Whole-genome assembly and annotation of little yellow croaker (*Larimichthys polyactis*) provide insights into the evolution of hermaphroditism and gonochorism

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Abstract

The evolutionary direction of gonochorism and hermaphroditism is an intriguing mystery to be solved. The special transient hermaphroditic stage makes the little yellow croaker (*Larimichthys polyactis*, *L. polyactis*) an appealing model for studying the formation of hermaphrodites. On the other hand, as the most famous commercial fish species in East Asia, the origin and evolutionary relationship of *L. polyactis* and *Larimichthys crocea* remain unclear. Here, we report the genome sequence of *L. polyactis*, with a size of ~706 Mb (contig N50 = 1.21 Mb and scaffold N50 = 4.52 Mb) and 25,233 protein-coding genes. Phylogenomic analysis suggests that *L. polyactis* diverged from the common ancestor of *Larimichthys crocea* ~25.4 million years ago. Our high-quality genome assembly enabled comparative genomic analysis, which revealed a number of within-chromosome rearrangements and translocations without major chromosome fission or fusion events between the two species. The *dmrt1* gene was identified as the candidate sex determination gene in *L. polyactis*. The expression of *dmrt1* and its upstream regulatory gene *rnf183* were both sexually dimorphic in the transcriptome analysis. *Rnf183*, unlike its two paralogues *rnf223* and *rnf225*, is only present in *Larimichthys* but not in other teleost species, suggesting that it originated from a lineage-specific duplication or was lost in other teleosts. Phylogenetic analysis shows that the hermaphrodite stage in male *L. polyactis* may be explained by the sequence evolution of *dmrt1*. Decoding the *L. polyactis* genome not only provides insight into the genetic underpinnings of hermaphrodite evolution but also provides valuable information for enhancing fish aquaculture.

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Key Words: little yellow croaker, whole-genome sequence, gonadal transcriptome, *dmrt1*, *rnf183*, hermaphrodite evolution

Introduction

Due to the diversity of ecological niches in the water environment, fishes exhibit a variety of sex determination systems and reproductive patterns. Gonochorism and hermaphroditism are the most important reproductive

patterns in sexually reproducing organisms and occur commonly in teleost fish (Renner and Ricklefs, 1995; Jarne and Auld, 2006; Bachtrog et al., 2014; Tree of Sex consortium, 2014; Lau et al., 2016); in particular, hermaphroditism has been documented in more than 1,500 species (Sadovy de Mitcheson and Liu, 2008). In fact, males and females are determined by diverse mechanisms that evolve rapidly in fish, yet this diversity in primary sex-determining signals is coupled with conserved molecular pathways that trigger male or female development. The nine taxonomic orders of teleosts in which hermaphroditism has been documented are scattered widely across the phylogeny of teleost fishes, and no order consists solely of hermaphroditic species but instead also contains gonochoristic (separate-sex, or dioecious) species. Interestingly, across all groups, only Perciformes have all types of reproductive patterns and sex differentiation methods, including gonochorism, hermaphrodite protandry, hermaphrodite protogyny, and simultaneous hermaphroditism (Bachtrog et al., 2014; Tree of Sex consortium, 2014; Avise and Mank, 2009). Therefore, whole-genome sequencing of fishes with different reproductive patterns and sexual differentiation methods in Perciformes, especially genome-wide studies of transitional species, will be a powerful tool for the analysis of the evolution of hermaphroditism and gonochorism in fishes.

Little yellow croaker (*Larimichthys polyactis*, *L. polyactis*), also named the small yellow croaker or yellow corvina, belongs to the genus *Larimichthys*, family Sciaenidae and order Perciformes. It is a croaker native to the western Pacific and one of the most famous fish species with great economic importance in China, Korea, and Japan. Little yellow croaker (*L. polyactis*) was found to be a good model for investigating the direction of evolution of hermaphroditism and gonochorism due to its special sexual reproduction process, in which males undergo a male-specific transient hermaphroditic stage (Xie et al., 2021). Briefly, *L. polyactis* is a gonochoristic fish species. When they reach sexual maturity, the sexual functions of males and females are separated, and the sex ratio is close to 1:1. However, in the juvenile stage, EPOs (early primary oocytes) will appear and degenerate between 43 and 80 dph (days post hatching) in the testis, forming an intersex gonad. This unique pattern of testis differentiation is neither typical hermaphroditism nor gonochorism. Because hermaphroditism is extremely rare in the Sciaenidae family, and the closely related species *Larimichthys crocea* (*L. crocea*) is a differentiated gonochorist with XX/XY sex determination (You et al., 2012), we speculate that this gonadal differentiation pattern in *L. polyactis* evolved from a gonochoristic ancestor. As the most closely related species in *Larimichthys*, these species demonstrate two different reproduction systems.

The underlying molecular mechanisms warrant further study. This system may serve as a transitional type from gonochorism to hermaphroditism. The male-specific nature of this transient stage is also fascinating, as it is different from the zebrafish gonadal development process, in which all individuals of zebrafish need to experience the juvenile ovary stage, with no sex differences in morphology and histology (Takahashi, 1977; Orban et al., 2009; Lau et al., 2016). Therefore, whole-genome sequencing of *L. polyactis* is a necessary approach to determine the evolutionary divergence of sexual reproduction patterns at the genome level.

The abundance of wild *L. polyactis* has severely declined since the 1980s, and its commercial value has increased significantly due to overfishing, seawater pollution, and ocean current and water quality changes (Zhang et al., 2016; Han et al. 2010). Although the resources of *L. polyactis* have gradually recovered (Cheng et al. 2004), overfishing has caused sexual precocity, gender imbalance, and growing miniaturization in *L. polyactis* populations (Jin 1996; Lin and Cheng 2004). Therefore, artificial breeding techniques are urgently needed to restore fishery resources. In 2015, an artificial breeding programme was successfully established by artificial spawning of wild *L. polyactis* and larval culture (Chen et al. 2016; Liu et al., 2019; Xie et al., 2021). A previous report of a genetic linkage map and karyotype analysis revealed that *L. polyactis* is a diploid ($2n = 48$) (Liu et al., 2020; Park and Gil, 2019). A high-quality assembled genome of *L. polyactis* will provide the necessary toolkit for studies of genome structure and evolution in its reproductive patterns and sex differentiation methods. In this study, to elucidate the role of genomic architecture in hermaphrodite evolution, we produced a high-quality reference genome (706 Mb) of *L. polyactis*. Transcriptome data were also obtained from 13 major organs and 18 gonadal development time points from both sexes. We have established an excellent model for studying the relationship between hermaphroditism and gonochorism in teleosts, and the discovery of a transient juvenile intersex stage in males also enriches the theory of teleost

sex determination and differentiation.

Materials and Methods

Samples and sequencing

The little yellow croaker *L. polyactis* (National Center for Biotechnology Information [NCBI] Taxonomy ID: 334908; Fishbase ID: 1877) female and male individuals (Figure 1A) were collected from an artificially bred F2 generation (2016) in Zhoushan, Zhejiang Province, China. The *L. polyactis* genome file has been submitted to NCBI (National Center for Biotechnology Information, <https://www.ncbi.nlm.nih.gov>), and the accession number is JAFMOB000000000. High-quality genomic DNA was extracted from the muscle and blood of a male fish using a Qiagen GenomicTip100 (Qiagen, Hilden, USA) for sequencing on the PacBio platform. To build a 20-kb insert size library, the first step was template preparation, which involved DNA concentration, damage repair, end repair, ligation of hairpin adapters, and template purification using AMPure PB Magnetic Beads. Then, the sequencing primer was annealed, and sequencing polymerase was bound to the SMRTbell template. Finally, the library was sequenced using the Pacific Biosciences RSII instrument, yielding approximately 48 Gb PacBio data (~69-fold coverage of the genome). At the same time, we constructed a short-insert (350 bp) paired-end library using a sample preparation kit following the manufacturer's recommendations, and the library was sequenced on the Illumina HiSeq platform with a 150 bp read length, obtaining approximately 128 Gb of data (~183-fold coverage of the genome). In addition, other mapping technologies, including BioNano genome mapping and 10× Genomics linked reads, were also used to generate a high-quality reference sequence of *L. polyactis*. A total of ~431 Gb of sequencing data was obtained (~616-fold coverage of the genome). Animal experiments were conducted in accordance with the regulations of the Guide for Care and Use of Laboratory Animals, which was approved by the Committee of Laboratory Animal Experimentation at Zhejiang Academy of Agricultural Sciences.

Estimation of genome size

To estimate the genome size of *L. polyactis*, we used reads from paired-end libraries to determine the distribution of K-mer values. The distribution of K-mers depends on the characteristics of the genome and follows Poisson's distribution (Li et al., 2010). A total of 128 Gb of high-quality short-insert reads (350 bp) were used to estimate the *L. polyactis* genome size based on the 17-mer frequency distribution using the following formula: Genome size = (total number of 17-mers)/(position of peak depth) (Liu et al., 2013).

Genome assembly

PacBio long-read *de novo* assembly

First, the PacBio reads were subjected to error correction according to the rate of insertions, deletions, and sequencing errors between the base pairs to obtain preassembled reads using *daligner* (Myers, 2014). Then, the preassembled reads were assembled by the consensus algorithm called *Overlap-Layout-Consensus* (DBG2OLC) to obtain contigs using *FALCON* (v1.2.4) (Pendleton et al., 2015). Finally, the assembled contigs were subjected to error correction with the PacBio data by *Quiver* (v5.0.1) (Chin et al., 2013), and the short-insert size (350 bp) reads were corrected by *pilon* (v1.22) (Walker et al., 2014).

Scaffold assembly

To construct superscaffolds, the PacBio contigs were first scaffolded using *fragScaff* (Adey et al., 2014) according to the location and distance information among linked reads produced from the 10x Genomics libraries, and then, the resulting scaffolds were connected to superscaffolds using optical map data. A high-density genetic map was built by *chromonomer* (version 1.07) (Catchen et al., 2020) to anchor the scaffolds into a chromosome level genome.

Genome evaluation

BWA evaluation

To evaluate the quality of the genome assembly, we mapped the short-insert size reads back to the scaffolds using BWA (Li et al., 2009) with the following parameters: “-o 1 -i 15”. The sequencing depth distribution follows a Poisson distribution, which indicates the uniformity of the genome sequencing data.

CEGMA and BUSCO evaluation

CEGMA (Core Eukaryotic Genes Mapping Approach) (Parra et al., 2007) uses a highly reliable set of 248 conserved protein families that occur in a wide range of eukaryotes to evaluate genome assembly. It has a series of procedures that can accurately identify the exon-intron structures of conserved genes in a novel genomic sequence. BUSCO (Benchmarking Universal Single-Copy Orthologs) (Simão et al., 2015) was combined with TBLASTN (Altschul et al., 1997), Augustus (Stanke and Waack, 2003; Burkhard et al., 2006) and HMMER (Mistry et al., 2013) to assess the completeness of the genome assembly.

Genome annotation

Repeat detection

After genome assembly, repeat annotation was carried out for the *L. polyactis* genome. We identified transposable elements (TEs) with a combination of homology and *de novo* prediction methods using multiple software packages. First, we built the *de novorepeat* library using RepeatModeler (Smit et al.), RepeatScout (Price et al., 2005), and LTR_FINDER (Xu et al., 2007). Second, we performed homology-based prediction using RepeatMasker with default parameters against Repbase and *de novo* repeat libraries at the DNA level. In addition, we used RepeatProteinMask (Tarailo-Graovac and Chen, 2009) with the default parameters to identify repeat sequences at the protein level. The tandem repeats were searched using Tandem Repeats Finder (Benson, 1999) with the parameters “Match=2, Mismatch=7, Delta=7, PM=80, PI=10, Minscore=50, and MaxPeriod=2000”.

Protein-coding gene annotation

We predicted the protein-coding genes with a combination of homology-based gene prediction, *de novo* methods, and RNA-seq data, and then, all predicted genes were integrated by EVIDENCEModeler (Haas et al., 2008) to obtain nonredundant data.

For homology-based gene prediction, we used the longest protein-coding sequences of the genes from eleven species, including *L. crocea*, *Oreochromis niloticus*, *Gasterosteus aculeatus*, *Oryzias latipes*, *Takifugu rubripes*, *Cynoglossus semilaevis*, *Cyprinus carpio*, *Danio rerio*, *Homo sapiens*, *Mus musculus* and *Branchiostoma floridae*, for analysis (Wang et al., 2014). First, we aligned those protein sequences against our assembled genome with an E-value < 1e-5 using TBLASTN (Altschul et al., 1997) and linked the alignment results with SOLAR (Darriba et al., 2012). Then, Genewise (version 2.2.0) (Birney et al., 2004) was used to predict gene models based on the alignment sequences. The homology-based gene structures were obtained.

We used Augustus (version 3.0.2) (Stanke & Waack, 2003; Stanke et al., 2006), Genescan (version 1.0) (Salamov and Solovyev, 2000), Geneid (version 1.4) (Parra et al., 2000), GlimmerHMM (version 3.0.2) (Majoros et al., 2004) and SNAP (Ian, 2004) for *de novo* gene prediction on the repeat-masked genome. Finally, we obtained five results from the five methods.

In addition, RNA-seq data from different tissues were used to assist in gene prediction. On the one hand, we mapped the RNA-seq data to the *L. polyactis* genome using TopHat (version 2.0.13) (Trapnell et al., 2009) and obtained gene structures by cufflinks (version 2.1.1) (Trapnell et al., 2010) based on alignment positions. On the other hand, we assembled the RNA-seq data by Trinity (version 2.1.1) (Grabherr et al., 2011) and then obtained the gene structures by PASA (Hass et al., 2003) software. We merged all gene structures obtained by the three approaches using EVIDENCEModeler (Haas et al., 2008) to generate the nonredundant gene sets. Finally, we used PASA (Hass et al., 2003) to acquire the final gene structures after adjusting the gene models generated from EVIDENCEModeler with the transcripts assembled by Trinity (Grabherr et al., 2011).

Functional annotation

We carried out gene function annotation to better understand the biological adaptation of the species. First, we used our coding proteins to search against InterPro (Mulder et al., 2007) databases, including Pfam (Finn et al., 2000), PRINTS (Attwood and Beck, 1994), PROSITE (Hulo et al., 2006), ProDom (Bru et al., 1998), SMART (Letunic et al., 2012) and PANTHER (Mi et al., 2005), by InterProScan (version 4.7) (Mulder et al., 2007) to obtain motifs and domains. Moreover, we obtained the Gene Ontology (GO) (Ashburner, 2000) terms for each gene from the corresponding InterPro35 descriptions. We also searched KEGG (Kyoto Encyclopedia of Genes and Genomes) (Hiroyuki et al., 2000) databases to determine which pathway the gene might be involved in, as well as SwissProt and TrEMBL (Bairoch and Apweiler, 2000) databases to find the gene symbols.

Noncoding RNA annotation

Noncoding RNA (ncRNA) is a kind of RNA molecule that cannot be translated into a protein. Here, we annotated four types of ncRNAs, including microRNAs (miRNAs), transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), and small nuclear RNAs (snRNAs). We searched for rRNAs by blasting the rRNA database and searched for siRNAs and snRNAs against the Rfam (Griffiths-Jones et al., 2005) database by INFERNAL (Nawrocki et al., 2009) software. Finally, we used tRNAscan-SE (Lowe and Eddy, 1997) to search for tRNAs in the genomes.

Genome comparison

Gene family cluster

Orthologues are homologous sequences resulting from a single ancestral sequence in the last common ancestor of an examined species by a speciation event (Yang et al., 2016). We carried out gene family clustering using OrthoMCL (Li et al., 2016) with 18 species, *L. polyactis*, *L. crocea*, *Oreochromis niloticus*, *Gasterosteus aculeatus*, *Oryzias latipes*, *Takifugu rubripes*, *Cynoglossus semilaevis*, *Danio rerio*, *Homo sapiens*, *Mus musculus*, *Gallus gallus*, *Branchiostoma floridae*, *Lepisosteus oculatus*, *Callorhinchus milii*, *Gadus morhua*, *Ciona intestinalis*, *Anolis carolinensis* and *Xenopus tropicalis*. First, we obtained the longest transcript of the genes and removed the genes whose protein length was less than 30 amino acids. Then, we found possible matches using the 18 species' protein sequences by running an all-vs-all blast with an E-value cutoff of 1e-7. Finally, we clustered the alignments into gene families using OrthoMCL (Li et al., 2016).

Phylogenetic analysis at the gene and genome levels

Phylogenetic relationships at the genome level were analysed for eighteen species, *L. polyactis*, *L. crocea*, *Oreochromis niloticus*, *Gasterosteus aculeatus*, *Oryzias latipes*, *Takifugu rubripes*, *Cynoglossus semilaevis*, *Danio rerio*, *Homo sapiens*, *Mus musculus*, *Gallus gallus*, *Branchiostoma floridae*, *Lepisosteus oculatus*, *Callorhinchus milii*, *Gadus morhua*, *Ciona intestinalis*, *Anolis carolinensis*, and *Xenopus tropicalis*. First, the amino acid sequences from the 590 single-copy genes among the 18 species were aligned individually by MUSCLE (Edgar and Robert, 2004). Then, we concatenated one supergene with the single-copy gene alignments and constructed a phylogenetic tree using the maximum likelihood (ML) method in RAxML software (version 8.0.19) (Stamatakis, 2006; Stamatakis et al., 2008). The phylogenetic relationships of RING-finger protein (*rnf*) family members on chromosome 2, *rnf183*, *rnf223*, and *rnf225*, and *dmrt1* were analysed among the following species: *Homo sapiens*, *Mus musculus*, *Alligator sinensis*, *Chelonia mydas*, *Xenopus tropicalis*, *Xenopus laevis*, *Xenopus tropicalis*, *Gallus gallus*, *Trachemys scripta*, *Danio rerio*, *Takifugu rubripes*, *L. crocea*, *L. polyactis*, *Oreochromis niloticus*, *Lates calcarifer*, *Amphiprion ocellaris*, *Oryzias latipes*, *Kryptolebias marmoratus*, *Acanthopagrus schlegelii*, *Labrus bergylta*, *Epinephelus merra*, *Epinephelus coioides*, *Epinephelus lanceolatus*, *Centropomus striata*, *Monopterus albus*, *Sparus aurata*, *Oncorhynchus mykiss*, *Gadus morhua*, and *Anolis carolinensis*. The GenBank accession number of each gene and species is listed in Supplementary Table S1. The evolutionary history was inferred by using the maximum likelihood method and general time reversible model (Nei and Kumar, 2000). The tree with the highest log-likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying neighbour-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum

composite likelihood (MCL) approach and then selecting the topology with a superior log-likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites. The rate variation model allowed some sites to be evolutionarily invariable. The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. This analysis involved several nucleotide sequences. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018; Stecher et al., 2020). According to the results of the phylogenetic tree, we corrected the nomenclature of *rnf225* and *rnf183* in the following species: *rnf225* has been wrongly named *rnf183* in *Danio rerio*, *Oryzias latipes*, *Kryptolebias marmoratus*, *Oreochromis niloticus*, *Amphiprion ocellaris*, and *Epinephelus lanceolatus*.

Divergence time estimation

Divergence time estimation was implemented based on the phylogenetic tree and CDSs by the mcmctree program of PAML (Yang, 2007) (<http://abacus.gene.ucl.ac.uk/software/paml.html>) among 18 species with the main parameters "burn-in=10000, sample-number=100,000 and sample-frequency=2". Seven calibrating time points were selected from the TimeTree (Hedges et al., 2015) website (<http://www.timetree.org>): *Homo sapiens* and *Mus musculus*, 61~100 million years ago (Mya); *Gallus gallus* and *Anolis carolinensis*, 225~239 Mya; *Homo sapiens* and *Gallus gallus*, 312~330 Mya; *Gasterosteus aculeatus*, *L. crocea*, *L. polyactis*, and *Takifugu rubripes*, 97~151 Mya; *Gadus morhua*, *Cynoglossus semilaevis*, *Gasterosteus aculeatus*, *L. crocea*, *L. polyactis*, *Takifugu rubripes*, *Oreochromis niloticus*, and *Oryzias latipes*, 139~158 Mya; *Danio rerio* and nine other species, 149~165 Mya; and *Callorhynchus milii* and fifteen other species, 422~463 Mya.

Gene family contraction and expansion

We explored gene families that underwent a significant change among *L. polyactis* and other species based on the abovementioned phylogenetic tree and divergence time. We performed expansion and contraction analysis using the CAFÉ (Version 2.1) (De et al., 2006) program after filtering the families that had more than 200 copies in one species had less than 2 copies in other species. By comparing each branch with their ancestor branch, the *P*-value was calculated using Fisher's exact test and was then adjusted by the false discovery rate (FDR). Families with an adjusted p-value less than 0.05 were considered to have a significant change, which means they underwent contraction or expansion during the process of evolution (Jeffery et al., 2006). The *L. polyactis* genes included in the contracted or expanded families were extracted and subjected to GO/KEGG enrichment analyses.

Genes with accelerated evolutionary rates

Positive selection analysis was carried out at the DNA sequence level by estimating the ratio (ω) of non-synonymous nucleotide substitutions (dN) to synonymous nucleotide substitutions (dS) between orthologous genes used in the phylogenetic analysis (Sun et al., 2012). We calculated different ω values in the phylogenetic tree, including ω_0 , ω_2 , and ω_1 , representing the whole tree, foreground branch, and background branch, respectively, with the branch-site model (Zhang et al., 2005; Zhang et al., 2013). P-values were computed using the χ^2 statistic and adjusted by the FDR method. Genes with an adjusted p-value less than 0.05 were thought to have undergone positive selection. We ran three groups of positive selection analyses. In the first group, we set *L. polyactis* as the foreground branch and *L. crocea* as the background branch, and 955 positively selected genes were found in the *L. polyactis* genome. In the second group, we set *L. polyactis* as the foreground branch and *Oryzias latipes*, *Oreochromis niloticus*, *Takifugu rubripes*, *L. crocea*, and *Gasterosteus aculeatus* as the background branch, and 761 positively selected genes were found in the *L. polyactis* genome. In the third group, we set *L. polyactis* as the foreground branch and *Takifugu rubripes*, *L. crocea*, and *Gasterosteus aculeatus* as the background branch, and 1080 positively selected genes were found in the *L. polyactis* genome.

Comparison of the *L. polyactis* and *L. crocea* genomes

Similar to *L. crocea*, *L. polyactis* has 24 chromosomes. To visualize the concordance with the *L. crocea* genome, the 24 chromosomes were aligned to *L. crocea* using blastp (blastp -e 1e-10 -b 5 -v 5 -m 8) (BLASTP, RRID:SCR_001010). Gene alignments of both nucleic acid and amino acid sequences

were concatenated and submitted to RAxML for maximum likelihood (ML) inference. The amino acid phylogeny was inferred using the GAMMA AUTO model, which enables RAxML to automatically determine the best-scoring protein substitution model (Stamatakis, 2014). The *L. croceagenome* was obtained using GenBank: CM011695.1 from NCBI (<https://www.ncbi.nlm.nih.gov/nucleotide/1523709032/>). The results were plotted using circos (version 0.69) (Krzywinski et al., 2009) and mcscan ([https://github.com/tanghaibao/jcvi/wiki/MCscan-\(Python-version\)](https://github.com/tanghaibao/jcvi/wiki/MCscan-(Python-version))) (Tang et al., 2008). The synteny inference of sex chromosomes was constructed by using JCVI utility libraries (Tang et al., 2008), which is a python package. The alignment of the two genomes was carried out by using the MUMMER alignment algorithm (MUMMER).

Gonadal RNA sequencing

Total RNA was extracted from thirteen kinds of tissues, including gill, muscle, kidney, head kidney, liver, spleen, brain, eye, heart, skin, testis, ovary, and intestine, and genomic DNA contamination was removed. The integrity of the RNA was evaluated on an agarose gel stained with ethidium bromide (EB), and its quality and quantity were assessed using a NanoDrop and Agilent 2100 Bioanalyzer. Then, cDNA libraries were constructed following the manufacturer's recommendations and sequenced on the Illumina HiSeq platform, generating 150-bp paired-end reads. A summary of the clean data from different tissues is shown in Supplementary Table S2. These RNAseq libraries were also sequenced to assemble transcriptomes and annotate genes.

Total RNA was extracted from three mixed pools of whole-fish samples at 7, 10, 20, and 30 dph, where each pool had six fishes; from three mixed pools of indistinguishable gonads at 40 dph, where each pool had six gonads; and from thirteen pairs of ovary and testis mixed pools at 50, 60, 70, 80, 100, 130, 180, 240, 270, 340, 360, 400, and 720 dph, where each pool had three samples. The genomic DNA contamination was removed. The integrity of the RNA was evaluated on an agarose gel stained with ethidium bromide (EB), and its quality and quantity were assessed using a NanoDrop and Agilent 2100 Bioanalyzer. Then, cDNA libraries were constructed following the manufacturer's recommendations and sequenced on the Illumina HiSeq platform, generating 150-bp paired-end reads. High-quality reads from each sample were mapped onto the assembled transcript sequences using Bowtie software (Langmead and Salzberg, 2012) with default parameters. The RSEM program (Li and Dewey, 2011) was then used to estimate the expression abundance of the transcripts. The total number of mapped reads for each transcript was determined and then normalized to determine RPKM (reads per kilobase of transcript per million mapped reads).

Statistical Analysis

Statistical software uses SPSS statistics v17.0 (Chicago, USA). Independent sample t-test, one-way ANOVA, and Duncan's post hoc test were performed to determine the statistical significance at $P < 0.05$.

Results

Genome sequencing assembly and annotation

We sequenced the *L. polyactis* (Figure 1A) genome using a combination of different approaches, including single-molecule real-time (SMRT) sequencing technology, Illumina short-insert size (350 bp) sequencing technology, and new mapping technologies (BioNano genome map and 10× Genomics linked reads). A total of ~431 Gb of data was obtained (~616-fold coverage of the genome) (Table 1). A total of 715 scaffolds were assembled, covering a total length of the final assembly of ~706 Mb. The calculated contig N50 and scaffold N50 were 1.21 Mb and 4.52 Mb, respectively (Table 2). The G-C content of the assembly was 41.45% (Supplementary Table S3, S4). Based on the constructed genetic map, scaffolds were mapped to 24 chromosomes, accounting for 88.55% of the total length (Table 2).

To evaluate the consistency and completeness of the genome assembly, we performed short-insert read mapping by BWA. The mapping rate was 97.49%, covering 99.36% of the genome with a homologous SNP rate of 0.03%. To assess the completeness of the genome assembly (Supplementary Table S5), 248 ultraconserved

eukaryotic genes were examined by CEGMA9, and 93.95% were found in our assembly, indicating high integrity of the core genes (Supplementary Table S6). BUSCO analysis was also conducted, a total of 4,304 out of the 4,584 searched BUSCO groups (93.9%) had been completely assembled in our genome, suggesting a high level of completeness of the de novo assembly (Supplementary Table S7). A total of 157 Mb (~22%) of genome sequences was predicted to be repetitive elements, including 38 Mb (5.34%) of long-terminal repeat (LTR) retrotransposons and 67 Mb (9.57%) of DNA transposons (Supplementary Table S8-S9).

For the chromosome-level assembly, a high-density linkage map was used to anchor the scaffolds. A total of 309 scaffolds were anchored to 24 pseudochromosomes, representing 88.55% of the total genome (Table 1).

A total of 25,233 protein-coding genes were predicted using a standard annotation pipeline, and 84.3% of the genes were supported by homologous protein sequences or transcriptomic data (Table 1; Figure 1B; Supplementary Table S10-S11). The sequence divergence rate for each family of TEs was calculated (Figure 1C).

Gene family analysis of *L. crocea*, *Gasterosteus aculeatus*, *Danio rerio*, and *L. polyactis* identified a core set of 10,215 gene families, 26,313 gene families, and 590 single-copy gene families among *L. polyactis* and seventeen other species; we counted and plotted the number of orthologous genes across the eighteen species in a Venn diagram (Figure 1D-E). On average, the genes had an exon length of 166.59 bp and intron length of 1,270.49 bp (Supplementary Table S10). Approximately 95.5% (24086) of the predicted genes were assigned functional annotations by alignment against various databases, including InterPro35, KEGG43, SwissProt45, and TrEMBL45 (Supplementary Table S12). Four types of noncoding RNAs (ncRNAs), including microRNAs (miRNAs), transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), and small nuclear RNAs (snRNAs), are annotated in Supplementary Table S13.

Genome evolution analysis

Phylogenetic analysis based on 590 selected 1:1 orthologous genes suggests that the *L. polyactis* lineage diverged from the common ancestor of the *L. crocea* lineage ~25.4 million years ago. The ancestor of *L. polyactis* and *L. crocea* separated from the ancestor of *T. fuqua* approximately 90.1 million years ago. Gene-family analysis among the 18 species found 320 expanded gene families and 544 contracted families on the *L. polyactis* branch (Figure 2). The three groups of positive selection analyses found that more than 700 genes underwent accelerated evolution. The *L. polyactis* genes included in the contracted or expanded families were extracted and subjected to GO/KEGG enrichment analyses. The expanded gene families and the positively selected genes were all probably important for *L. polyactis* adaptations (Figure 2, Supplementary Tables S14-S23).

Comparative genomic analysis of the sex chromosomes

Comparison of the genomes of *L. polyactis* and *L. crocea* revealed that 24 chromosomes of *L. polyactis* could be unambiguously aligned to single chromosomes of *L. crocea* (Figure 3). The results reflected the high quality of the *L. polyactis* genome assembly. Although these chromosomes did not experience chromosome fission and fusion events, a number of rearrangements and translocations were found in pairwise comparisons (Figure 3).

Syntenic analysis of the sex chromosomes of *L. crocea* (linkage group 22, LG22) and *L. polyactis* (chromosome 2, Chr. 2) revealed that one scaffold (scaffold 8) was inserted into Chr. 2 of *L. polyactis* and that the other sequences had high collinearity. Interestingly, six members of the ring finger protein (*rnf*) family and several genes involved in sex differentiation and gonadal development, including the sex determination candidate *dmrt1*, were identified in the sex determination region (Figure 4A). A 4.62 Mb region surrounding *dmrt1* (from *piwi2* to *rnf122*) in *L. polyactis* was compared to the corresponding syntenic region in *L. crocea* (4.73 Mb). Duplication of a protein-coding gene in the cilia- and flagella-associated protein family, *cfap157*, was also found in *L. polyactis* (Figure 4B). Since a large number of *rnf* genes were not observed in any other fish clade, we analysed the phylogenetic relationship of these sex-linked *rnf* genes and autosomal members in *Larimichthys*. The results showed that *rnf* family members have a high degree of homology between these

two species, *L. polyactis* and *L. crocea* (Figure 4C).

Identification and expression analysis of the sex-determining candidate gene *dmrt1*

Transcriptome analysis was performed on thirteen *L. polyactis* tissues, gill, muscle, kidney, head-kidney, liver, spleen, brain, eye, heart, skin, testis, ovary, and intestine. Gonad gene expression profiling was also conducted at 18 developmental stages in whole-fish samples at 7, 10, 20, and 30 dph, indistinguishable sex gonads at 40 dph, and testes and ovaries at 13 later stages (see Methods). The early-stage gonads before 40 dph clustered together; indistinguishable sex gonads at 40 dph and testes before meiosis (50-180, 400 and 720 dph) clustered together; and initiation of meiosis to form the functional testis of sexual maturity (240-360 dph) clustered together. From 50 dph, all stages of ovarian development clustered together (50-720 dph). A panel of sex determination- and differentiation-related genes, including *cyp19a1a*, *figla*, *rnf183*, *foxl2*, *rnf225*, *gsdf*, *amh*, and *dmrt1*, were annotated in the genome and transcriptome data (Figure 5 and Supplementary Figure S1). Among them, *cyp19a1a*, *figla*, *rnf183*, and *foxl2* showed an ovary expression bias, while *rnf225*, *gsdf*, *amh*, and *dmrt1* showed a significant testis bias. If we restricted the genes in the sex determination region, *dmrt1* was testis-specific in all stages (Figure 6), and *rnf183* (Figure 7) and *figla* (Figure 8) were significantly ovary biased. Therefore, we conclude that *dmrt1* is the best sex determination gene candidate.

A phylogenetic tree was constructed from *dmrt1* nucleotide sequences. The results showed that most of the fish species analysed could be clustered in one main group (Figure 6A). Most of them are Perciformes, except for *Oryzias latipes*, *Kryptolebias marmoratus*, and *Monopterus albus*. Similarly, the closest relative of *L. polyactis* based on *dmrt1* is still *L. crocea*. The fish group contains gonochorist, juvenile hermaphrodite, hermaphrodite protandry and hermaphrodite protogyny. Interestingly, the branches of *L. polyactis* and *L. crocea* were located between species with hermaphrodite protandry and hermaphrodite protogyny, including *Epinephelus merra*, *Epinephelus coioides*, *Labrus bergylta*, *Centropomus striata*, *Acanthopagrus schlegelii*, *Lates calcarifer* and *Monopterus albus*. *Oreochromis niloticus*, *Oryzias latipes* and *Takifugu rubripes* are typical gonochorists, and they are scattered in different branches. *Danio rerio* belongs to the juvenile hermaphrodite group and is distributed in a separate branch. The other main group includes mammals, reptiles, birds, and amphibians, which are gonochorists. The reproductive patterns of these species are shown in the diagram (Figure 6A). These results indicated that *dmrt1* was correlated with the evolution of gonochorism and hermaphroditism.

A gene locus map showed that the gene orientation of *dmrt1* and its downstream genes *dmrt3*, *dmrt2*, and *smarca2* is conserved among mammals, reptiles, amphibians, and other teleosts, except *Larimichthys*. The gene order was rearranged, with an *rnf183* gene insertion immediately upstream of *dmrt1* in *Larimichthys*. Between the closely related *T. rubripes* and *Larimichthys*, the upstream gene contents were different, suggesting rapid genomic evolution in this region (Figure 6B). The results indicated that *rnf183* is present only in *Larimichthys* and not in any other teleost species.

The gene structure of *dmrt1* contains 5 exons, resembling that of other teleost species, including *D. rerio*, *O. latipes*, *T. rubripes*, *O. niloticus* and *L. crocea*. Compared with that of its close relative *L. crocea*, the fourth exon has an 18-bp insertion (Figure 6C). Further analysis of the transcription profiles in its gene neighbourhood identified an immediate upstream gene, *rnf183*, whose expression was also sexually dimorphic. Analysis of the transcripts of *rnf183* revealed that this gene has two exons and is only 3197 bases away from *dmrt1* (Figure 6D).

Rnf183 and its close paralogues show a pattern of rapid evolution in teleosts

Rnf183 is 3 kb upstream of *dmrt1*, and it displayed an opposite sex-biased pattern in gonad expression compared to *dmrt1*. Its ovary-specific expression may result in testis-specific expression of *dmrt1* through a collateral damage effect. We investigated the evolutionary history of *rnf183* and found that two autosomal genes, *rnf223* and *rnf225*, are closely related paralogues. Orthologues for all three genes could be found in mammals, reptiles, and amphibians. Although *rnf223* and *rnf225* are present in most fish species we examined, *rnf183* can only be found in Sciaenidae, Latidae, Pomacentridae, Labridae, Sparidae, Salmonidae, and Cichlidae, suggesting gene loss during evolution (Figure 7A).

A gene locus map showed that the gene orientation of *rnf183* and its upstream and downstream genes is relatively conserved among mammals, amphibians, and reptiles but not among teleosts. *rnf183* is next to *rnf223* in *Xenopus*, suggesting that they may have been derived from an ancient duplication event. The sex-linked location of *rnf183* is only found in *Larimichthys*, and its gene neighbourhood is very different from that of other fish species with *rnf183* orthologues (Figure 7B).

The tissue distribution and gonadal expression of *rnf183*, *rnf223* (on Chr. 11), and *rnf225* (Chr. 24) were analysed. *rnf183* was expressed at low levels in gills/testes and was extremely highly expressed in ovaries (RPKM >120). *rnf225* was highly expressed in gills (RPKM > 120), with only a very low level of expression detected in skin and other tissues. *rnf223* was expressed broadly in multiple tissues, with a moderate level in the testis (RPKM > 10) but no expression in the ovary (Figure 7C). These results indicated the functional divergence of *rnf183*, *rnf223*, and *rnf225*, presumably due to subfunctionalization after gene duplication. While *rnf223* and *rnf225* maintain their expression and function in the gill and other tissues, *rnf183* specializes in sex determination, differentiation or maintenance in the ovary of *L. polyactis*.

Sex-related gene expression profiles in *L. polyactis*

A few well-identified and studied sex-related genes were screened to analyse expression profiles. First, *Amh* (anti-Müllerian hormone, on Chr. 6) and *gsdf* (gonadal soma-derived factor, on Chr. 16) are gonad-specific and highly expressed in the testis. *Amh* initiated its expression from 40 dph before testicular meiosis (50-180 dph), and it was downregulated during spermatogenesis to mature testis. In contrast to *amh*, *Gsdf* was expressed as early as 7 dph and maintained high expression during the juvenile (7-40 dph) and later stages (after 50 dph). However, high expression of *gsdf* was also detected in the ovary when entering the breeding season (360 and 720 dph). *Cyp19a1a* (cytochrome P450, family 19, subfamily A, polypeptide 1a, on Chr. 10), *figla* (factor in germline alpha, on Chr. 2) and *foxl2* (Forkhead Box L2, in Chr. 15) are considered tightly related to oestrogen synthesis, oocyte formation, and ovarian differentiation. Although *cyp19a1a* was widely expressed in many tissues and expressed as early as 7 dph, in some cases, its expression was significantly higher in ovaries than in testes. *Figla* is mainly expressed in the gonads, especially in the ovary. It was first detected at 40 dph and maintained a significantly higher level in the ovary than in the testis. *Foxl2* was mainly expressed in the gill, eye, brain, and gonad, whereas it was more highly expressed in the ovary than in the testis at a few stages. Interestingly, these three genes were all expressed in the testis in the transient hermaphrodite stage (43-80 dph) in *L. polyactis*, and even at this stage, there were no significant differences in the expression levels of *cyp19a1a* and *foxl2* in the testis and ovary, except *foxl2* at 50 dph (Figure 8). These results indicated that the transient hermaphrodite stage (43-80 dph) in *L. polyactis* has a complex formation basis and molecular mechanism.

Discussion

A high-quality genome assembly of *L. polyactis*

L. polyactis is a commercially important Sciaenidae fish species. Adult fish show sexual dimorphism, with females remarkably larger than males. A reference genome is crucial for biological studies of *L. polyactis*. In the present study, we assembled the most contiguous genome of *L. polyactis* via the integration of nanopore long-read sequencing, Bionano, and 10x Genomics technologies. This is the first high-quality *de novo* assembled genome in Sciaenidae using third-generation sequencing.

Our assembly benefited from long-read sequencing technologies. The combination of long-read assembly and Illumina short-read correction ensured the accuracy and completeness of the genome assembly. The contig N50 is much longer with a smaller number of contigs than the genome assembly of several fish species using third-generation sequencing (Table S17) (Gong et al., 2018; Cai et al., 2019; Conte et al., 2019; Ge et al., 2019; He et al., 2019; Jiang et al., 2019; Yang et al., 2019). Physical mapping using Bionano and 10x Genomics linked reads further improved the quality of the genome assembly to the pseudochromosome level. *rnf183* and its paralogues can be identified and annotated, which provides another indication of the high quality of gene assembly.

A total of 25,233 protein-coding genes were annotated in the *L. polyactis* genome, 23,991 of which had functional annotations. A total of 5,671 non-coding RNAs were also identified. In addition, the genome contained 22.28% repetitive sequences, which was lower than the genomes of *Danio rerio* (52.5%), *Epinephelus lanceolatus* (41.01%), *Epinephelus akaara* (43.02%), *Glyptosternon maculatum* (33.96%), and *Gasterosteus aculeatus* (25.2%).

Most teleosts have undergone teleost-specific genome duplication (TGD), and their haploid genomes contain 24 to 25 chromosomes. The number of chromosomes assembled in the genome of *L. polyactis* was 24, which was consistent with the *L. crocea* and karyotype analysis (Liu et al., 2020; Park and Gil, 2019). By compared the genomes of these close species, although these chromosomes did not experience chromosome fission and fusion events, a number of rearrangements and translocations were found in pairwise comparisons.

A model for studying the evolution of hermaphroditism and gonochorism in fish

Theoretically, gonochorism and simultaneous hermaphroditism are the two evolutionary endpoints on a reproductive spectrum (Avisé and Mank, 2009), where gonochorism could evolve from hermaphroditism either by gradually increasing sex-specific investment or the occurrence of male- or female-sterility mutations (Bachtrog et al., 2014). The opposite also exists; hermaphroditism could evolve from gonochorism due to sexual reproduction, and sex determination genes or loci have been lost and re-evolved many times in plants (Schaefer H, Renner SS, 2010).

In fish, the direction of evolution between hermaphroditism and gonochorism remains unclear due to the lack of transitional species between the above two reproductive methods. Perciformes have all types of reproductive systems, including gonochorism, hermaphrodite protandry, hermaphrodite protogyny, and simultaneous hermaphroditism (Avisé and Mank, 2009), and these fish could serve as excellent models to study the evolution of hermaphroditism and gonochorism. As reported in the literature, hermaphroditism is widespread across teleost fish phylogeny, and no order consists solely of hermaphroditic species, suggesting that teleost fish evolved their own sexual reproduction independently in the process of adapting to environmental changes. After experiencing stress from various environmental changes, perhaps fishing pressure, larger individuals are less likely to escape capture and are forced to mature earlier; alternatively, environmental pollution from environmental endocrine disruptors may cause feminization, resulting in a population-level sex ratio imbalance. Mutations in some sex-related genes may contribute to accelerated sexual maturation, such as females always initiating meiosis before males, or may help to resist the effects of environmental endocrine disruptors to maintain a balanced sex ratio throughout the population. These may be reasons for the transition from gonochorism to hermaphroditism.

Finding a transitional species in the evolution of hermaphroditism and gonochorism in Perciformes is very important for the study of the evolution of the fish reproductive spectrum. Our previous study demonstrated that *L. polyactis* ' unique pattern of testis differentiation is neither typical hermaphroditism nor gonochorism (Xie et al., 2021). We speculate that this gonadal differentiation pattern in *L. polyactis* evolved from a gonochoristic ancestor. As mentioned above, *L. polyactis* has experienced severe overfishing and pollution of environmental endocrine disruptors in spawning grounds in recent decades (Jin 1996; Lin and Cheng 2004; Lu et al., 2020). Therefore, *L. polyactis* can be regarded as a transitional species in the process of evolution from gonochorism to hermaphroditism.

The availability of the *L. crocea* genome adds value to research on sex determination evolution. *L. polyactis* and *L. crocea* are the closest relatives that can interbreed (Liu et al., 2019), but they display remarkable differences in reproductive patterns and gonad development processes. *L. crocea* needs two years to reach sexual maturity, whereas *L. polyactis* is sexually mature at one year of age. Male *L. polyactis* needs to undergo a transient hermaphrodite stage, while *L. crocea* does not. Collinearity analysis of the whole genome of these two closely related species showed that almost all chromosomes of *L. crocea* and *L. polyactis* had high homology. The sex chromosome LG22 of *L. crocea* is also highly homologous with Chr. 2 of *L. polyactis*, except *L. polyactis* Chr. 2 has an inserted scaffold. In conclusion, *L. polyactis* is a very good model for studying the evolutionary direction between hermaphroditism and gonochorism. Whether the sexual

reproduction type of *L. polyactis* will change from gonochorism to complete hermaphroditism is still an open question. The underlying molecular mechanisms warrant further study.

dmrt1* and its upstream gene *rnf183* are candidate sex determination genes in *L. polyactis

Dmrt1 is a conserved and critical sex determination- and differentiation-related transcription factor. *Dmrt1* mainly inhibits the expression of oestrogen synthesis signalling pathway factors through antagonism with Foxl2 (Forkhead box protein L2), thus inhibiting the synthesis of oestrogen to achieve testis maintenance and development (Li et al., 2013). The medaka sex determination gene *Dmy* is a unique copy of *dmrt1* on the Y chromosome (Matsuda et al., 2002; Nanda et al., 2002), and half-smooth tongue sole also uses *dmrt1* (Chen S et al., 2014) as a sex-determination gene. In addition, the *dmrt1* gene region has been reported as a sex-specific molecular marker found in *Nibeia albiflora*, *L. crocea*, and *Scatophagus argus* (Sun et al., 2018; Lin et al., 2017; Mustapha et al., 2018).

It is interesting to note that *rnf183* is only 3 kb upstream of *dmrt1*, and it displayed an opposite sex-biased pattern in gonad expression compared to *dmrt1*. The RING (Really Interesting New Gene) finger protein (*rnf*) family is the largest family of E3 ubiquitin ligases and contains a RING-finger domain. These members act as regulators in most cases and are widely involved in diverse aspects of growth and development, biological processes, and stress or environmental responses (Chasapis and Spyroulias, 2009; Sun et al., 2019). In mammals, RNF12 causes REX1 breakdown through dose-dependent catalysis, thereby representing an important pathway to initiate X-chromosome inactivation (Gontan et al., 2012). In humans, RING-finger protein 183 (*RNF183*) is a kidney-specific ubiquitin ligase (Kaneko et al., 2016). The abnormal expression of RNF183 is thought to be involved in several diseases, not only tumorigenesis but also inflammatory conditions such as inflammatory bowel disease (IBD). The molecular mechanism underlying the development and pathophysiology of this disease has not yet been fully elucidated (Yu et al., 2016; Okamoto et al., 2020). Although only a few studies on the role of RNFs have been reported in fish, it is clear that RNF is a regulator of downstream gene expression and molecular pathways (Yu et al., 2016). The present study revealed rapid evolution of *rnf183* in fish and lineage-specific loss in some species, such as zebrafish. *rnf183* is ovary-specific in *L. polyactis* and could drive the testis-specific expression of *dmrt1* through a collateral damage effect. More interestingly, *rnf183* is upstream of *dmrt1* only in *Larimichthys* but not in any other teleost species, suggesting that this innovation in gene regulation has occurred recently. The expression of *rnf183* shows obvious sexual dimorphism in the gonads, suggesting that this gene family plays an important role in sex determination, differentiation, and/or maintenance. The molecular mechanism requires further investigation.

Compared to the sex chromosomes in *L. crocea*, the results showed that the sex determination candidate gene *dmrt1* was also identified in *L. polyactis*. Transcriptome analysis showed that *dmrt1* is the most important sex-specific gene in *L. polyactis*, which is expressed in the testis and not in the ovary at almost any time point. Moreover, the expression of the *dmrt1* upstream gene *rnf183* also showed obvious sexual dimorphism. Phylogenetic analysis showed that hermaphroditism might be related to *dmrt1* evolution. Therefore, we speculated that *dmrt1* and *rnf183* are sex determination candidate genes in *L. polyactis*, and further research on sex-specific molecular markers is needed.

***Figla*, *foxl2* and *cyp19a1a* expression patterns responsible for the male-specific transient hermaphroditic stage**

Figla, *foxl2* and *cyp19a1a* were considered to be three of the most important genes for ovarian differentiation. FIGLA is an oocyte-specific transcription factor that controls the expression of oocyte-specific zona pellucida (ZP) proteins (ZP1, ZP2, and ZP3) by binding to the E-box (CANNTG) in their promoters (Liang et al., 1997). Targeted mutagenesis of *Figla* in mouse resulted in ovaries that were small owing to massive depletion of oocytes and failure of primordial follicle formation (Soyal et al., 2000). In the hermaphroditic wrasse (*Halichoeres poecilopterus*), which displays a female-to-male sex change, *figla* was abundantly expressed in the ovary, and its level decreased during the sex change with oocyte degeneration. Moreover, the male-specific gene *dmrt1* showed an opposite change in expression during sex reversal (Miyake et al., 2012). Overexpression of *figla* in XY male Nile tilapia (*Oreochromis niloticus*) led to a lack of meiotic spermatocytes as well as

spermatids (Qiu et al., 2015). In zebrafish, *figla* was expressed from 26 dpf, which was likely the time when gonadal differentiation occurred (Jørgensen et al., 2008). *figla* mutant zebrafish showed that the germ cells were clustered in cysts and underwent meiosis, forming oocytes at the prefollicular chromatin nucleolar (CN) stage (stage IA). However, the subsequent transition from cystic CN oocytes to individual follicular perinucleolar oocytes (stage IB) was blocked, resulting in an all-male phenotype in the mutant (Qin et al., 2018).

As a natural inducer of ovarian differentiation, oestrogen is considered a key factor in sex differentiation (Wang et al., 2007; Nagahama, 2000). It is well documented that *foxl2* and *cyp19a1a* (Cytochrome P450 Family 19 Subfamily A Member 1) play a vital role in promoting ovarian development and maintaining feminization afterward in both hermaphroditic and gonochoristic teleosts (Wu and Chang, 2018; Wang et al., 2007; Guiguen et al., 2010; Li et al., 2013; Li et al., 2019). In tilapia, *Cyp19a1a* is expressed as early as 5 dph in XX female fish to drive the differentiation of the ovary (Xie et al., 2016). *Foxl2* and *Dmrt1* play antagonistic roles in sex differentiation by regulating *cyp19a1a* expression and oestrogen production (Li et al., 2013). In zebrafish, oestrogen E2 or bisphenol A (BPA) primarily bind *Esr2a* to inhibit the expression and action of *dmrt1* (Song et al., 2020). Loss of function of *cyp19a1a* by CRISPR/Cas9 and TALENs will lead to complete masculinization in zebrafish and sexual reversion from female to male in XX tilapia, respectively (Lau et al., 2016; Zhang et al., 2017).

In *L. polyactis*, *figla*, *foxl2* and *cyp19a1a* were also abundantly expressed in the ovary and expressed at lower levels in the testis. Interestingly, *figla*, *foxl2* and *cyp19a1a* were also expressed in the testis in the male-specific transient hermaphroditic stage; however, the *figla* level decreased with oocyte degeneration after 80 dph, whereas *foxl2* and *cyp19a1a* expression even reached a very high level at 100 dph, but there was no significant difference between ovary and testis samples before 130 dph. These results demonstrated that *figla*, *foxl2*, and *cyp19a1a* are responsible for EPO formation and the male-specific transient hermaphroditic stage.

In summary, the high-quality genome of *L. polyactis* not only provides insight into the genetic underpinnings of hermaphrodite evolution but also provides a solid foundation for sex-related gene expression dimorphism in *L. polyactis*. This is valuable information for enhancing fish aquaculture.

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Data Accessibility

The raw genome and RNA sequencing data have been deposited in the Sequence Read Archive (SRA) database under Bioproject number PRJNA705840. The final chromosome assembly of *L. polyactis* has been submitted to NCBI (National Center for Biotechnology Information, <https://www.ncbi.nlm.nih.gov>) Assembly under accession number JAFMOB000000000. The version described in this paper is version JAFMOB000000000.

Conflict of Interest

The authors declare that they have no competing interests.

Author Contributions

Conceived and designed the experiments: Q.X., X.W. and B.L.; collected sample: Q.X., X. H, W.Z., F.L.; performed the experiments: Q.X., X. H. and J. S.; performed the data analyses: Q.X, J. S., B.N., Y. X., M. L., Q. L., P. X. and X.W; wrote the paper: Q.X. X.W. and B.L. All authors contributed to manuscript revision, read and approved the submitted version.

Figure legends

Figure 1. Characteristics of the *Larimichthys polyactis* genome assembly.

(A) A picture showing female and male little yellow croaker, *L. polyactis* . (B) Comparison of the gene sets obtained using different prediction methods (*de novo* , homology-based, RNA-seq-based). Over 84.3% of 25233 genes were supported by at least one prediction method. (C) Divergence distribution of classified families of TEs. Red indicates DNA transposons (DNAs), dark yellow indicates long interspersed nuclear elements (LINEs), green indicates long terminal repeats (LTRs), light blue indicates short interspersed nuclear elements (SINEs), and purple indicates unknown TEs.(D) The Venn diagram shows common and unique gene families among the four species. The four species include *Larimichthys crocea* (Lcr), *Gasterosteus aculeatus* (Gac), *Danio rerio*(Dre) and *Larimichthys polyactis* (Lpo). (E) Protein orthology comparison among the genomes of eighteen species. The eighteen species include *Larimichthys polyactis* (Lpo), *Larimichthys crocea* (Lcr), *Oreochromis niloticus* (Oni), *Gasterosteus aculeatus* (Gac), *Oryzias latipes* (Ola), *Takifugu rubripes*(Tru), *Cynoglossus semilaevis* (Cse), *Danio rerio* (Dre), *Homo sapiens* (Hsa), *Mus musculus* (Mmu), *Gallus gallus* (Gga), *Branchiostoma floridae* (Bfl), *Lepisosteus oculatus* (Loc), *Callorhinchus milii* (Cmi), *Gadus morhua*(Gmo), *Ciona intestinalis* (Cin), *Anolis carolinensis* (Aca) and *Xenopus tropicalis* (Xtr). Single-copy orthologues are marked with pink, multiple-copy orthologues are marked with yellow, unique genes are marked with dark yellow, and other orthologues are marked with green.

Figure 2. Phylogenetic and gene expansion and contraction analysis of *Larimichthys polyactis*.

Phylogenetic tree of 18 vertebrate genomes constructed using 592 single-copy orthologous genes. The numbers on the branches indicate the estimated diverge times in millions of years ago, and red circles indicate the calibration time from fossil *Homo sapiens* (Hsa), *Mus musculus* (Mmu), *Gallus gallus* (Gga), *Anolis carolinensis* (Aca), *Xenopus tropicalis* (Xtr), *Danio rerio* (Dre), *Gadus morhua* (Gmo), *Cynoglossus semilaevis* (Cse), *Gasterosteus aculeatus* (Gac), *Larimichthys polyactis* (Lpo), *Larimichthys crocea* (Lcr), *Takifugu rubripes* (Tru), *Oryzias latipes* (Ola), *Oreochromis niloticus* (Oni), *Lepisosteus oculatus* (Loc), *Callorhynchus milii* (Cmi), *Ciona intestinalis* (Cin) and *Branchiostoma floridae* (Bfl).

Figure 3. Genome comparisons between *Larimichthys crocea* and *Larimichthys polyactis*.

Twenty-four chromosomes of *L. polyactis* show a one-to-one relationship with 24 *Larimichthys crocea* chromosomes. Linkage groups 1-24 on the left of the circle represent *Larimichthys crocea* chromosomes, and chromosomes 1-24 on the right represent *L. polyactis* chromosomes.

Figure 4. Sex chromosome comparisons between *Larimichthys crocea* and *Larimichthys polyactis*.

(A) Genome collinearity analysis of sex chromosomes in *Larimichthys crocea* and *L. polyactis*. The lines between the two horizontal lines link the alignment blocks. Plus and minus represent the direction of gene transcription. The positions of genes related to sex and reproduction are marked on chromosomes. (B) Enlarged view of the region between *piwi2* and *rnf122* on sex chromosomes. (C) Phylogenetic tree of *rnf* family members on sex chromosomes of *Larimichthys crocea* and *L. polyactis*. The evolutionary history was inferred by using the maximum likelihood method and general time reversible model. The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. Evolutionary analyses were conducted in MEGA X.

Figure 5. Transcriptome correlation and heat map analysis.

Transcriptome analysis of three mixed pools of whole-fish samples of *L. polyactis* at 7, 10, 20, and 30 dph, where each pool had six fishes; from three mixed pools of indistinguishable gonads at 40 dph, where each pool had six gonads; and from thirteen pairs of ovary and testis mixed pools at 50, 60, 70, 80, 100, 130, 180, 240, 270, 340, 360, 400, and 720 dph, where each pool had three samples. The results showed that the correlation of gonads of different sexes at different periods had high quality and good correlation clustering. The positions of sex determination, differentiation or maintenance genes, including *cyp19a1a*, *figla*, *rnf183*, *foxl2*, *rnf225*, *gsdf*, *amh* and *dmrt1*, are marked in the picture.

Figure 6. *dmrt1* gene is involved in the hermaphrodite intersex stage in *L. polyactis*.

(A) Phylogenetic tree of *dmrt1* in hermaphroditic and gonochorial vertebrates. The evolutionary history was inferred by using the maximum likelihood method and general time reversible model. Evolutionary analyses were conducted in MEGA X. For the different sexual reproduction patterns, please refer to the legend in the figure. (B) Syntenic analyses of *dmrt1* and its adjacent genes in humans (*Homo sapiens*), mice (*Mus musculus*), lizards (*Anolis carolinensis*), frogs (*Xenopus laevis*), tilapia (*Oreochromis niloticus*), zebrafish (*Danio rerio*), fugu (*Takifugu rubripes*), seabass (*Lates calcarifer*), and large (*Larimichthys crocea*) and little yellow croaker (*Larimichthys polyactis*). The NCBI gene browser (<https://www.ncbi.nlm.nih.gov>) was used to make this figure. Gene symbols are described according to the browser. *L. polyactis*' gene symbols are based on the genome annotation. (C) Gene structure of *dmrt1* in different teleosts. The yellow rectangle represents exon 1, the light green rectangle represents exon 2, the dark green rectangle represents exon 3, the blue rectangle represents exon 4 and the red rectangle represents exon 5. The numbers below the black line represent the number of bases. (D) The gene structure of *dmrt1* and its upstream gene *rnf183*. *rnf183* has two exons and is 3197 bp away from *dmrt1*; the brown rectangle represents *rnf183* transcripts. (E) Tissue distribution and expression profiles of *dmrt1* in different developmental stages of the ovary and testis. Five indistinguishable sex stages and thirteen pairs of ovaries and testes from 7, 10, 20, 30, 40, 50, 60, 70, 80, 100, 130, 180, 240, 270, 340, 360, 400 and 720 dph (days post hatching) were sequenced using Illumina 2000 HiSeq

technology in the present study. A normalized measure of RPKM (reads per kilobase per million reads) was used to normalize the expression profiles of *dmrt1*. Different uppercase and lowercase letters above the error bars indicate significant differences in females or males at different time points, respectively, at $P < 0.05$, as determined by one-way ANOVA followed by Duncan's post hoc test. * and ** represent a significant difference at $P < 0.05$ and $P < 0.01$ between females and males, respectively, by independent t-test.

Figure 7. Phylogenetic analysis and expression profile of *rnf183* and its paralogues *rnf223* and *rnf225* in *L. polyactis*

(A) Phylogenetic tree of *rnf183*, *rnf223*, and *rnf225* in vertebrates. The evolutionary history was inferred by using the maximum likelihood method and general time reversible model. Evolutionary analyses were conducted in MEGA X. (B) Syntenic analyses of *rnf183* and its adjacent genes in humans (*Homo sapiens*), mice (*Mus musculus*), lizards (*Anolis carolinensis*), frogs (*Xenopus laevis*), tilapia (*Oreochromis niloticus*), zebrafish (*Danio rerio*), seabass (*Lates calcarifer*), and large (*Larimichthys crocea*) and little yellow croaker (*Larimichthys polyactis*). The NCBI gene browser (<https://www.ncbi.nlm.nih.gov>) was used to make this figure. Gene symbols are described according to the browser. *L. polyactis*' gene symbols are based on the genome annotation. (C) Tissue distribution and expression profiles of *rnf183*, *rnf223* and *rnf225* and in different developmental stages of the ovary and testis. The results are presented as the means $\pm$ SD from a sample of three individuals for each data point. Different lowercase and uppercase letters above the error bar indicate significant differences in females and males at different time points, respectively, at $P < 0.05$, as determined by one-way ANOVA followed by Duncan's post hoc test. * and ** represent a significant difference at $P < 0.05$ and $P < 0.01$ between females and males at the same time point, respectively, by independent t-test.

Figure 8. Sex-related gene expression profile in *L. polyactis* gonads.

Expression profiles of *amh*, *gsdf*, *cyp19a1a*, *figla* and *foxl2* based on tissue distributions and different developmental stages of the ovary and testis. The results are presented as the means $\pm$ SD from a sample of three individuals for each data point. Different lowercase and uppercase letters above the error bars indicate significant differences in females and males at different time points, respectively, at $P < 0.05$, as determined by one-way ANOVA followed by Duncan's post hoc test. * and ** represent a significant difference at $P < 0.05$ and $P < 0.01$ between females and males at the same time point, respectively, by independent t-test.

Tables

Table 1 The statistics of sequencing data for *L. polyactis* genome Assembly

Sequencing types	Insert size	Total data (G)	Read length (bp)	Sequence coverage (X)
Pacbio reads	20 Kb	48.12	—	68.74
Illumina	350bp	128.37	150	183.39
10X Genomics	—	137.28	150	196.11
bionano	—	117	—	167.6
Total	—	430.77	—	615.84

Table 2 Summary of *L. polyactis* genome assembly statistics

Total length	706,148,513 bp
Number of scaffolds	715
Number of contigs	3,134
Longest scaffold	20,429,779 bp
Contig N50 length	1,208,045 bp
Scaffold N50 length	4,519,237 bp
Scaffold N90 length	389,991 bp

Total length	706,148,513 bp
Genome characteristics	Genome characteristics
Percent assembled	102%
GC content	41.49%
Contents of repetitive elements	22.28%
Content of transposable elements	21.26%
Heterozygous SNP number and rate (SNPs per kb)	3,895,963 (0.575%)
Predicted protein-coding gene number	25,233
Predicted non-coding RNA gene number	5,671
Quantity of scaffolds anchored on chromosomes	309
Proportion of the genome is anchored to chromosomes	88.55%
Length of scaffolds anchored on chromosomes	625,267,019 bp

GC Guanine/Cytosine

Figures

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Cluster analysis of differentially expressed genes

Figure 6.

Figure 7.

Figure 8.