAGTGCAAG
              ********** **** *** ** ** ** *** ******** **  **** ************* ** ***

Query         TCATTCATTTTTTGGCTTCAGTAAAAAGCACAT----GACATATTTAACAGAAAATTACACAGAAATTTCAA
Subject       TTATTTATTTTTCAGTGTCAGAAAATAATGCAGCAAACATATATTTAACACAAAATTACACAAAAACCTCCA
              * *** ******  *  **** *** *   **      * ********** *********** ***  ** *

Query         CAACTAAATCTAATATATTTTTTGTTTAGTATTTATTGTGTCCACACTTTGCCTTTATTTCAGTGTCCATTC
Subject       CAACTATGTACAATATCAAATCT-TTCAGTATTTAGTGTGTCCACCCTTTGCCTTTATTACAGCTTCCATTC
              ******  *  *****    * * ** ******** ********* ************* ***  *******

Query         TTTTCAGGACACTTGCTCT----------CAGGAATCTGCAAGGATATTTTTCCACACTCTG----------
Subject       TTTTC-GGAGACTTGCTTTGAGTTTTTCNAAGAAATCTGCAGGGATNTTTTTCCACGCTTCTTGTAAACGCC
              ***** *** ******* *           ** ******** **** ********* **
Query         ---AAAGTTCAGTCTTAGAAGTTGGTTGCATTTTCTGCTTTTC
Subject       TCCAAAGTTCAGTCTTAGAAGTTGGTTGCATTTTCTGCTTCTC
                 ************************************ **
```

```
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Strand=Minus/Plus

Query       ACATATAGTACTGTGCGAAAGACAGTGAACATCC-TTCATTTATTTAATTAACAGTCAAAACAGCCATTAT
Subject     ACATACAGTACTGTGCAAAAGTCAGAGACCACCCTTTCTTTTATTCACTTTCCAGTGAAAACAGCCATTAA
            ***** ********** **** *** ** ** ** *** ****** * **  **** *************

Query       GTACAACTCATTCATTTTTTGGCTTCAGTAAAAAGCACAT----GACATATTTAACAGAAAATTACACAGA
Subject     GTGCAAGTTATTTATTTTTCAGTGTCAGAAAATAATGCAGCAAACATATATTTAACACAAAATTACACAAA
            ** *** * *** ******  *  **** *** *   **      * ********** *********** *

Query       AATTTCAACAACTAAATCTAATATATTTTTTGTTTAGTATTTATTGTGTCCACACTTTGCCTTTATTTCAG
Subject     AGCCTCCACAACTACGTACAATATCAAATCT-TTCAGTATTTAGTGTGTCCACCCTTTGCCTTTATTACAG
            *   ** *******  *  *****    * * ** ******** ********* ************* ***

Query       TGTCCATTCTTTTCAGGACACTTGCT
Subject     CTTCCATTCTTTTCGGGAGACTTGCT
              ************ *** *******
```
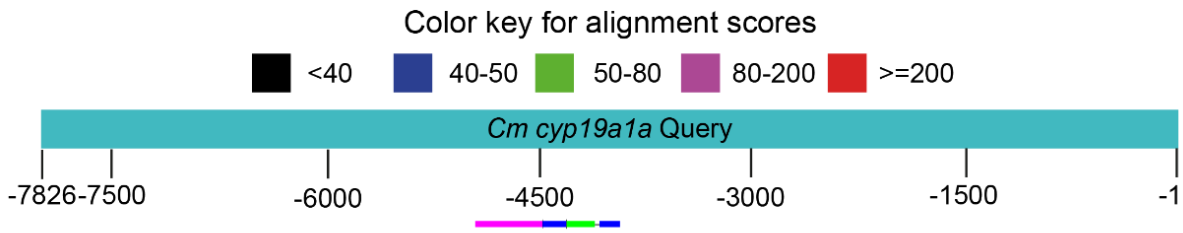

Fig. S2.C



TCB1 transposase [*Polypterus senegalus*]
Sequence ID: MBN3292004.1Length: 340Number of Matches: 4

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Range 1: 76 to 185
Alignment statistics for match #1
Score: 7/112(6%)      Expect: 8e-51    Method: Compositional matrix adjust.
Identities: 58/112(52%)     Positives:78/112(69%)
Gaps: 100 bits(248)   Frame: +2

Query  2    CIKNRYDSLVEITAWAQKHF*NPLRVNTTHHCIHkckskl*ntkkkPYINRIQK--RCHL  175
            C  NR  ++ EIT WAQ++F  PL VNT H  I +C+ KL + KKKP++++I K  RCH
Sbjct  76   CTTNRNATVKEITEWAQEYFQKPLSVNTIHRAIRRCQLKLYSAKKKPFLSKIHKLRRCH-  134

Query  176  LWARAHIKWTEGKWKSVLWSDESTFEILFGNHGQSVLWA---EDHSACYQGS  322
             WAR H+KW+  KWK+VLWSDES FE+LFGN G+ V+     +D+ +CYQ S
Sbjct  135  -WARDHLKWSVAKWKTVLWSDESRFEVLFGNLGRHVIRTKEDKDNPSCYQRS  185

Range 2: 219 to 271
Alignment statistics for match #2
Score: 79.0 bits(193) Expect:8e-51    Method: Compositional matrix adjust.
Identities: 34/53(64%)     Positives: 41/53(77%)
Gaps: 0/53(0%)   Frame: +1
```

```
Query  415  IYMFCQHMLPSRRHLFQGRP*LFQQDNVKPHSACITTALLCFKTVRMLNWPAC  573
            I +  QHMLPSRRHLFQGRP +FQQDN +PHSA ITT+ L  + +R+L WP C
Sbjct  219  IQVLEQHMLPSRRHLFQGRPCIFQQDNARPHSASITTSWLRRRRIRVLKWPVC  271

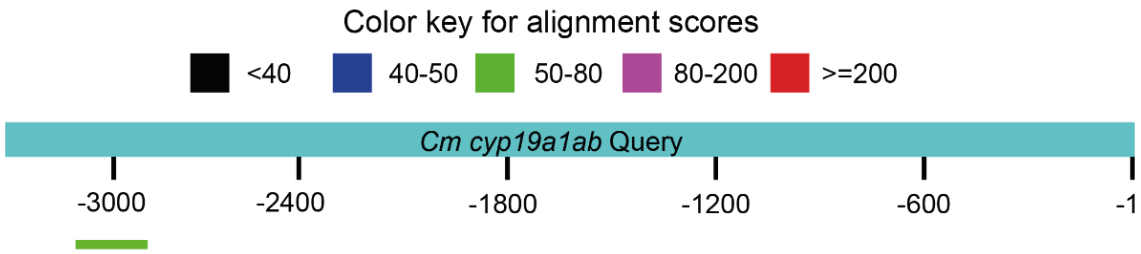

Range 3: 186 to 225
Alignment statistics for match #3
Score: 47.0 bits(110) Expect: 8e-51    Method: Compositional matrix adjust.
Identities: 22/40(55%)      Positives: 29/40(72%)
Gaps: 0/40(0%)   Frame: +3


Query  318  VQKPVFMMV*GGISAHDMGNVHICEGTINAE*YIHVLSTY  437
            VQKP  +MV G +SA  MG++H+ +GTINAE YI VL  +
Sbjct  186  VQKPASLMVWGCMSACGMGSLHVWKGTINAEKYIQVLEQH  225


Range 4: 279 to 311
Alignment statistics for match #4
Score: 47.0 bits(110) Expect: 8e-51    Method: Compositional matrix adjust.
Identities: 22/33(67%)      Positives: 29/33(87%)
Gaps: 0/33(0%)   Frame: +2


Query  596  ENIWRIMKLKIRQRRP*TDEQLKSCIKQEQKNI  694
            ENIWRI+K K+RQRRP T EQL++CI+QE ++I
Sbjct  279  ENIWRIIKRKVRQRRPKTIEQLEACIRQEWESI  311
```

Fig. S2.D



PREDICTED: uncharacterized protein LOC101236062 [*Hydra vulgaris*]
Sequence ID: XP_004213182.1Length: 116Number of Matches: 1

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Range 1: 13 to 81
Alignment statistics for match #1
Score: 52.4 bits(124) Expect: 6e-04    Method: Compositional matrix adjust.
Identities: 27/69(39%)      Positives: 42/69(60%)
Gaps: 0/69(0%)   Frame: -3

Query  411  IHLHKEG*SQKNITEKNGVSKSGVQKCIRGYRENGTPNDHARPGRPPKLSPLEKHYSKLS  232
            I LH+EG SQ  I+ K G S + +++ I+ +RE G+  +  + GRPPKL+  +  Y K
Sbjct  13   IVLHEEGYSQPQISSKTGYSMTAIREIIKKFRETGSLRNRKKSGRPPKLTKDDNKYLKTL  72

Query  231  SLKRRESFS  205
            SL+ R+  S
Sbjct  73   SLRNRKKIS  81
```

Figure S3. Predicted transcription binding sites in *Colossoma macropomum cyp19a1a* (A) and *cyp19a1b* (B) regulatory region sequences. Colours are matching the comparative graphical scheme on Figure 3. The predictions are shown on the corresponding position of the query sequence (above), including the name of the factor that binds to the motif and the dissimilarity index (below), except for manually predicted sites such as ERE, Foxl2 and Sox9.

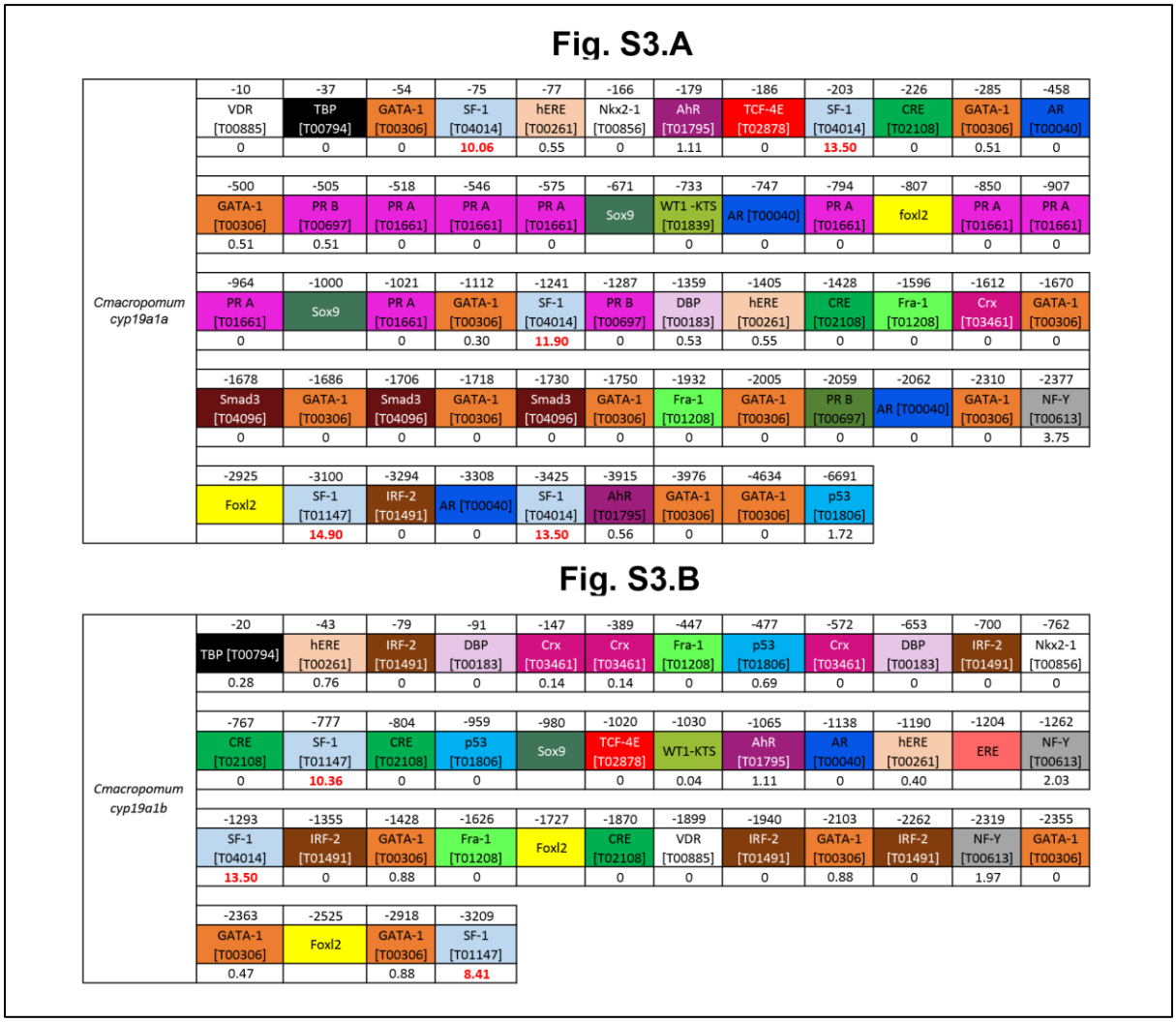

Figure S4. Infograph of Cyp19 sequences from different vertebrates, with corresponding taxonomic groups and genbank sequence ID.

Aromatase protein sequences across vertebrates										
Cephalochordata							Amphioxiformes	*Bfloridae*	XP_035672840, XP_035669280	CYP19A1
Cyclostomata							Myxiniformes	*Eptatretus burgeri*	ENSEBUP00000001770	
Sarcopterygii							Coelacanths	*Latimeria chalumnae*	XP_006001985.1	
							Amphibians	*Xenopus tropicalis*	NP_001090630.1	
							Birds	*Gallus gallus*	NP_001001761.2	
							Reptiles	*Anolis carliolinensis*	XP_016852003.1	
							Mammals	*Homo sapiens*	NP_000094.2	
Chondrichthyes							Chimaeriformes	*Callorhinchus milii*	XP_042199999.1	
								Amblyraja radiata	XP_032906075.1	
								Rinchodon typus	XP_020386362.1	
Non-teleost fishes	Cladistia						Polypteriformes	*Erpetoichthys calabaricus*	XP_028678241.1	
	Chondrostei						Acipenseriformes	*Acipenser ruthenus*	XP_034757780, XP_033905095	
	Holostei						Lepisosteiformes	*Lepisosteus oculatus*	XP_015198515.1	
Teleost fishes	Basal teleosts	Elopomorpha	Elopiformes					*Megalops cyprinoides*	XP_036391574	Cyp19a1
			Anguilliformes					*Anguilla anguilla*	Q6QHT9	
								Anguilla japonica	XP_035252576	
		Osteoglossomorpha	Osteoglossiformes					*Sclerophages formosus*	XP_029109168.1	
								Arapaima gigas	VFQV01000034	
								Heterotis niloticus	CM030889.1	
								Gymnarchus niloticus	JAACJT010005334.1	
								Mormyrus iriodes	JAAGVU010005045.1	
								Paramormyrops kingsleyae	XP_023672946.1	
								Brevimyrus niger	JAABNY01000140.1	
								Mormyrus lacerda	JAABNX010000007.1	
	Clupeocephala		Otocephala	Clupeiformes				*Denticeps clupeoides*	XP_028812927	Cyp19a1a
								Clupea harengus	XP_012670226.2	
								Coilia nasus	CM017723	
								Limnothrissa miodon	AGFZM010001022.1	
				Ostariophysi	Anotophysa	Gonorynchiformes		*Alosa alosa*	KAG5270649.1	
								Chanos chanos	XP_030648858	
					Otophysa	Cypriniformes		*Danio rerio*	NP_571229.3	
								Carassius auratus	XP_026087132.1, XP_026143636.1	
								Cyprinus carpio	XP_042630894.1, XP_042599607.1	
						Characiphysi	Siluriformes	*Tachysurus fulvidraco*	XP_026999292.1	
								Pangasianodon hypophthalmus	XP_034161197.1	
								Ictalurus punctatus	XP_017321613.1	
								Ameiurus melas	Not detected	
							Gymnotiformes	*Electrophorus electricus*	XP_026865672.2	
							Characiformes	*Hepsetus odoe*	CM029375.1	
								Astyanax mexicanus	XP_022542742.1	
								Colossoma macropomum	XP_036420492	
								Pygocentrus nattereri	XP_017574997.2	
			Euteleostei					*Gasterosteus aculeatus*	XP_040022997.1	
								Takifugu rubripes	XP_011608039.2	
								Dicentrarchus labrax	CAC43178.1	
								Monopterus albus	XP_020461421.1	
								Oreochromis niloticus	NP_001266515.1	
								Oryzias latipes	NP_001265808.1	
								Esox lucius	XP_012992178.2	
								Gadus morhua	XP_030231952.1	
								Salmo salar	XP_014030724.1, XP_013982947.1	
								Onchorhynchus mykiss	XP_036819945, XP_036837015	
	Clupeocephala		Otocephala	Clupeiformes				*Denticeps clupeoides*	XP_028813650.1	Cyp19a1b
								Clupea harengus	XP_031420590.1	
								Coilia nasus	CM017737	
								Limnothrissa miodon	Not detected	
								Alosa alosa	KAG5273686.1	
				Ostariophysi	Anotophysa	Gonorynchiformes		*Chanos chanos*	XP_030630778.1	
					Otophysa	Cypriniformes		*Danio rerio*	NP_571717.2	
								Carassius auratus	XP_026110034.1, XP_026057650.1	
								Cyprinus carpio	XP_042608630.1	
						Characiphysi	Siluriformes	*Tachysurus fulvidraco*	AKK31592.1	
								Pangasianodon hypophthalmus	XP_026775731.2	
								Ictalurus punctatus	XP_017340012.1	
								Ameiurus melas	KAF4081567.1	
							Gymnotiformes	*Electrophorus electricus*	XP_026877222.2	
							Characiformes	*Hepsetus odoe*	CM029372	
								Astyanax mexicanus	KAG9280783.1	
								Colossoma macropomum	XP_036432374.1	
								Pygocentrus nattereri	XP_017562184.1	
			Euteleostei					*Gasterosteus aculeatus*	XP_040017705.1	
								Takifugu rubripes	NP_001166967.1	
								Dicentrarchus labrax	AAM95455.1	
								Monopterus albus	XP_020479848.1	
								Oreochromis niloticus	XP_005450866.1	
								Oryzias latipes	BAJ78778.1	
								Esox lucius	XP_019912080.2	
								Gadus morhua	XP_030221819.1	
								Salmo salar	XP_014025761.1	
								Onchorhynchus mykiss	XP_036824875.1	

CAPÍTULO III

THE REPERTOIRE OF THE ELONGASES OF VERY LONG-CHAIN FATTY ACIDS GENE FAMILY IS CONSERVED IN TAMBAQUI (*COLOSSOMA MACROPOMUM*): EXPRESSION PROFILES OFFER INSIGHTS INTO THE SEXUAL DIFFERENTIATION PROCESS

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The repertoire of the elongation of very long-chain fatty acids (Elovl) protein family is conserved in tambaqui (*Colossoma macropomum*): Gene expression profiles offer insights into the sexual differentiation process

Renato B. Ferraz [a,b,c], Rômulo V. Paixão [d,e], Mónica Lopes-Marques [f,g,h], André M. Machado [c,h], Ana L. Salaro [a], L. Filipe C. Castro [c,h,*], Óscar Monroig [i,**], Fernanda L.A. O'Sullivan [e,**]

[a] Department of Animal Biology, Federal University of Viçosa, Brazil
[b] Department of Entomology, Federal University of Viçosa, Brazil
[c] CIIMAR - Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Portugal
[d] PPGCARP Programa de Pós-Graduação em Ciência Animal e Recursos Pesqueiros, Department of Fisheries, Faculty of Agricultural Sciences, Federal University of Amazonas, Brazil.
[e] Embrapa Amazônia Ocidental, Rodovia AM - 010, km 29, Manaus, Brazil
[f] i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal
[g] IBMC - Instituto de Biologia Molecular e Celular, Universidade do Porto, Porto, Portugal
[h] Department of Biology, Faculty of Sciences, University of Porto, Portugal
[i] Instituto de Acuicultura Torre de la Sal, Consejo Superior de Investigaciones Científicas (IATS-CSIC), Ribera de Cabanes 12595, Castellón, Spain

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ABSTRACT

Elongation of very long-chain fatty acids (Elovl) proteins are critical players in the regulation of the length of a fatty acid. At present, eight members of the Elovl family (Elovl1–8), displaying a characteristic fatty acid substrate specificity, have been identified in vertebrates, including teleost fish. In general, Elovl1, Elovl3, Elovl6 and Elovl7 exhibit a substrate preference for saturated and monounsaturated fatty acids, while Elovl2, Elovl4, Elovl5 and Elovl8 use polyunsaturated fatty acids (PUFA) as substrates. PUFA elongases have received considerable attention in aquatic animals due to their involvement in the conversion of C_{18} PUFAs to long-chain polyunsaturated fatty acids (LC-PUFA). Here, we identified the full repertoire of *elovl* genes in the tambaqui *Colossoma macropomum* genome. A detailed phylogenetic and synteny analysis suggests a conservation of these genes among teleosts. Furthermore, based on RNAseq gene expression data, we discovered a gender bias expression of *elovl* genes during sex differentiation of tambaqui, toward future males. Our findings suggest a role of Elovl enzymes and fatty acid metabolism in tambaqui sexual differentiation.

1. Introduction

Elongation of very long-chain fatty acid (ELOV) proteins are widely present in the genomes of animals, plants and microorganisms, regulating the length of fatty acids (FA) (Guillou et al., 2010). All ELOVL proteins contain a characteristic domain and a highly conserved HXXHH motif, which is conserved in yeast, mouse, rat and human (Jakobsson et al., 2006). The number of Elovl genes differs markedly among species. In teleost fish, seven Elovl were previously described, with Elovl1, Elovl3, Elovl6 and Elovl7 preferring saturated and monounsaturated fatty acids (SFA and MUFA) substrates; while Elovl2, Elovl4 and Elovl5 are selective for display a capacity to convert the C_{18} polyunsaturated fatty acids (PUFA) linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3) to long-chain ($C_{20–24}$) polyunsaturated fatty acids (LC-PUFA) (Guillou et al., 2010). These enzymes, referred to herein as "PUFA elongases", have received significant attention due to the health relevance of LC-PUFA such as arachidonic acid (ARA, 20:4n–6), eicosapentaenoic acid (EPA, 20:5n–3) and docosahexaenoic acid (DHA, 22:6n–3), which are essential, playing many biological roles such as growth, development and reproduction of vertebrates (Tocher and Dick,

* Corresponding author at: CIIMAR - Interdisciplinary Centre of Marine and Environmental Research, Av. General Norton de Matos S/N, 4450-208 Matosinhos, University of Porto, Portugal.
** Corresponding authors.
E-mail addresses: filipe.castro@ciimar.up.pt (L.F.C. Castro), oscar.monroig@csic.es (Ó. Monroig), Fernanda.almeida@embrapa.br (F.L.A. O'Sullivan).

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Available online 22 April 2022

2000). Recently, a new member of Elovl family has been unveiled in fish genomes, Elovl8, and studies have shown that Elovl8 has the ability to elongate PUFA to LC-PUFA (Oboh, 2018; Li et al., 2020; Sun et al., 2021).

The elongation of SFA and MUFA is catalyzed by four elongases, namely Elovl1, Elovl3, Elovl6 and Elovl7. Studies in mammals have shown that these enzymes often have overlapping substrate preference (Guillou et al., 2010; Kihara, 2012; Sherry et al., 2019). Briefly, Elovl1 is involved in the production of SFA up to C_{26} in length (Ofman et al., 2010). Elovl3 participate in the synthesis of SFA and MUFA of up to C_{24} (Zadravec et al., 2010). Elovl6 is involved in the elongation of SFA and MUFA up to C_{18}, while Elovl7 is reported to elongate SFA and MUFA of 18–22 carbons (Sherry et al., 2019; Naganuma et al., 2011). Although these FA are essential barrier components of the plasma membranes, where they play important roles in several aspects of cellular growth (Guillou et al., 2010), few studies have been done with SFA and MUFA elongases in teleost fish.

Elovl involved in the biosynthesis of LC-PUFA such ARA, EPA and DHA, have been widely studied and functionally characterised in several species of teleost fish (Li et al., 2020; Monroig et al., 2016; Monroig et al., 2018; Xie et al., 2020; Monroig et al., 2022). Elovl2 and Elovl5 are highly conserved across vertebrates (Monroig et al., 2016). In teleosts, most species studied have a single *elovl5* gene with the ability to elongate C_{18} and C_{20} PUFA substrates, with relatively low efficiency toward C_{22} PUFA (Castro et al., 2016). While sharing a common evolutionary origin with Elovl5 (Monroig et al., 2016), Elovl2 has specificity toward C_{18} and C_{20} PUFA but, importantly, has high affinity toward C_{22} PUFA (Morais et al., 2009; Carmona-Antoñanzas et al., 2013). Indeed, teleost Elovl2 has demonstrated the capacity to elongate docosapentaenoic acid (DPA, 22:5n-3) to tetracosapentaenoic acid (TPA, 24:5n-3), a key elongation step required for DHA synthesis through the so-called "*Sprecher pathway*" (Sprecher, 2000; Oboh et al., 2017b). Another significant functional player in PUFA elongation is Elovl4. Similarly, to Elovl2, teleost Elovl4 can also contribute to DHA biosynthesis via the Sprecher pathway since they have the ability to elongate DPA to TPA. Moreover, Elovl4 plays a crucial role in the biosynthesis of the so-called "very long-chain ($>C_{24}$) polyunsaturated fatty acids (VLC-PUFA), compounds that accumulate in vertebrate photoreceptors that can contain up to C_{44} (Serrano et al., 2021). Teleost Elovl4 have been functionally characterised from a variety of species (Monroig et al., 2011; Carmona-Antonanzas et al., 2011; Monroig et al., 2012; Monroig et al., 2010; Kabeya et al., 2015; Oboh et al., 2017a; Zhao et al., 2019).

In various cases, each elongase family displays two paralogs in teleost fish species. In zebrafish, two *elovl1* genes (*elovl1a* and *elovl1b)* are highly expressed in the swim bladder; *elovl1b* is also expressed in the kidney, being a key determinant on the development of these organs (Bhandari et al., 2016). Another example is *elovl4*, which exhibits two gene copies, *elovl4a* and *elovl4b,* with characteristic substrate specificity and expression pattern (e.g., Monroig et al., 2010; Oboh et al., 2017a; Yan et al., 2017). In addition, elovl7 in fish also has two copies (elovl7a and elovl7b) (Xue et al., 2014). And, also elovl8, designated elovl8a and elovl8b (Oboh, 2018; Li et al., 2020; Sun et al., 2021). However, a detailed analysis in the Teleostei lineage is still missing. The origin of these duplicated genes is most likely linked to the teleost specific whole-genome duplication (TGD) (Pasquier et al., 2016). Genome duplications have clear impacts in the evolution of species, since they provide redundant genes facilitating diversifying selection often leading to the development of novel functions (Lynch and Conery, 2000). Unsurprisingly, in teleosts a great plasticity on the mechanisms of sex determination and differentiation is encountered in this animal class (Herpin and Schartl, 2015; Moore and Roberts, 2013). However, in spite of having different systems defining the sexual identity of teleosts, the involvement of steroids on the differentiation of fish gonads is highly conserved (Li et al., 2019). Steroidogenesis is based on the presence and transport of cholesterol into the mitochondria, which, in some fish, is dependent on the activity of arachidonic acid (ARA, 20:4n-6) (Castillo et al., 2006), indicating that some FA have direct effects on sex steroid production in teleosts (Berkes et al., 2006). Moreover, lipids are necessary for testis development, representing fundamental structural components during testicular growth and maturation (Shi et al., 2018; Oresti et al., 2013; Bogevik et al., 2020). Despite the importance of membrane lipids biosynthesis and remodelling on male germ cell differentiation, the function of FA in testis development and maturation is not fully understood yet in fish, being restricted to certain species (e.g., Bogevik et al., 2020).

Tambaqui (*Colossoma macropomum*) is a neotropical teleost fish native to the Amazon and Orinoco River basins, representing over a quarter of the total aquaculture production in Brazil (IBGE., 2020). To date, there are few information on the sex determination and differentiation molecular mechanisms of tambaqui. In this study, to further expand and identify the full repertoire of the Elovl family present in the tambaqui genome, we identified, for the first time, all *elovl* genes (1 to 8), and performed a detailed phylogenetic and synteny analysis that display the conservation of these genes when compared to other species. Additionally, to address the expression and possible function of this gene family, we analyzed the tambaqui transcriptome data of pre-differentiating males and females.

2. Materials and methods

2.1. Sequence and phylogenetic analysis of Elovl

Pygocentrus nattereri elovl gene sequences were used to blast search a *C. macropomum* genome to identify tambaqui's *elovl* genes (*elovl1a, elovl1b, elovl2, elovl3, elovl4a, elovl4b, elovl5, elovl6, elovl7a, elovl7b, elovl8a* and *8b*) (Ferraz et al., 2020; Hilsdorf et al., 2021). The deduced amino acid sequences were aligned to the corresponding orthologs from other species. Alignment performed in MAFFT (Katoh and Toh, 2008) with the L-INS-i method. The resulting sequence alignments were inspected and stripped of 90% columns containing gaps. A phylogenetic analysis was carried out by constructing phylogenetic trees using the Maximum Likelihood method and the JTT matrix-based model (Jones et al., 1992). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The proportion of sites where at least one unambiguous base is present, in at least one sequence for each descendent clade, is shown next to each internal node in the tree. This analysis involved 51 amino acid sequences. There was a total of 388 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

2.2. Synteny maps

Synteny maps were created using the annotated genomes available in NCBI, more specifically *C. macropomum* (GCF_904425465.1), *A. mexicanus* (GCF_000372685.2), *P. nattereri* (GCA_001682695.1) and *Danio rerio* (GCF_000002035.6). All synteny maps are centred on the target gene and four neighboring genes were collected up and downstream, when possible.

2.3. Gene expression analysis

In silico searches of available transcriptomic libraries (NCBI bioproject: PRJNA541929) from tambaqui sexually undifferentiated juveniles were used to screen for the *elovl* gene transcripts. This approach includes six tambaqui juveniles that were selected before the first evidence of histological sexual differentiation. Total RNA was extracted from their trunks for sequencing and a subsequent de novo transcriptome assembly. Further, Principal Component Analysis (PCA) of the whole transcriptome data was used to cluster samples in two distinct groups: Male Like Group (MLG) and Female Like Group (FLG).

Differential gene expression, functional annotation and gene enrichment were used to identify genes and pathways related to sex differentiation in tambaqui (Lobo et al., 2020). In this context, the expression values of *elovls* were retrieved as TPM (Transcripts Per Kilobase Million) for differential expression analysis between groups. We also assessed the transcriptomic libraries of immature ovary and testis to obtain insights of *elovl* expression at differentiated gonadal level. Heatmaps and bar graphs were performed using Graphpad Prism 8.

3. Results

3.1. Sequence analysis and phylogenetics of Elovl

We identified a total of 12 *elovl*-like sequences in two available

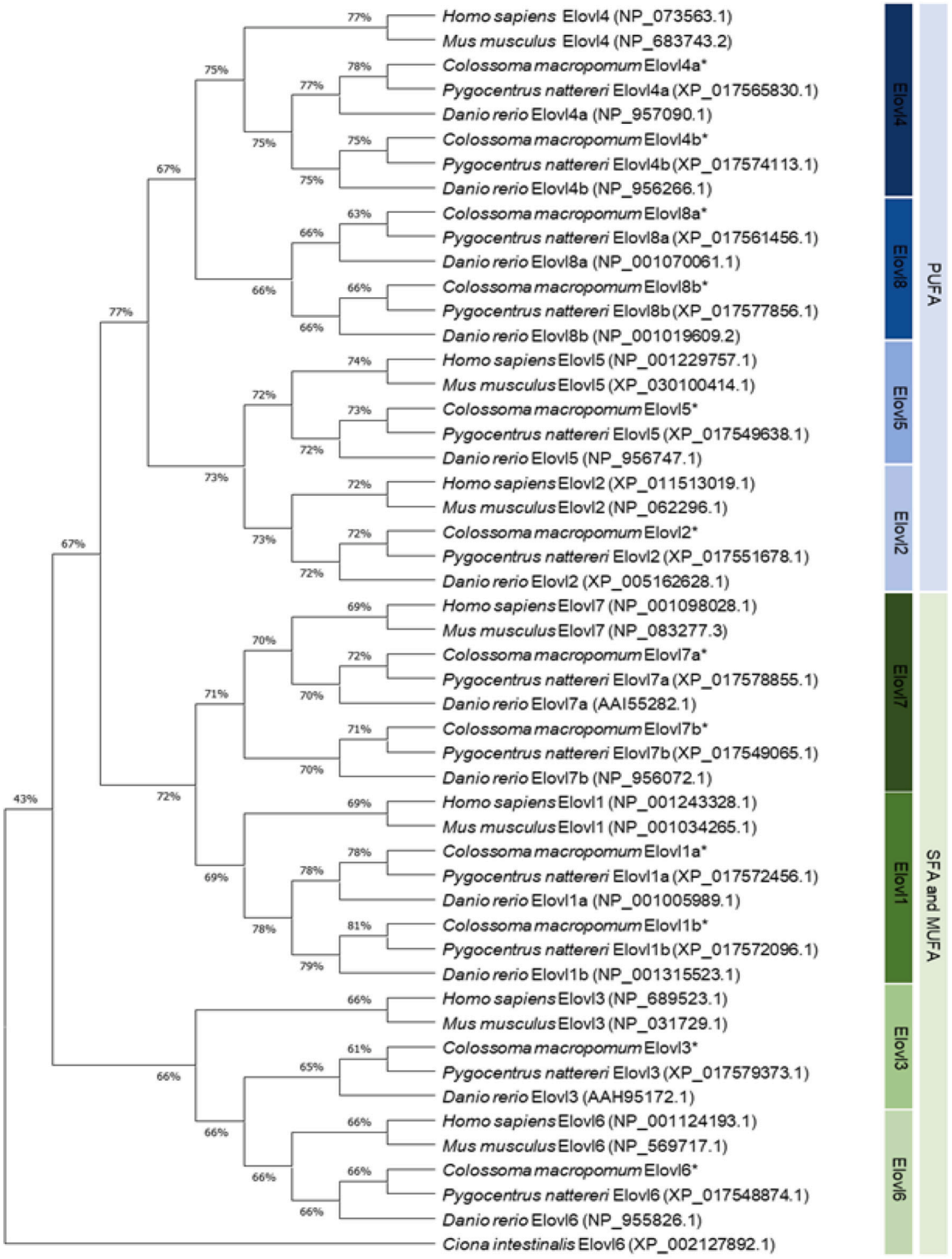

Fig. 1. Phylogenetic tree comparing the deduced amino acid sequences of *Colossoma macropomum* (Elovl1a, Elovl1b, Elovl2, Elovl3, Elovl4a, Elovl4b, Elovl5, Elovl6, Elovl7a, Elovl7b, Elovl8a and Elovl8b), and sequences from teleost species and mammalian. The *Ciona intestinalis* Elovl6 was included in the analysis as an outgroup sequence to construct the rooted tree. Where "*" represents the protein identified in this work.

genome assemblies (Ferraz et al., 2020; Hilsdorf et al., 2021). To determine the orthology relationships of the identified sequences, we next undertook phylogenetic analysis. The phylogenetic analysis of *C. macropomum elovl* sequences resulted in the construction of a phylogenetic tree with the highest log likelihood (−13978.46) and inclued all the genes previously identified in other teleost species, including the new members from the *elovl8* class (Fig. 1). The topology of the tree showed two clades, one containing Elovl involved in the elongation of SFA and MUFA, and another including Elovl involved in the LC-PUFA biosynthetic pathways ("PUFA elongases") (Fig. 1). Among the latter, two distinct clusters were identified, one consisting of Elovl2 and Elovl5 sequences, and the other consisting of Elovl4 and Elovl8 sequences (Fig. 1). More distantly related, two main clusters could be distinguished including Elovl3/Elovl6, Elovl1/Elovl7. Additionally, the identified aa sequence of tambaqui Elovl8a and Elovl8b showed high identity scores with *D. rerio* Elovl8 proteins in alignment, demonstrating conserved HXXHH histidine box motif where "*" represents "Q" (glutamine) in position −5 from the HXXHH, and the ER retrieval signal (Fig. 2).

3.2. Gene annotation and synteny

To support the phylogenetic analysis in assigning orthology to the tambaqui *elovl* genes, we performed a comparative synteny analysis on the premise that true orthologous genes are located in locus that are conserved across species. The elongases *elovl1a, elovl1b, elovl2, elovl3, elovl4a, elovl4b, elovl5, elovl6, elovl7a, elovl7b, elovl8a* and *elovl8b* were located in scaffolds that included, besides the target elongase, at least one neighboring gene. For comparative purposes, synteny maps were created for tambaqui and for two additional Characidae species (*A. mexicanus* and *P. nattereri*), and for the model teleost *D. rerio*.

Tambaqui *elovl2* is located (NW_023494894.1) together with a set of neighboring genes (*tmem14ca, mak, gcm2, sycp2l, gnaI, mppe1, tnfaip3-like*), the analysis of the *elovl2* locus in *A. mexicanus, P. nattereri* and *D. rerio* revealed that this locus is highly conserved (Fig. 3). Tambaqui *elovl5* was also identified in a highly conserved region (NW_023494791.1) with adjacent genes (*cox20, fbxo9, gclc, klhl31*), which were also observed in the corresponding locus of the other species investigated (Fig. 3). Regarding the tambaqui *elovl8a* and *elovl8b,* these genes were identified in scaffolds NW_023494807.1 and NW_023494793.1. The comparative analysis of these loci with the corresponding locus in *A. mexicanus*, *P. nattereri* and *D. rerio*, showed once again a high degree of conservation of neighboring genes in the analyzed species (Fig. 3). *Elovl4a* was located in scaffold NW_023494785.1 with the neighboring genes, namely *sox4b, cdkal1, echdc1, soga3, dctn3, ttk, bckdhb* and *tenta5a*, all of which are also found in the same genomic locus of *elovl4a* in the other teleost species analyzed herein (Fig. 3). Regarding *Elovl4b* (located in scaffold NW_023495330.1), we found a high degree of conservation of its locus when compared to the same locus in *P. nattereri* and *D. rerio*. However, when compared *A. mexicanus*, we found a single conserved neighboring gene, namely *soga3b*. We found teleost specific duplicates in neighboring *elovl4a* and *elovl4b,* more specifically *soga3, tent5aa* and *soga3b, tenta5ab.*

Tambaqui *elovl1a* was identified in scaffold NW_023494807.1, together with a set of neighboring genes (*pbx1-like, glula, tmem275, kncn, cdc20, cc2d1b* and *zyyve9a-like*), which are conserved with the remaining species investigated here (Fig. 4). Regarding tambaqui *elovl1b*, synteny maps showed a high conservation of neighboring genes in a comparative analysis of this locus with those of *A. mexicanus*, *P. nattereri* and *D. rerio* (Fig. 4). In addition to *elovl1a* and *elovl1b*, we identified other teleost-specific genome duplicates in this region, such as *zfyve9a-like* neighboring *elovl1a* and *zfyve9b* next to *elovl1b*. Tambaqui *elovl7a* and *elovl7b* were identified in scaffolds NW_023494793.1 and NW_023494804.1 respectively (Fig. 4). Additionally, teleost specific duplicates (*rab3c, pde4d* and *rab3c-like, pde4d-like*) were identified in both loci. Synteny analysis of tambaqui *elovl6 locus* showed a high degree of conservation among the analyzed species. Tambaqui *elovl3* was located in scaffold NW_023494790.1, with the following neighboring genes *acad-like* and *hmx3a-like* on one side, and *uros, mmp21, ctbp21* and *zranb1a,* on the other (Fig. 4). When comparing this region with the corresponding region in *A. mexicanus*, *P. nattereri* and *D. rerio* one side of the locus is conserved and therefore supporting orthology, while the other side is not conserved having been rearranged. In *D. rerio* and several other teleost species a duplicate named *elovl6-like* (NP_958908.1) has been identified in public databases. Although this

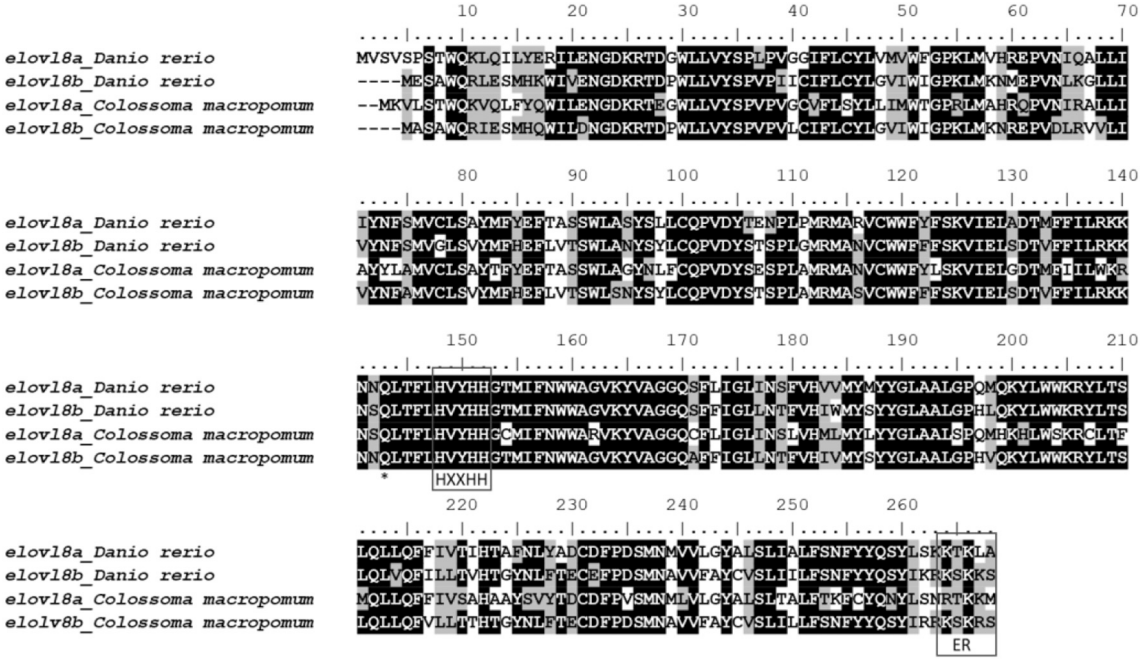

Fig. 2. Comparison of the deduced amino acid (AA) sequences of Elovl8a and Elovl8b from *Colossoma macropomum* with Elovl8a *Danio rerio* (NP_001070061.1) and Elovl8b *D. rerio* (NP_001019609.2). The AA sequences were aligned using BioEdit. Identical residues are shaded black and similar residues are shaded grey. Indicated are the conserved HXXHH histidine box motif, where "*" represents "Q" (glutamine) in position −5 from the HXXHH, and the ER retrieval signal.

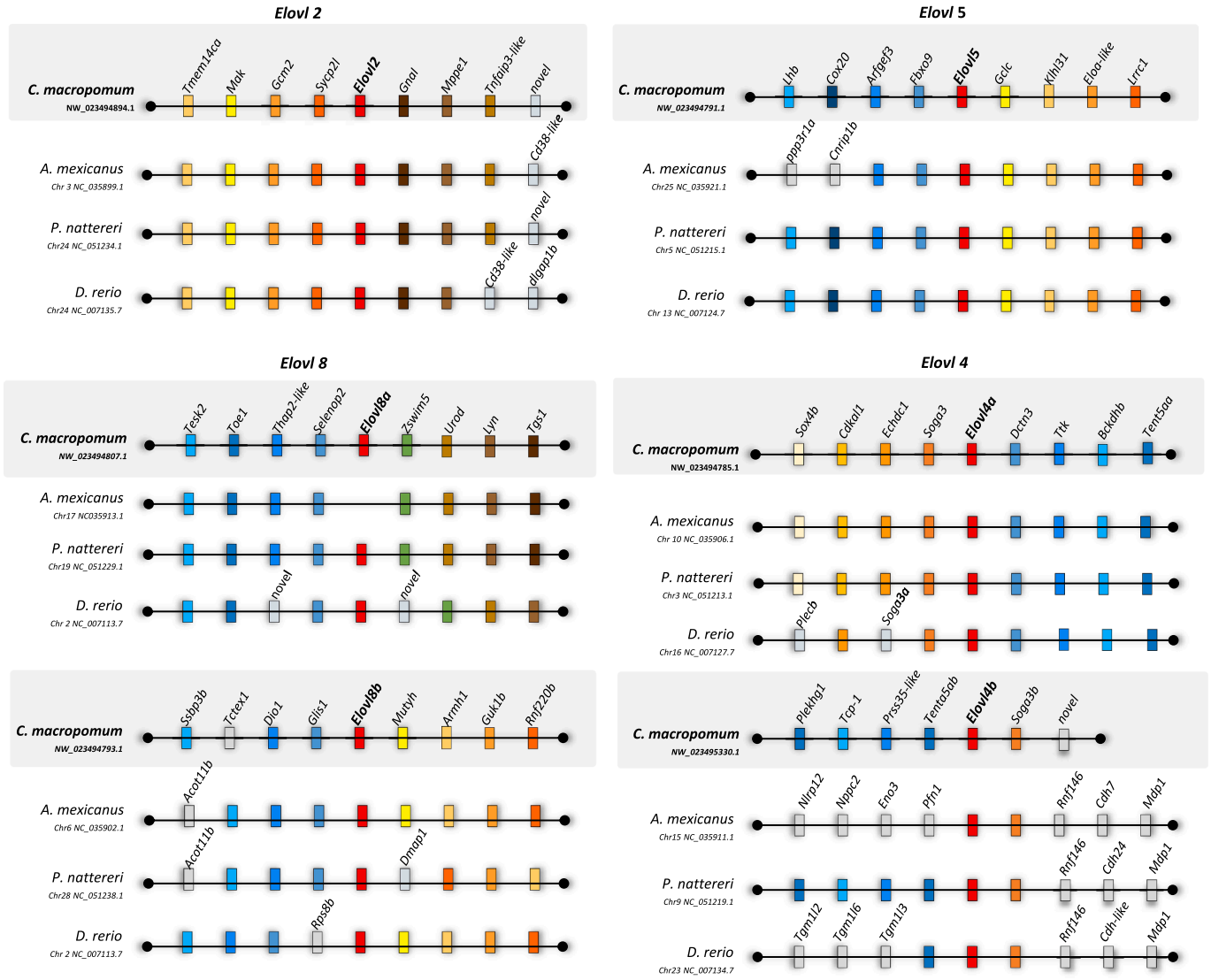

Fig. 3. Gene annotation of *Colossoma macropomum* genomic scaffold and comparative synteny maps. Synteny maps of gene involved in PUFA biosynthesis *Elovl2, Elovl5, Elovl4a, Elovl4b, Elovl8a* and *Elovl8b,* respectively. Target elongase is represented in red, neighboring cross-species conserved genes are represented in colour. Genes related by teleost genome duplication are underlined. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

gene has been termed "*elovl6-like*", an extended analysis of the locus with the identification of the *nfkb2, glud1, shld2* and *psd* gene, suggests that it may correspond to an *elovl3* 3R duplicate (Fig. S1). The investigation of the corresponding locus in tambaqui, *A. mexicanus* and *P. nattereri* revealed that this locus has split and subsequently rearranged, thus we were unable to identify the corresponding duplicate in these species (Fig. S1). Nevertheless, poor genome assembly or coverage may underlie the non-identification of this duplicate in these species.

3.3. Gene expression of elovl in juveniles from different sexes

We identified 10 *elovl*-like gene transcripts in the transcriptome of sexually undifferentiated tambaqui juveniles, including *elovl1b, elovl2, elovl3, elovl4a, elovl4b, elovl5, elovl6, elovl7b, elovl8a* and *elovl8b* (Fig. S2). The *elovl1a* transcripts, which were not detected in undifferentiated juveniles, were detected in ovary and testis. However, no *elovl7a* transcripts were found in undifferentiated juveniles and gonad tissues. Among PUFA elongases, *elovl5* transcripts were significantly more abundant in males (MLG) than females (FLG), $P < 0.0001$ (Fig. 5). Despite the variation among male individuals, *elovl2* levels were significantly higher ($P < 0.01$; Mean 36.82 ± 20.23) than in the FLG (Mean $= 1.252 \pm 0.47$) (Fig. 5). At the baseline, both *elovl4* duplicates and *elovl8a* reached the lowest levels in both sex groups. In addition, the *elovl4b* was detected exclusively in FLG, while *elovl8a* was detected exclusively in MLG (Fig. 4). Otherwise, at the gonadal level, *elovl4a* transcripts were the most abundant in ovary and testis, in comparison to *elovl2* and *elovl5*. No *elovl8a* transcripts were detected in the ovary and testis transcriptome (Fig. 5). Among Elovl with affinity toward SFA and MUFA, *elovl1b* was the most abundant in both sex groups and displayed differential expression ($P < 0.003$), with *elovl3* ($P < 0.01$), *elovl6* ($P < 0.0001$) and *elovl8b* ($P < 0.001$) transcripts being more abundant in males (Fig. 4). Transcripts of *elovl7b*, despite the variation within female individuals, were more abundant in FLG (Mean $= 6.327 \pm 6.309$) than MLG (0.706 ± 0.16). In contrast, at the gonad level, the *elovl7b* was overexpressed in ovary (TPM $= 156.56$) and testis (TPM $= 183.13$).

4. Discussion

Recent comparative genomic studies have addressed the gene repertoire of the *elovl* gene family, mainly those that participate in the

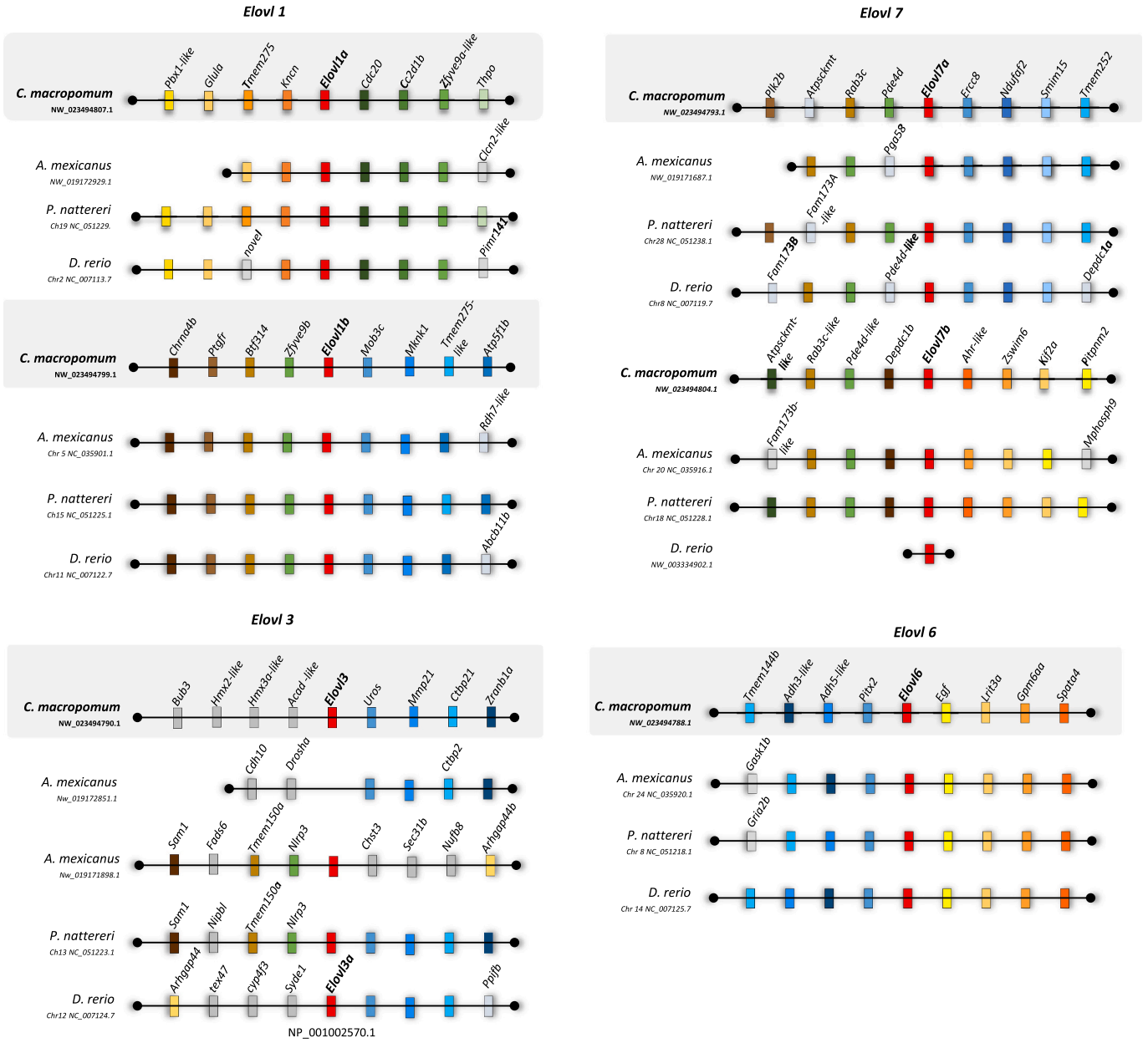

Fig. 4. Gene annotation of *Colossoma macropomum* genomic scaffold and comparative synteny maps. Synteny maps of gene involved in MUFA and SFA biosynthesis *Elovl1a, Elovl1b, Elovl7a, Elovl7b, Elovl3* and *Elovl6*, respectively. Target elongase is represented in red, neighboring cross-species conserved genes are represented in colour. Genes related by teleost genome duplication are underlined. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

LC-PUFA biosynthesis in chordates (e.g. Castro et al., 2016; Monroig et al., 2018). Overall, various approaches have highlighted the role of whole genome duplications, specifically expanding the *elovl* gene catalog (Oboh et al., 2017a; Kabeya et al., 2015). For example, studies on the biosynthesis of LC-PUFA indicate that *elovl2* and *elovl5* emerged after genome duplication, from a common *elovl2/5* of the invertebrate chordate amphioxus (Monroig et al., 2016). Here, we identified *elovl* 1–8 gene orthologs in the tambaqui genome. Phylogenetic analysis confirmed that the all *elovl* genes identified in tambaqui genome are orthologous to the corresponding *elovl* identified in fish and mammalians. Our approach further recovered the recently described *elovl8* in teleosts (Li et al., 2020; Sun et al., 2021). Sequence alignment of tambaqui Elovl8 with Elovl8 from *D. rerio* showed a high degree of conservation, confirming that the identified genes are true *elovl8* gene orthologs (Fig. 2).

Specifically, *elovl2* and *elovl5* have already been studied in tambaqui, including their functional characterization (Ferraz et al., 2019). Their encoded enzymes have well demonstrated functions in the LC-PUFA synthesis from C_{18} PUFA precursors. Similarly, both *elovl4* paralogs (*a* and *b*) were previously characterised from tambaqui (Ferraz et al., 2020), displaying elongase activities consistent with their participation in the biosynthesis of VLC-PUFA (Kabeya et al., 2015; Li et al., 2017; Yan et al., 2017; Oboh et al., 2017a; Jin et al., 2017; Zhao et al., 2019; Betancor et al., 2020). In the present study, we further identified the duplication of three other genes in tambaqui, more specifically *elovl1*, *elovl7* and *elovl8*. Phylogenetic analyses grouped the newly identified sequences into the corresponding clade with other teleost *elovl* sequences. The strongest evidence came from the conserved synteny analysis, where *elovl1a*, *elovl1b*, *elovl7a*, *elovl7b*, and *elovl8a* are conserved in the same position as in other teleosts. In vivo and in vitro

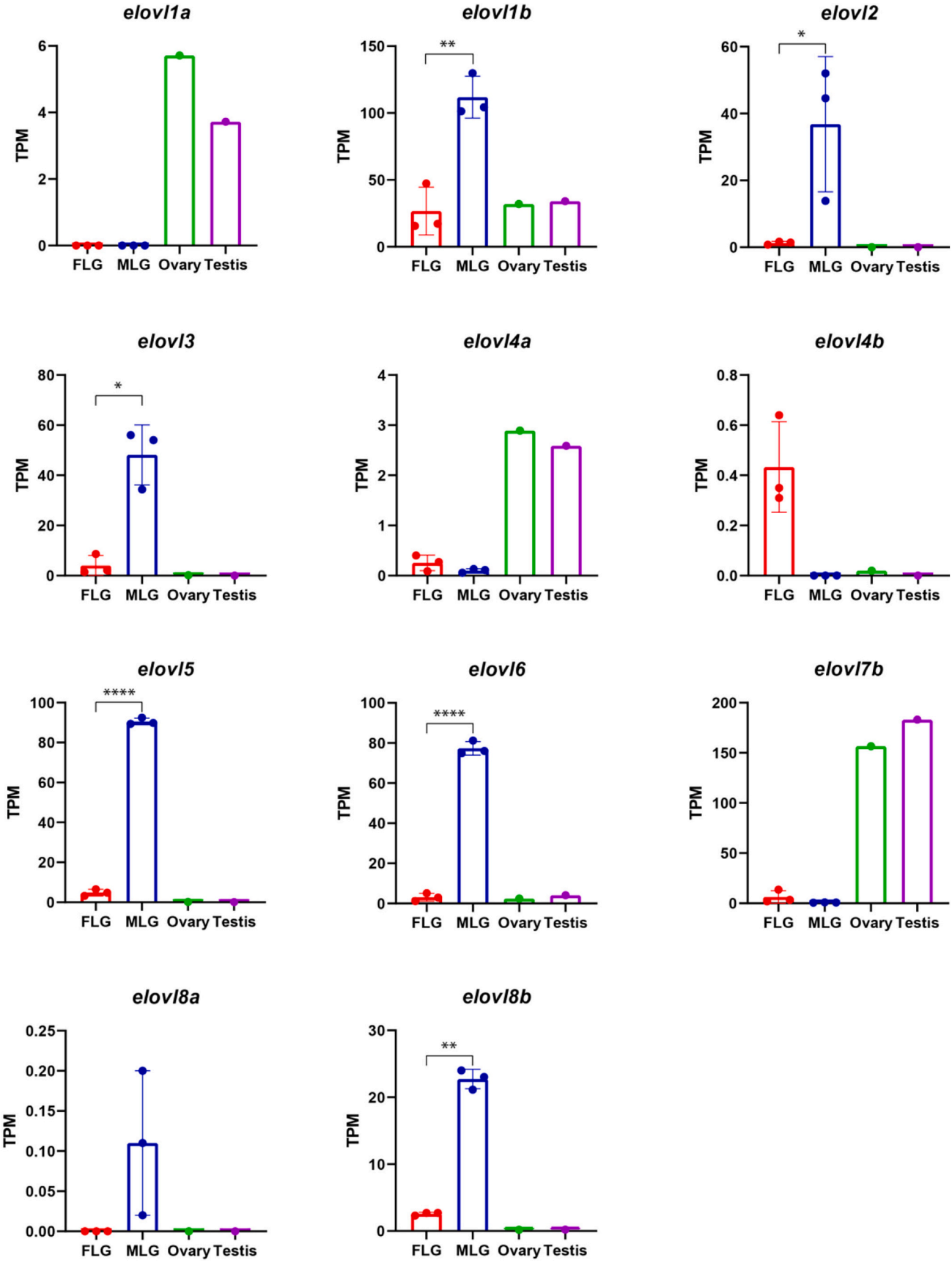

Fig. 5. Gene expression analysis. (B) Expression pattern of *elovl* genes presented as bar graphs with individual expression values displayed as dots. Asterisks denote statistical significance for the differential expression between Female Like Group (FLG) and Male Like Group (MLG). **** = $P < 0.0001$; ** = $P < 0.001$; * = $P < 0.05$.

experiments with fish species confirmed that *elovl8a* activity was specific to C18– C20 PUFAs, whereas *elovl8b* activity was specific to 18:0 and the 20:1 MUFA. These results suggested that *elovl8a* has similar functions compared to *elovl4* and *elovl5*, while *elovl8b* shares some functions with *elovl1*, *elovl3*, and *elovl7* (Li et al., 2020; Oboh, 2018; Sun et al., 2021). Here, the deduced aa sequence of the Elovl8 isoforms from tambaqui contain all the features of the vertebrate Elovl protein family members, including ER retrieval signal at the C terminus containing lysine (K) residues, and the diagnostic histidine box (HXXHH) (Jakobsson et al., 2006). Moreover, the histidine (H) box and its N-terminal side (QLTFLHVYHH) show a typical aa pattern of the PUFA elongase subfamily of eukaryotic elongases, with a glutamine (Q) at position −5 and a leucine (L) at position −1 from the first H (Hashimoto et al., 2008). This similarity suggests that, like the Elovl8 isolated in *S. canaliculatus*, tambaqui Elovl8 plays a role in the LC-PUFA cascade, thus contributing to the biosynthesis of VLC-PUFA. Interestingly, these results suggest a flexibility of tambaqui regarding the endogenous capacity of PUFAs biosynthesis, which fits with the species trophic level. The Amazonian fish species Tambaqui has natural habitat with variable dietary source, depending on the life stage and time of the year (Oliveira et al., 2006). The Amazon River is a complex system, with the natural floods and droughts. Therefore, tambaqui exploits the diverse resources exhibiting different feeding habits, impacting his ability to use both animal and plant food sources (Silva et al., 2000). Since the feed varies, the constituents in the FA profile must also shift, changing the biosynthesis of LC-PUFA. Therefore, it is very likely that the retention of all *elovls* and the maintenance of their activities in LC-PUFA biosynthesis pathway in tambaqui during the evolutionary process allows them to explore all these ecological niches.

The functional mapping of the *elovl* genes in sexually undifferentiated juvenile tambaqui transcriptomes revealed that this species expresses all the enzymatic arsenal required to elongate SFA, MUFA (*elovl1b*, *elovl3*, *elovl6*, *elovl7b* and *elovl8b*), PUFA including LC-PUFA (*elovl5*, *elovl2*) and VLC-PUFA (*elovl4a* and *elovl4b*) substrates prior to gonadal differentiation. The sex bias differential expression of certain *elovl* genes, namely *elovl2*, *elovl3*, *elovl*5, *elovl6* and *elovl8b*, in undifferentiated tambaqui indicates striking differences in FA biosynthesis capability at this stage. The elevated *elovl5* transcript abundance in both sex groups may reflect a high activity toward elongation of LA (18:2n-6) and ALA (18:3n-3), which results in efficient synthesis of ARA (20:4n-6) and EPA (20:5n-3), respectively. ARA, DHA and EPA, are selectively transferred and conserved, indicating the crucial role of these fatty acids during sexual maturation of fish species (Li et al., 2018). For example, ARA has been reported to play an essential role in steroidogenesis of Atlantic Salmon during testicular maturation, in which ARA likely stimulates sex steroid production via increased gonadal *star* expression and cholesterol transport to the mitochondrial steroidogenesis pathway (Bogevik et al., 2020). Similarly, in tambaqui, the differentiating males present high expression of genes involved in the biosynthesis of steroid hormones (including the synthesis of testosterone; Lobo et al., 2020).

Another possible role of Elovl on differentiating testis is the fundamental function of lipids and cholesterol for the Sertoli cell activity and testis cellular modelling in vertebrates (reviewed in Shi et al., 2018). In mouse, in vitro studies showed that ARA is especially important in membranes of testicular Leydig cells for improved cholesterol transport (Castillo et al., 2006). DHA is also required for cellular membrane structure and function, as they are integral elements of phospholipids that are fundamental components of lipid bilayers (Tocher, 2010). Knockout zebrafish models for *elovl2* and *elovl*5 revealed that Elovl2 dominantly mediates elongation from EPA to DHA in teleost fish (Liu et al., 2020). Additionally, the deficiency of DHA in the *elovl2*−/− zebrafish females resulted in an all-male offspring (Sun et al., 2021), evidencing the critical role of endogenouos n-3 PUFAs in fish sex differentiation and gamete quality. In tambaqui differentiating males, the transcript abundance of *elovl2* was significantly higher than females. The significance of a sex bias endogenous production of LC-PUFAs during tambaqui differentiation and elucidating whether some *elovl* support male development via ARA-androgen stimulation, are very intriguing questions that remain to be investigated, as well as the integration of additional gene expression analysis including qPCR (Betancor et al., 2017).

The sex biased *elovl*6 transcript abundance detected in tambaqui juvenile trunks prior to sex differentiation ($P < 0.0001$) indicates different accumulation and, subsequently, overall balance of FA composition, which are commercially relevant and needs further investigation. Differential expression of *elovl6*, enzyme involved in elongation of SFA and MUFA, has been associated with hepatic and plasma sex differences in Sprague–Dawley rats SFAs/MFAs balance (Marks et al., 2013). Moreover, sex differences in concentration of SFA and MUFA has been reported in rodents (Burdge et al., 2008; Extier et al., 2010) and human (Marangoni et al., 2007) whole blood. Nevertheless, *elovl6*$^{-/-}$ zebrafish presented significant higher content of whole-body lipid and lower content of fasting blood glucose than WT (Wang et al., 2020).

Among Elovl with chain lengths of 18–24 carbons, *elovl1b* was the main transcript during tambaqui sex differentiation and together with *elovl3*, were significantly more abundant in males. Production of C20 and C22 Coenzyme As (CoAs) by Elovl1 is essential for C24 sphingolipid synthesis (Ohno et al., 2010). Knockout of *elovl1* causes defects in swim bladder and kidney development in zebrafish (Bhandari et al., 2016). Otherwise *elovl7b,* which was more abundant in undifferentiated females at basal levels compared to *elovl1* and *elovl3*, was the main *elovl* in the gonads.

In conclusion, we have shown that tambaqui has all the elongases previously described in fish. A detailed phylogenetic and synteny analysis was carried out to confirm the conservation and synteny of these genes compared to other species. We confirmed that there are four duplicated genes in Elovl family in tambaqui, namely *elovl1a / elovl1b, elovl4a / elovl4b, elovl7a / elovl7b,* and *elovl8a / elovl8b*, probably resulting from the specific genome duplication occurred in teleosts. We also identified the novel member of the Elovl family (Elovl8) in tambaqui. Along to the previously characterised fatty acyl desaturase (*fads2*) and elongases (*elovl2*, *elovl4* and *elovl5*), the existence of Elovl8 ensures even more the full capacity for LC-PUFA biosynthesis from C_{18} PUFA in tambaqui. Finally, we demonstrate for the first time, a possible involvement of *elovl* in the sex differentiation of a teleost species.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpb.2022.110749.

Declaration of Competing Interest

The authors have no competing and conflicting of interests to declare.

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Supplementary material

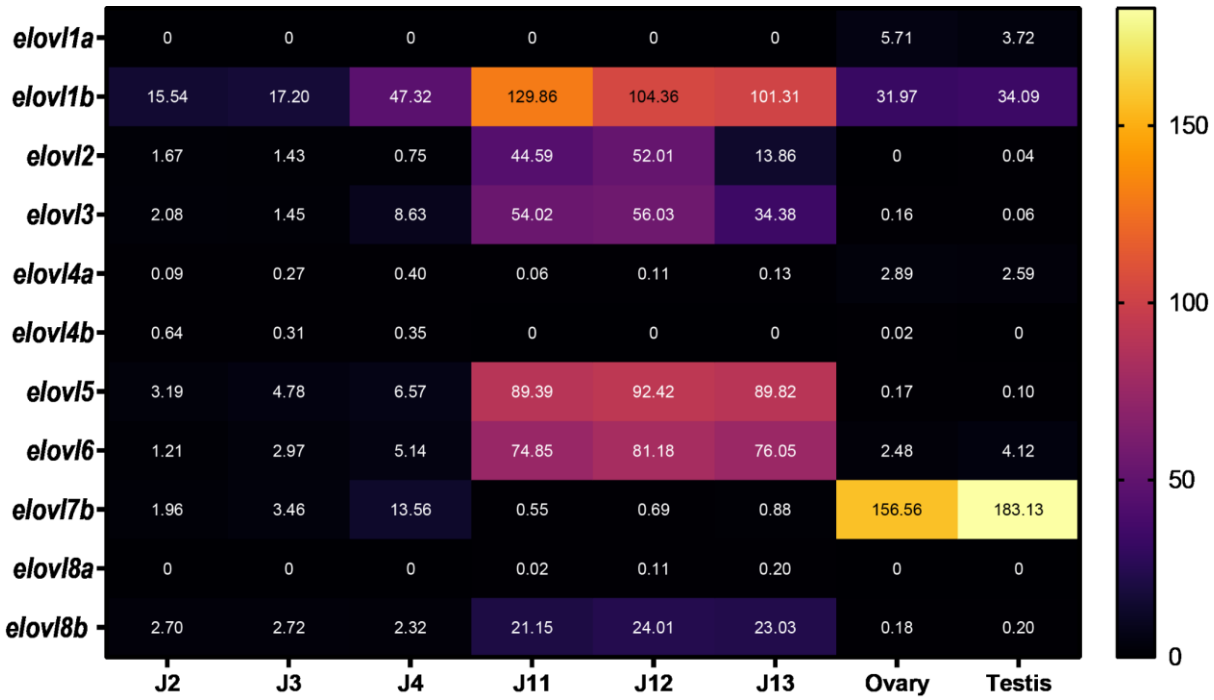

Figure S1: Heat map illustrating RNA-Seq differential expression data of elovl genes between tambaqui female-like group (FLG; J2, J3, J4) and male-like group (MLG; J11, J12, J13) at sex undifferentiated stage, and differentiated gonads (ovary and testis). The values are displayed as Transcripts Per Kilobase Million (TPM).

CONCLUSÃO

Este trabalho caracterizou os aspectos moleculares relacionados à biossíntese e regulação de estrógenos e biossíntese de ácidos graxos durante a diferenciação sexual em tambaqui, que tem sido reportada como crucial para o desenvolvimento ovariano em peixes no geral. No primeiro capítulo, realizamos uma revisão sobre as vias gênicas envolvidas no processo de diferenciação sexual de teleósteos, possibilitando a identificação e mapeamento de mecanismos de determinação sexual e diferenciação sexual a nível molecular baseados nas tecnologias de nova geração. A partir disso, foi possível observar que além da variabilidade de genes envolvidos no topo da cascata molecular da determinação sexual, os genes envolvidos na diferenciação sexual também variam substancialmente a nível de expressão gênica. Além disso, variações na função dos genes parálogos envolvidos na biossíntese de estradiol, *cyp19a1a* (aromatase gonadal) e *cyp19a1b* (aromatase cerebral), evidenciados tanto por análises expressão gênica quanto pela inativação dos genes, confrontam o papel crucial do estradiol na diferenciação sexual em espécies não modelo como o tambaqui, frente ao que vem sendo classicamente reportado para a maioria das espécies de peixes. No capítulo II, a identificação da ausência de um dos parálogos em peixes Otocephala, revelados pela primeira vez, mostra que as aromatases podem ter destinos evolutivos distintos, além da sub funcionalização, nos diferentes táxons após a duplicação do genoma inteiro específico dos teleósteos. Além disso, os padrões não convencionais de expressão gênica das aromatases detectados em tambaqui, sugerem que a versão cerebral (*cyp19a1b*) tenha um papel importante nos estágios iniciais do desenvolvimento, regulado pela expressão do receptor de estrógeno beta (*esr2a*). No entanto, o perfil de expressão da aromatase gonadal em tambaqui, tanto nos estágios iniciais quanto em adultos, assim como a ausência de efeito dos inibidores de aromatase na razão sexual, apontam para uma mudança na expressão de ovário-específico para testículos e ovários nesta espécie, que pode estar relacionada com as drásticas variações na região regulatória decorrente da ação de transposons, como diferenças no tamanho da sequência intergênica, baixa conservação de genes vizinhos e ausência do sítio de regulação correspondente ao receptor órfão SF-1, principal fator de transcrição da aromatase nas gônadas. Adicionalmente no capítulo III, mostramos que a expressão diferencial de genes relacionados à biossíntese de ácidos graxos da família gênica *elovl-like*, mais expressos em machos no transcriptoma de juvenis sexualmente indiferenciados, corrobora com o possível papel das aromatases na diferenciação sexual de machos em tambaqui. Embora a relação entre função reprodutiva e metabolismo lipídico tenha sido bem estudada, os efeitos do metabolismo lipídico na determinação e diferenciação do sexo são pouco compreendidos. Nossos achados sugerem um papel do metabolismo de Elovl e ácidos graxos na diferenciação sexual do tambaqui. Dessa forma. pode-se esperar que o conceito de mudanças no metabolismo lipídico que afetam o sexo contribua grandemente para o controle artificial do sexo nesta espécie de grande importância econômica. No geral, esta tese contribui para o entendimento dos aspectos moleculares da diferenciação sexual em espécies neotropicais de água doce, com informações que poderão ser utilizadas como pontos de referência em estudos comparativos futuros.