Transcriptome Sequencing and Analysis of Male Fourfinger Threadfin, *Eleutheronema tetradactylum* (Shaw, 1804)

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Abstract

Fourfinger threadfin, *Eleutheronema tetradactylum* (Shaw, 1804), is one of the country's most popular table fish species. This is a priority species for mariculture in India. Reproductive biology, life cycle, culture, and management are some budding research areas, while there is a lacuna in the species' genetic information. Using Illumina next-generation sequencing technique, 82,072 mRNA transcripts in the mature testis of fourfinger threadfin were obtained. The present study attempted to understand the expression of genes that facilitate sex determination, sex differentiation and gonadal maturation. From 82,072 transcripts, 50,943 were predicted by comparing them to proteins in UniProt using BLASTX. A total of 41 genes involved in sex determination and sex differentiation, spermatogenesis, steroid receptors in testis, steroidogenesis and gonadotropin-releasing hormone (GnRH) regulation are reported for the first time in *E. tetradactylum*. The entire raw sequencing reads of transcripts were made available in NCBI under BioProject PRJNA770837.

Keywords: fourfinger threadfins, Polynemidae, next-generation sequencing, *de novo* assembly

Introduction

Fourfinger threadfin, *Eleutheronema tetradactylum* (Shaw, 1804), is a perciform fish of the family Polynemidae with four pectoral filaments. It is also commonly known as blue threadfin, tassel fish and locally as Indian salmon. Fourfinger threadfin is predominantly seen in the shallow muddy bottom of the coastal waters, whereas the juveniles are known to occur in estuaries (Nesarul et al., 2014). They are widely distributed from the Persian Gulf to the west coast of India and Sri Lanka, from the east coast of India and the Andaman Islands to Penang, Thailand, Malacca, China, Taiwan, the Philippines, and North and West Australia (Fischer and Bianchi 1984, Hena et al., 2011). In Indian waters, *E. tetradactylum* dominated the Mumbai coast, Chilka Lake in Orissa and Hooghly-Matlah estuary of West Bengal (Barman and Mishra, 2010).

Numerous researchers have considered this an economically valid species (Fischer and Bianchi, 1984; Rainboth, 1996, Nesarul et al., 2014, Qu et al., 2020). Fourfinger threadfin was reported as the second-largest target species group for Northern Australia's net fisheries (Welch, 2010). Central Marine Fisheries Research Institute (CMFRI), Kochi, prioritised *E. tetradactylum* for mariculture in India in 2017 (Ranjan et al., 2017). Small villages along the coast of Pulicat, Tamilnadu, India, such as Old Pulincheri, Ellaikallu, Palaya Arangam, Nadukuppam and Vairavankuppam serve as a potential fishing ground for the species (Vinoth, 2014). Such a vital table fish species was classified as endangered in the Persian Gulf by IUCN in 2015 (Motomura et al., 2015). Listing a commercially important fish in the IUCN red list might be due to the lack of adequate information on reproductive biology and the life cycle of *E. tetradactylum*, which eventually are the limitations in both culturing and conserving them. Henceforth the research on biology, culture and management started budding. Currently, considerable

information is available on the breeding biology (Nesarul et al., 2014) of *E. tetradactylum* in Indian waters, while the exploration of the genetic profile of the species is scarce.

In recent years advances in RNA and DNA sequencing approaches have contributed to significant findings in organism physiology, phylogeny and ecology (Miller and Maclean, 2008; Miller et al., 2014; Alvarez et al., 2015; De Wit et al., 2015; Evans, 2015). Next-generation sequencing (NGS) is one of the common platforms that aid in sequencing all RNA transcripts, generating the transcriptome data. The transcriptome library serves as a pool of information that can be accessed to explore for answering biological questions.

Over the past decade, studies using RNA-Seq technologies in both model species and commercially important fish species have increased drastically (Qian et al., 2014). Transcriptome analysis in the gonads of various marine and freshwater fishes has been reported over the past few years. These marine species include tongue sole *Cynoglossus semilaevis* (Günther, 1873) (Lin et al., 2021a), yellowfin seabream *Acanthopagrus latus* (Houttuyn, 1782) (Li et al., 2020), Chinese sturgeon, *Acipenser sinensis* (Gray, 1835) (He et al., 2020), silver sillago, *Sillago sihama* (Forsskål, 1775) (Tain et al., 2019), Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) (Tao et al., 2018 and Tang et al., 2019) and bastard halibut *Paralichthys olivaceus* (Temminck & Schlegel, 1846) (Zhang et al., 2016). Next-generation sequencing data of freshwater fishes such as common carp, *Cyprinus carpio* (Linnaeus, 1758) (Liu et al., 2021), Hong Kong catfish, *Clarias fuscus* (Lacepède, 1803) (Lin et al., 2021b), blotched snakehead, *Channa maculata* (Lacepède, 1801) (Ou et al., 2020) and Siberian sturgeon, *Acipenser baerii*. (Brandt, 1869) (Klopp et al., 2020) are available.

The present study aimed to construct a transcriptome library for the mature testis of *E. tetradactylum* using Illumina HiSeq X Ten to bridge the gap between the species' reproductive biology and genetic information. On constructing the transcriptome library, the study attempted to identify the gene involved in sex determination, sex differentiation, spermatogenesis, steroid receptors in testis, steroidogenesis and GnRH regulation.

Materials and Methods

Sampling of tissue

Live fish were collected with the help of local fishers at Vairavan Kuppam (13.3241°N, 80.1673°E). It is a small village or hamlet in Pulicat (Pazhaverkadu), Thiruvallur district, Tamil Nadu, India. The fish were brought to the laboratory with the support of aerators and their gonad was dissected in the ice-cold environment and immediately stored in RNAlater (Sigma Aldrich, USA).

RNA isolation, cDNA library construction and Illumina sequencing

Total RNA was isolated from 100 mg of the testis sample using TRIzol (Thermo Fisher Scientific, USA), a ready-to-use reagent to isolate high-quality total RNA. The quality of the RNA was checked using the 2200 TapeStation system (Agilent Technologies, USA), and the concentration of RNA was checked using Qubit 3.0 fluorometer (Thermo Fisher Scientific, USA). mRNA sample with RNA Integrity Number (RIN) value 7.4 was used to prepare Illumina sequencing libraries. Library preparation of the sample was performed using 1 µg of total RNA with the help of TruSeq RNA Library Prep Kit v2 (Illumina, USA). The steps involved in library preparation were (1) mRNA purification and fragmentation where the polyA containing mRNA molecules were separated using magnetic bead technology. (2) Synthesis of the first strand of cDNA using random hexamers and random primers, followed by the second strand of cDNA. (3) The overhangs of ds cDNA were cut by 3' to 5' exonuclease activity to form blunt ends using an end-repair mix. (4) The trimmed cDNA was adenylated at the 3' end to prevent ligation of fragments, followed by adapter ligation to provide complementary overhanging. (6) DNA fragments with the adapter molecule at both ends were selectively amplified. (5) Amplified DNA was sequenced on the Illumina HiSeq X Ten sequencing platform at Agrigenome Labs Pvt. Ltd., Kerala, India. After the sequencing run, Illumina RNA-Seq data was processed to generate FASTQ files.

Analysis using bioinformatics

The bioinformatics analysis pipeline for the reference-free *de novo* assembly comprises the following steps.

Data pre-processing

Raw reads that were obtained from the sequencer are stored in FASTQ. The pre-processing procedure includes adapter sequence removal, quality filtering and trimming. The AdapterRemoval-V2 tool was applied for adapter sequence removal and quality filtering (Schubert et al., 2016). Only the raw reads with a quality score of at least Q30 were retained for further analysis. Further, rRNA sequences were removed from the library by mapping with the SILVA database, a comprehensive database of ribosomal sequences.

De novo assembly

De novo assembly was performed using Trinity programme, followed by clustering and generating a list of unique transcripts using CD-HIT-EST (Li and Godzik, 2006). The unique transcripts obtained were further filtered by identifying the coding regions within the transcripts using Transdecoder (Haas et al., 2013). Thereby more valid transcripts were used for the analysis.

Transcriptome annotation

The following steps annotated the predicted genes.

Matching with the UniProt database using the BLASTX program

The transcripts were compared to the UniProt database using the BLASTX program with an E-value cutoff of 10^{-3}. The best BLASTX hit based on query coverage, per cent identity, similarity score and description of each gene was filtered out. For instance, 70 % query coverage and 60 % identity were used as a threshold for obtaining the qualified unique transcripts.

Gene ontology annotation

Gene ontology (GO) annotation was performed using UniProt Knowledgebase through in-house custom scripts and the functionally annotated transcripts were grouped into three categories, namely molecular function (MF), cellular component (CC) and biological process (BP).

Results

Reads assembly

Raw reads obtained from the sequencer were 13,539,970 paired-end reads with 150 bp mean read length. After pre-processing, 9,805,495 clean paired-end reads were assembled, resulting in 162,452 transcripts. After generating unique transcripts by CD-HIT-EST, a total of 113,016 transcripts were available for checking the coding regions. The software transdecoder filtered the transcripts resulting in 82,072 unique transcripts with potential coding regions. At the end of the assembly process, 82,072 transcripts were available to prepare the RNA-seq data library of the testis sample in fourfinger threadfins, *E. tetradactylum*. Among them, the smallest transcript was 201 base pairs (bp) in length and the largest was 15,395 bp in length. There were about 13 transcripts with more than 10000 bp and 16,648 transcripts with more than 1000 bp in length.

Gene annotation

BLASTX predicted the function of 50,943 out of 82,072 transcripts. The annotated transcripts were categorised based on the result of GO analysis. The possible functions of the annotated transcripts were clustered into three main categories: cellular component, molecular function, and biological processes (Mehinto et al., 2012). The GO annotation showed that 53,834, 40,426, and 39,869 GO terms belonged to molecular function, biological process, and cellular components categories, respectively. The genes involved in the 'integral component of membrane' were the most commonly assigned GO terms in the cellular component category, followed by the genes involved in the 'ATP binding' in the molecular function and the genes involved in 'signal transduction' for the 'biological processes' category, respectively.

Identification of genes

From the transcriptome library of *E. tetradactylum*, genes involved in sex determination and sex differentiation, spermatogenesis, steroid receptors in testis, steroidogenesis and GnRH regulation were identified in *E. tetradactylum* genome for the first time (Table 1).

List of genes potentially have differential expression in testis

Because only mature testes were analysed, no differential expression analysis could be performed to identify the sex-specific genes. However, the list of differentially expressed genes in the testis could be generated by collecting information from other fish species' reports (Wang et al., 2017; Boonanuntanasarn et al., 2020; Li et al., 2020). Thus, the list of expressed genes in this study that was shown to be differentially expressed in other fish species' testis is shown in Table 2.

Data availability in NCBI

The raw sequencing reads of transcripts are available in NCBI sequence read archive (SRA) under accession SRS10547193 as part of BioProject PRJNA770837.

Discussion

Next-generation sequencing

Ismail et al. (2019) performed the whole genome sequencing in three tissue samples excised from the caudal peduncle of *E. tetradactylum*, which resulted in 8,390,317; 7,085,775 and 8,461,589 raw reads, a total of 30,209; 25,107 and 29,943 genes were annotated against the NCBI non-redundant nucleotide sequence database. While the genomic analysis from muscle tissue of *E. tetradactylum* studied by Qu et al. (2020) resulted in 37,683 protein-coding genes. In comparing the statistical data of the previous sequencing experiments of *E. tetradactylum*, the present study had more raw reads (9,805,495) and the annotated gene (50,943). Though the studies were carried out in *E. tetradactylum*, the higher number of raw reads in the present study probably suggested a higher breadth of coverage, which generated a higher number of protein-coding genes than in the previous studies. Due to the unavailable reference genome sequence, the *de novo* assembly of transcripts was applied to analyse transcriptome data obtained in the present study.

Table 1. Genes identified from the transcriptome library of the mature testis in *Eleutheronema tetradactylum*.

Gene name	Seq ID	Protein name	GO terms	GO category
I. Sex determination and sex differentiation				
dmrt3	TRINITY_DN71501_c0_g1_i1	Doublesex and mab-3 related transcription factor 3	Biological	regulation of transcription, DNA-templated [GO:0006355]
			Molecular	metal ion binding [GO:0046872]; sequence-specific DNA binding [GO:0043565]
			Cellular	nucleus [GO:0005634]
dmrta1	TRINITY_DN78182_c0_g1_i1	DMRT like family A1 DMRTA1/ Doublesex- and mab-3-related transcription factor 1-like	Biological	regulation of transcription, DNA-templated [GO:0006355]
			Molecular	metal ion binding [GO:0046872]; sequence-specific DNA binding [GO:0043565]
			Cellular	nucleus [GO:0005634]
dmrta2	TRINITY_DN48905_c0_g1_i1	DMRT like family A2	Biological	regulation of transcription, DNA-templated [GO:0006355]
			Molecular	metal ion binding [GO:0046872]; sequence-specific DNA binding [GO:0043565]
			Cellular	nucleus [GO:0005634]
dmrt2 like	TRINITY_DN19487_c0_g1_i2	Doublesex- and mab-3-related transcription factor 2-like	Biological	regulation of transcription, DNA-templated [GO:0006355]
			Molecular	metal ion binding [GO:0046872]; sequence-specific DNA binding [GO:0043565]
			Cellular	nucleus [GO:0005634]
dmrt2a	TRINITY_DN19487_c0_g1_i1	Doublesex and mab-3 related transcription factor 2a	Biological	apoptotic process [GO:0006915]; determination of left/right symmetry [GO:0007368]; regulation of transcription, DNA-templated [GO:0006355]
			Molecular	metal ion binding [GO:0046872]; sequence-specific DNA binding [GO:0043565]
			Cellular	integral component of membrane [GO:0016021]; nucleus [GO:0005634]
dmrta2	TRINITY_DN78970_c0_g1_i1	Doublesex-and mab-3-related transcription factor A2	Biological	regulation of transcription, DNA-templated [GO:0006355]
			Molecular	metal ion binding [GO:0046872]; sequence-specific DNA binding [GO:0043565]
			Cellular	nucleus [GO:0005634]
sox 5	TRINITY_DN38486_c0_g1_i1	SRY-box transcription factor 5		
foxl2	TRINITY_DN50720_c0_g1_i1	Forkhead box B1; Forkhead box protein B2	Molecular	DNA-binding transcription factor activity [GO:0003700]; sequence-specific DNA binding [GO:0043565]
			Cellular	nucleus [GO:0005634]
foxj3	TRINITY_DN99881_c0_g1_i1	Forkhead box protein J3-like	Molecular	DNA-binding transcription factor activity [GO:0003700]; sequence-specific DNA binding [GO:0043565]
			Cellular	nucleus [GO:0005634]

Table 1. Continued.

Gene name	Seq ID	Protein name	GO terms	GO category
amh	TRINITY_DN6868_c0_g1_i1	Anti-Müllerian hormone	Biological	gonad development [GO:0008406]
			Molecular	growth factor activity [GO:0008083]
			Cellular	extracellular region [GO:0005576]
amhr2	TRINITY_DN748_c1_g1_i1	Anti-Müllerian hormone receptor type II	Molecular	ATP binding [GO:0005524]; transmembrane receptor protein serine/threonine kinase activity [GO:0004675]
			Cellular	integral component of membrane [GO:0016021]
II. Spermatogenesis				
piwil1	TRINITY_DN3074_c0_g1_i1	Piwi like RNA-mediated gene silencing 1	Biological	anatomical structure morphogenesis [GO:0009653]; gene silencing by RNA [GO:0031047]; piRNA metabolic process [GO:0034587]; regulation of translation [GO:0006417]; spermatid development [GO:0007286]
			Molecular	piRNA binding [GO:0034584]
			Cellular	P granule [GO:0043186]
sycp1	TRINITY_DN498_c0_g1_i2	Synaptonemal complex protein 1	Biological	synaptonemal complex assembly [GO:0007130]
			Cellular	synaptonemal complex [GO:0000795]
sycp2	TRINITY_DN35_c0_g1_i21	Synaptonemal complex protein 2 isoform X1	Cellular	chromosome [GO:0005694]; nucleus [GO:0005634]
sycp3	TRINITY_DN867_c0_g1_i11	Synaptonemal complex protein 3	-	
odf2	TRINITY_DN1183_c0_g2_i1	Outer dense fiber protein 2 isoform X1	Cellular	cell projection [GO:0042995]; cytoplasm [GO:0005737]; microtubule organising centre [GO:0005815]
III. Steroid receptors in testis				
er2a	TRINITY_DN75202_c0_g1_i1	Estrogen receptor 2a	Biological	cellular response to estradiol stimulus [GO:0071392]; intracellular estrogen receptor signalling pathway [GO:0030520]
			Molecular	estrogen receptor activity [GO:0030284]; nuclear receptor activity [GO:0004879]; sequence-specific DNA binding [GO:0043565]; steroid binding [GO:0005496]; zinc ion binding [GO:0008270]
			Cellular	nucleus [GO:0005634]
paqr3	TRINITY_DN10317_c0_g1_i4	Progestin and adipoQ receptor family member 3	Cellular	integral component of membrane [GO:0016021]
paqr9	TRINITY_DN110262_c0_g1_i1	Progestin and adipoQ receptor family member 9	Cellular	integral component of membrane [GO:0016021]
pgrmc1	TRINITY_DN532_c0_g1_i12	Membrane-associated progesterone receptor component 1	Cellular	integral component of membrane [GO:0016021]

Table 1. Continued.

Gene name	Seq ID	Protein name	GO terms	GO category
pgrmc2	TRINITY_DN63913_c0_g1_i2	Membrane-associated progesterone receptor component 2	Cellular	integral component of membrane [GO:0016021]
IV. Genes involved in steroidogenesis				
cyp17a1	TRINITY_DN77424_c0_g1_i1	17-alpha-hydroxyprogesterone aldolase	Biological	sex differentiation [GO:0007548]; steroid biosynthetic process [GO:0006694]
			Molecular	17-alpha-hydroxyprogesterone aldolase activity [GO:0047442]; heme binding [GO:0020037]; iron ion binding [GO:0005506]; steroid 17-alpha-monooxygenase activity [GO:0004508]
			Cellular	membrane [GO:0016020]
hsd3b1	TRINITY_DN7288_c1_g3_i5	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	Biological	steroid biosynthetic process [GO:0006694]
			Molecular	3-beta-hydroxy-delta5-steroid dehydrogenase activity [GO:0003854]
			Cellular	integral component of membrane [GO:0016021]
hsd11b1	TRINITY_DN57345_c0_g1_i1	Hydroxysteroid 11-beta dehydrogenase 1 like	Molecular	oxidoreductase activity [GO:0016491]
V. Genes regulating GNRH				
fstl3	TRINITY_DN84750_c0_g1_i1	Follistatin like 3	-	
inha	TRINITY_DN101_c1_g1_i1	Inhibin alpha chain	Molecular	growth factor activity [GO:0008083]; hormone activity [GO:0005179]
			Cellular	extracellular region [GO:0005576]
inhba	TRINITY_DN80088_c0_g1_i1	Inhibin beta A chain-like	Molecular	growth factor activity [GO:0008083]; hormone activity [GO:0005179]
			Cellular	extracellular region [GO:0005576]

GO: Gene ontology.

Analysis of annotated genes

Among the sex-determining genes, *dmrt* is the first identified gene family known to have sex-determining functions in fish (Matsuda et al., 2002). Its male-specific upregulation has been observed in fishes, amphibians and reptiles (Raymond et al., 1998, Smith et al., 1999, Kettlewell et al., 2000, Shibata et al., 2002, Kobayashi et al., 2004). In the present study, genes of the *dmrt* family such as *dmrt3, dmrta2, dmrt2-like* and *dmrt2a* were identified. Other sex-determination genes were also found, including *amh, fox and sox5*. *amh* is involved in gonadal differentiation in male and female fish (Kluver et al., 2007). *foxl2* gene is predominantly involved in sex determination and sex reversal in females (Wang et al., 2007). The presence of *foxl2* in the testis sample can be justified as *E. tetradactylum* was reported to exhibit protandrous hermaphroditism in Indian waters (Shihab et al., 2017). *Sox5* has a crucial role in regulating fish germ cell development, and reported to regulate *dmrt1* (Schartl et al., 2018).

Genes involved in gametogenesis such as *piwil1, sycp1, sycp2, sycp3, odf2* are identified in the transcriptome library of the mature testis of *E. tetradactylum*. *piwil1* plays a vital role in germline specification in teleost (Yi et al., 2014). Synaptonemal complex genes (*sycp1, sycp2, sycp3*) were found to express in preleptotene stage of primary spermatocyte in zebrafish *Danio rerio* (Hamilton, 1822) (Ozaki et al., 2011) and *odf2* is a protein constituting the sperm cytoskeleton in mammals (Brohmann et al., 1997; Shao et al., 1997; Turner et al., 1997; Schalles et al., 1998). Moreover, *piwil1, sycp1, sycp2, sycp3, odf2* were reported to be the markers for spermatogenesis in protandrous yellowfin seabream

Table 2. List of differentially expressed genes of the mature testis in *Eleutheronema tetradactylum*.

Gene name	Seq ID	Protein name	GO terms	GO category
1. Protandrous yellowfin seabream (*Acanthopagrus latus* (Houttuyn, 1782)) - Testis (Li et al., 2020). *Tektin2* is expressed in spermatozoa and might be associated with sperm motility (Xiong et al., 2018). Radial spoke head protein (RSPH) family contributes to sperm motility in humans (Jeanson et al., 2015).				
tekt	TRINITY_DN122_c0_g2_i1	Tektin	Biological	cilium assembly [GO:0060271] cilium movement involved in cell motility [GO:0060294]
			Cellular	microtubule cytoskeleton [GO:0015630]; motile cilium [GO:0031514]
rsph3	TRINITY_DN4297_c0_g1_i3	Radial spoke head 3	Cellular	cell projection [GO:0042995]; cytoplasm [GO:0005737]; cytoskeleton [GO:0005856]
rsph1	TRINITY_DN460_c0_g1_i1	Radial spoke head component 1		
rsph14	TRINITY_DN235_c0_g1_i3	Radial spoke head 14 homolog		
fabp	TRINITY_DN1609_c0_g1_i1	Fatty acid-binding protein	Molecular	fatty acid binding [GO:0005504]
			Cellular	cytoplasm [GO:0005737]
2. Japanese puffer (*Takifugu rubripes* (Temminck & Schlegel, 1850)) - Testis (Wang et al., 2017) Protein Wnt acts as an additional regulator that influences the differentiation of mesenchymal precursors. Dmrt3 is highly expressed in the testis when compared with the ovary.				
fgf2	TRINITY_DN57063_c0_g1_i1	Fibroblast growth factor receptor substrate 2-like	Cellular	membrane [GO:0016020]
wnt10b	TRINITY_DN3214_c0_g1_i2	Protein Wnt	Biological	multicellular organism development [GO:0007275]; Wnt signaling pathway [GO:0016055]
			Molecular	signaling receptor binding [GO:0005102]
			Cellular	extracellular region [GO:0005576]
cyp11a1	TRINITY_DN275_c0_g1_i12	Cholesterol side-chain cleavage enzyme, mitochondrial; Cholesterol desmolase	Biological	C21-steroid hormone biosynthetic process [GO:0006700]; cholesterol metabolic process [GO:0008203]
			Molecular	cholesterol monooxygenase (side-chain-cleaving) activity [GO:0008386]; heme binding [GO:0020037]; iron ion binding [GO:0005506]
			Cellular	mitochondrial inner membrane [GO:0005743]
cyp11b	TRINITY_DN20921_c0_g1_i2	Cytochrome P450 family 11 subfamily b		
dmrta1	TRINITY_DN78182_c0_g1_i1	DMRT like family A1	Biological	regulation of gene expression epigenetic [GO:0040029]

Table 2. Continued.

Gene name	Seq ID	Protein name	GO terms	GO category
			Molecular	metal ion binding [GO:0046872]; NAD-dependent histone deacetylase activity (H3-K14 specific) [GO:0032041]
			Cellular	cytoplasm [GO:0005737]; histone deacetylase complex [GO:0000118]
dmrt3	TRINITY_DN71501_c0_g1_i1	Doublesex and mab-3 related transcription factor 3	Biological	regulation of transcription, DNA-templated [GO:0006355]
			Molecular	metal ion binding [GO:0046872]; sequence-specific DNA binding [GO:0043565]
			Cellular	nucleus [GO:0005634]
3. Snakeskin gourami (*Trichopodus pectoralis* Regan, 1910) ovary (Boonanuntanasarn et al., 2020) These testis-upregulated genes appear to be involved in spermatogenesis and sperm motility function.				
nbl1	TRINITY_DN100618_c0_g1_i1	Neuroblastoma suppressor of tumorigenicity 1	Biological	intracellular protein transport [GO:0006886]
			Molecular	small GTPase binding [GO:0031267]
nr2e3	TRINITY_DN5841_c0_g1_i1	Photoreceptor-specific nuclear receptor-like	Molecular	DNA-binding transcription factor activity [GO:0003700]; sequence-specific DNA binding [GO:0043565]; zinc ion binding [GO:0008270]
			Cellular	nucleus [GO:0005634]
pih1d2	TRINITY_DN558_c0_g1_i6	PIH1 domain containing 2	-	- -
fbxo36	TRINITY_DN11972_c0_g1_i5	F-box protein 36	-	- -
mybl2	TRINITY_DN97386_c0_g1_i1	MYB proto-oncogene like 1	Cellular	nucleus [GO:0005634]

GO: Gene ontology.

(*A. latus*) (Li et al., 2020).

Several members of the progestin and adipoQ-Receptor (*paqr*) family were identified as potential mediators of these non-genomic effects of progestins (Zhu et al., 2003a, b; Thomas, 2008) and the progestins are found to mediate various steps in sperm maturation (Ueda et al., 1985; Miura et al., 1991, 1992). As the sample of the present study was carried out in mature testis, the progestin receptors such as *paqr3, paqr9, pgrmc1 and pgrmc2* were expressed. In addition to the progestin receptors, mature testis of *E. tetradactylum* expressed *er2a* as it correlates with the previous findings of *Er2* protein expression in the Leydig cells of mature testis in rainbow trout (*Oncorhynchus mykiss* (Walbaum, 1792)) (Bouma et al., 2001) and estrogen has a functional role in the final stages of spermatogenesis in Japanese huchen *Hucho perryi* (Brevoort, 1856) (Amer et al., 2001). Along with the steroid receptors, the genes encoding for important enzymes involved in steroidogenesis, such as *cyp17a1*, *hsd3b1* and *hsd11b1*, that has a key role in gonadal maturation were expressed in the testis of *E. tetradactylum*. In fishes, *cyp17a1* is indispensable for male fertility (Yang et al., 2021), *hsd3b1* particularly converts pregnenolone to progesterone, thereby increasing the steroid products which mediate gonadal maturation (Ijiri et al., 2008) and *hsd11b1* is necessary for normal testicular development (Ozaki et al., 2006).

Inhibin has a paracrine regulation on testicular function (Mather et al., 1992), while follistatin (*fst*) plays a key role in gonadal development and the regulation of

gonadal function (Marchetti et al., 2003). The present study revealed that the expression of *fst* and inhibin in the mature testis of *E. tetradactylum* corresponded to the above findings.

Further, the entire study opens a vast area of research in validating the genes responsible for the sex determination, sex differentiation and gonadal maturation of *E. tetradactylum*.

Differentially expressed genes

Transcriptome analysis of testis, ovotestis and ovary in protandrous yellowfin seabream (*A. latus*), genes such as *tekt, rsph3, rsph1. rsph14 and fabp* were upregulated in testis (Li et al., 2020). Similarly, comparative RNA-Seq analysis of ovary and testis in Japanese puffer (*Takifugu rubripes* (Temminck & Schlegel, 1850)), *fgf2, wnt10b, cyp11a1, cyp11b, dmrta1, dmrt3* genes were differentially expressed in testis (Wang et al., 2017). The male gonads of snakeskin gourami (*Trichopodus pectoralis* Regan, 1910) had higher expression of genes such as *nbl1, nr2e3, pih1d2, fbxo36, mybl2* in the testis compared to the ovary (Boonanuntanasarn et al., 2020). All the genes discussed above were identified in the transcriptome library of *E. tetradactylum* mature testis. The presence of similar genes in the annotated transcriptome data of *E. tetradactylum* helps to presume that these may have a sex-specific role in the species.

Conclusion

These findings offer valuable information to increase the existing genomic resources of fourfinger threadfin, *Eleutheronema tetradactylum*. This is the first study reporting the list of genes potentially involved in sex determination, sex differentiation and gonadal maturation in the testis of *E. tetradactylum*. Identification of genes in major biological processes related to testis development and function leads to understanding the threadfin fishes at the molecular level, which will eventually open various research outbreaks in aquaculture and wild fisheries. During the crucial period of gonads' maturation, many genes involving sex determination, sex differentiation, and sex-associated traits would be differentially expressed, facilitating the organism's propagation. The present study is the first attempt to reveal sex-specific genes that limited exploration only in male gonads. The follow-up endeavour would be decoding the transcripts of the mature ovary and exploring the differential expression of sex-dependent and sex-specific genes during the gonads' maturation.

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