Whole-genome sequencing of leopard coral grouper (Plectropomus leopardus) and exploration of regulation mechanism of skin color and adaptive evolution

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ABSTRACT

Leopard coral groupers belong to the Plectropomus genus of the Epinephelidae family and are important fish for coral reef ecosystems and the marine aquaculture industry. To promote future research of this species, a high-quality chromosome-level genome was assembled using PacBio sequencing and Hi-C technology. A 787.06 Mb genome was assembled, with 99.7% (784.57 Mb) of bases anchored to 24 chromosomes. The leopard coral grouper genome size was smaller than that of other groupers, which may be related to its ancient status among grouper species. A total of 22 317 protein-coding genes were predicted. This high-quality genome of the leopard coral grouper is the first genomic resource for Plectropomus and should provide a pivotal genetic foundation for further research. Phylogenetic analysis of the leopard coral grouper and 12 other fish species showed that this fish is closely related to the brown-marbled grouper. Expanded genes in the leopard coral grouper genome were mainly associated with immune response and movement ability, which may be related to the adaptive evolution of this species to its habitat. In addition, we also identified differentially expressed genes (DEGs) associated with carotenoid metabolism between red and brown-colored leopard coral groupers. These genes may play roles in skin color decision by regulating carotenoid content in these groupers.

Keywords: Leopard coral grouper; Genome; Evolution; Skin color; Immune

INTRODUCTION

Groupers (Epinephelidae, Perciformes) are predators found in coral reef ecosystems, and include more than 160 species in 16 genera (Zhuang et al., 2013). They are commercially important in aquaculture, with approximately 47 grouper species cultured in East and Southeast Asia (Rimmer & Fan, 2013). These important aquatic species are closely related to the adaptive evolution of this species to its habitat.

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Glamuzina, 2019) and market demand increasing sharply worldwide. In China, the annual grouper output reached 160 000 tons in 2018 (Yang et al., 2020). Groupers are protogynous hermaphrodites, whereby they firstly differentiate as females, with some later changing to males (Adams, 2003). They have a complicated social system and the occurrence of sex reversal can be triggered by a change in social status (Chen et al., 2019). Thus, groupers are a good model for studies on sex inversion, speciation, and social systems.

The leopard coral grouper (*Plectropomus leopardus*), also called common coral trout, is a valuable marine fish belonging to the *Plectropomus* genus of the Epinephelidae family (Wang et al., 2015a). This fish is mainly distributed in the western Pacific regions of Western Australia, eastward to the Caroline Islands and Fiji and from southern Japan to Queensland, Australia (https://www.iucnredlist.org/species/44684/100462709). Wild populations have decreased due to the destruction of spawning aggregations and overfishing, and international protection measures (e.g., IUCN) for this fish are being considered. In recent years, artificial breeding technology has been successfully established for the leopard coral grouper. Moreover, these fish are considered an important resource for intensive industrial farming in recirculating aquaculture systems due to their high nutritional value, tender flesh, beautiful skin color, and high breeding density.

The substantial commercial and research value of leopard coral groupers has attracted the attention of aquatic scientists, including exploration on breeding (Burgess et al., 2020; Chen et al., 2016; Melianawati et al., 2013), feed additives (Yu et al., 2018), reproductive biology (Adams, 2003; Bunt & Kingsford, 2014; Khasanah et al., 2019; Metcalfe et al., 2018), phylogenetic analysis (Chen et al., 2018; Craig & Hastings, 2007; Ma et al., 2016; Xie et al., 2016), and skin color (Maoka et al., 2017; Wang et al., 2015a; Xie et al., 2016). However, the lack of genomic resources severely hinders deeper investigation of leopard coral groupers. So far, only two transcriptome analyses have been carried out with relatively limited gene sequences and expression information (Mekuchi et al., 2017; Wang et al., 2015a). Furthermore, only two grouper species genomes, i.e., red-spotted grouper *Epinephelus akaara* (Ge et al., 2019) and giant grouper *Epinephelus lanceolatus* (Zhou et al., 2019a), have been published. However, *Epinephelus* and *Plectropomus* species diverged from their most recent common ancestor (MRCA) a long time ago. At present, no valid genome has been reported for *Plectropomus* species. The assembly of a leopard coral grouper genome is essential for further research on evolutionarily status, molecular-assisted selection, sex reversal, and quantitative trait locus (QTL) mapping of traits of interest in *Plectropomus* species.

Body color is an important economic trait in aquatic animals, especially for the leopard coral grouper. Its integument is mainly divided into black, brown, or red color (Maoka et al., 2017), which is an important factor in determining fish quality as Chinese markets prefer those fish with a bright red color, which sell for twice the price of black fish. Recent research has shown that different proportions of carotenoids, such as tunaxanthin, astaxanthin, and tunaxanthin, determine skin color in this species (Maoka et al., 2017). Several studies related to skin color in this fish have also been carried out in our laboratory. For example, 38 candidate genes underlying the mechanism of color and pigmentation were detected based on comparative transcriptome analysis (Wang et al., 2015a); a total of 74 single nucleotide polymorphisms and one indel were identified in the complete mitochondrial genome of red and gray fish (Xie et al., 2016); and clear differences between light-red and gray fish in COI, ND2, and D-loop sequences were observed via mitochondrial DNA analysis (Cai et al., 2013; Van Herwerden et al., 2009). Thus, to improve leopard coral grouper traits, it is essential that the molecular mechanism regulating skin color is explored.

An elaborate reference genome could provide a strong foundation for further research on the leopard coral grouper. Third-generation sequencing technologies have improved the extension of sequencing reads and provide superior platforms to produce complete and exact genomes (Quail et al., 2012). Here, we used PacBio long-read sequencing (Eid et al., 2009) and high-throughput chromosome conformation capture (Hi-C) technology (Lieberman-Aiden et al., 2009) to assemble a high-quality chromosome-level genome of the leopard coral grouper. This should provide important genomic resources for subsequent investigations on sex reversal, genetic evolution, physiological regulation, and molecular breeding. Based on the high-quality genome, comparative genomes and transcriptomes were used to analyze the adaptive evolution and regulation of skin color in leopard coral groupers.

**MATERIALS AND METHODS**

**Ethics statement**

All experiments in the present study were approved by the Animal Care and Use Committee of the School of Life Sciences, Sun Yat-Sen University, Guangdong, China.

**Animal preparation for genome sequencing**

One leopard coral grouper (Figure 1A) sampled from the Tropical Marine Aquatic Breeding Center, Wenchang, Hainan Province, China, was used for genome sequencing and assembly. The fish was immediately dissected after anesthesia with MS-222. White muscle tissue was sampled for DNA extraction, which was then used for genomic DNA sequencing and Hi-C library construction. To assist annotation of genes, 10 tissues, including skin, muscle, liver, kidney, brain, intestine, fat, spleen, heart, and gill, were collected, and then stored in RNAlater for RNA isolation.

**Library construction and genome assembly**

Total DNA was extracted from white muscle tissue with a TIANamp Marine Animals DNA Kit (Tiangen Biotech Co., Ltd., China). Quality and quantity of total DNA were determined by NanoDrop 2000 (Thermo Fisher Scientific Inc., USA). A paired-end sequencing library was constructed using a
seed reads, and low-quality reads were trimmed; the revised were set as seed reads, other PacBio data were aligned to the Canu v1.8 (Koren et al., 2017). Simply, the longest subreads polymerase reads (subreads) were used for assembly with polymerase reads linked with the barcode adapter, clean short polymerase reads, low-quality polymerase reads, and using 19-mer. For the PacBio sequencing data, after removing data were used to estimate genome size and heterozygosity harboring Phred quality scores of ≤20. The remaining clean nucleotides (N); and (c) low-quality reads with >50% bases with the barcode adapter; (b) reads with ≥10% unidentified stringent filtering standards: i.e., removing (a) reads linked with the barcode adapter; (b) reads with ≥10% unidentified nucleotides (N); and (c) low-quality reads with >50% bases harboring Phred quality scores of ≤20. The remaining clean data were used to estimate genome size and heterozygosity using 19-mer. For the PacBio sequencing data, after removing short polymerase reads, low-quality polymerase reads, and polymerase reads linked with the barcode adapter, clean polymerase reads (subreads) were used for assembly with Canu v1.8 (Koren et al., 2017). Simply, the longest subreads were set as seed reads, other PacBio data were aligned to the seed reads, and low-quality reads were trimmed; the revised seed reads were assembled into contigs based on overlap of seed reads. The contigs were corrected by alignment of PacBio data using pbmm2 v1.1.0 (https://github.com/PacificBiosciences/pbmm2) and GenomicConsensus: Arrow (https://github.com/PacificBiosciences/GenomicConsensus). Consensus correction was performed based on Illumina data using BWA v0.7.17 (Li & Durbin, 2010) and Pilon v1.16 (Walker et al., 2014). Lastly, redundant contigs were detected and selectively removed from de novo assembly using Redundans (Przybucz & Gabaldón, 2016).

For estimating genome completeness, Illumina data were first mapped back to the leopard coral grouper genome to calculate the mapping rate using BWA v0.7.17 (Li & Durbin, 2010). The orthologs of the leopard coral grouper were then aligned to a reference gene set from the Actinopterygii database (Actinopterygii_odb9), which was constructed from 20 fish species consisting of 4 584 orthologs using Benchmarking Universal Single-Copy Orthologs (BUSCO).

**Pseudochromosome construction**

Hi-C was performed to assist in the construction of the chromosome-level leopard coral grouper genome. Muscle samples were fixed with fresh paraformaldehyde and then DNA-protein bonds were created. The Mbo I restriction enzyme was used to digest the DNA and the overhanging 5’ ends of the DNA fragments were repaired with a biotinylated residue. The fragments close to each other in the nucleus during fixation were ligated and the denatured proteins were removed. The Hi-C fragments were further sheared by sonication into 350 bp fragments, which were then pulled-down with streptavidin beads. The library was sequenced on the Illumina HiSeq X Ten platform with PE150. To anchor scaffolds into pseudochromosomes, JUCER v1.6.2 (https://github.com/aidenlab/juicer) was first used to map and process the reads. The misjoins in the scaffolds were eliminated using 3D *de novo* assembly (3D-DNA) software (Dudchenko et al., 2017).

**Annotation of repeats**

Tandem repeats were predicted using TRF v4.07b (Benson, 1999) and transposable elements (TEs) were identified using LTR Finder (http://life.fudan.edu.cn/life/ltr_finder/) (Xu et al., 2010), RepeatScout v1.05 (http://www.repeatmasker.org), and PILER v1.0 (Edgar & Myers, 2005). Coding sequences were removed from the predicted repeat sequences through alignment to the SwissProt database using blastx with e-value ≤1e−4, identity ≥30, coverage ≥30%, and length ≥90 bp. In addition, non-coding RNA (ncRNA) was removed from the predicted repeat sequences via alignment in Rfam 11.0 using blastn with e-value ≤1e−10, identity ≥80, and coverage ≥50%. The predicted TEs were aligned to the RepBase and TE protein database using WU-BLAST to remove simple repeats, satellites, and ncRNAs. The TEs were classified using RepeatClassifier. For removing redundant repeat sequences, the predicted repeat sequences were aligned with each other using blastn with e-value ≤1e−10, identity ≥80, coverage ≥80%, and length ≥80bp. The repeat regions were searched

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**Figure 1 Leopard coral grouper (Plectropomus leopardus)**

A: Bright red fish (used for genome assembly). B: Brown fish. Scale bars: 10 cm.
against RepBase using RepeatMasker v3.3.0 and against the TE protein database using RepeatProteinMask v3.3.0. Finally, the repeat sequences in the scaffold were masked using RepeatMasker v3.3.0 and RepeatProteinMask v3.3.0.

ncRNA and mRNA annotation
Non-coding RNA includes ribosomal RNA (rRNA), transfer RNA (tRNA), long non-coding RNA (lncRNA), small nuclear RNA (snRNA), small nucleolar RNA ( snoRNA), micro RNA (miRNA), and other unknown RNA. Here, tRNA was predicted using rRNAscan-SE v1.3.1 (Lowe & Eddy, 1996) and rRNA, snRNA, snoRNA, and microRNA were predicted following alignment to Rfam (ftp://ftp.ebi.ac.uk/pub/databases/Rfam).

Protein-coding genes were predicted via alignment of homologous sequences and RNA-seq assisted methods. For predicting homologous sequences, protein sequences of Oreochromis niloticus, Cyprinus carpio, Salmo salar, Larimichthys crocea, and Oryzias latipes were downloaded from the NCBI database and aligned to the repeat masked genome using GeMoMa v1.4.2 (Keilwagen et al., 2016). Moreover, RNA-seq data from the 10 tissues were aligned to the genome using HISAT2 v2.0.4 (Kim et al., 2015) and assembled using Cufflinks v2.2.1 (Trapnell et al., 2012). Open reading frames (ORFs) were predicted using PASA v2.0.1. The gene structure was predicted based on assembled RNA-seq data using Augustus v3.2.2 (Stanke et al., 2006), SPNPAP (Leskovc & Sosić, 2016), GlimmerHMM v3.01 (Majoros et al., 2004), and GeneMark-ET v4.21 (Lomsadze et al., 2014). Gene predictions based on the above results were merged using EVM (http://evidencemodeler.sourceforge.net/) (Haas et al., 2008).

Functional annotations of the predicted genes in the leopard coral grouper genome were performed based on several public databases, including NCBI-NR, KEGG, eggNOG, and SwissProt using blast+ (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/) with e-value ≤1e–05. We performed gene ontology (GO) annotation using Blast2GO (Conesa et al., 2005). We also used InterProScan v4.7 (Jones et al., 2014) to obtain protein domain annotations in the InterPro database, which contains multiple protein databases including CATH-Gene3D, CDD, HAMAP, PANTHER, Pfam, PIRSF, PRINTS, ProDom, PROSITE, SMAT, SUPERFAMILY, and TIGRFAMs. Lastly, functional annotations of the best alignments in each database were used as the final consensus gene annotation results.

Genome evolution analysis
For identification of gene families, we downloaded 12 fish species genomes, including zebra fish (Danio rerio, GCF_000002035.6), large yellow croaker (Larimichthys crocea, GCF_000972845.2), Atlantic salmon (Salmo salar, GCF_000233375.1), Asian swamp eel (Monopterus albus, GCF_001952655.1), Atlantic cod (Gadus morhua, GCF_902167405.1), rainbow trout (Oncorhynchus mykiss, GCF_002163495.1), barramundi (Lates calcarifer, GCF_001640805.1), tilapia (Oreochromis niloticus, GCF_001858045.2), coelacanath (Latimeria chalumnae, GCF_000225785.1), medaka (Oryzias latipes GCF_002234675.1), lancelet (Branchiostoma belcheri, GCF_001625305.1), and brown-marbled grouper (E. fuscoguttatus, not published) for comparison using all to all BLAST with an e-value of 1e–5. The Markov Cluster Algorithm (MCL) was then used to cluster the alignments into gene families using OrthoMCL with inflation 1.5.

A phylogenetic tree was constructed using shared single-copy genes of the leopard coral grouper and other fish species mentioned above. Protein sequences of these single-copy genes were aligned using MUSCLE v3.7 (Edgar, 2004). The phylogenetic tree was constructed using PhyML software v3.0 (Guindon et al., 2010) with the maximum-likelihood (ML) algorithm. Collinearity analysis of the leopard coral grouper and brown-marbled grouper genomes was carried out using MUMmer v4 (Kurtz et al., 2004). The divergence times in each tree node were predicted using MCMAntree in the PAML package (Yang, 1997). Three calibration times were obtained from the TimeTree database (Kumar et al., 2017).

The expansion and contraction of orthologous gene families between the ancestor and other species were predicted using CAFE v1.6 (De Bie et al., 2008). A random death model was used to study changes in gene families along each lineage of the phylogenetic tree. A probabilistic graphical model (PGM) was introduced to the probability of transitions in gene family size from parent to child node phylogeny. Functional prediction of expanded and contracted genes was carried out based on the GO and KEGG pathway databases.

Detection of differentially expressed genes (DEGs) among leopard coral groupers with different body color using RNA-seq
The high-quality chromosome-level leopard coral grouper genome provides an important reference for transcriptome analysis. To determine transcriptome differences between bright red (Figure 1A, 517.7±82.47 g) and brown fish (Figure 1B, 504.3±95.6 g), the skins of three bright red and brown fish (Figure 1A, 517.7±82.47 g) and brown fish (Figure 1B, 504.3±95.6 g) were sampled from the Tropical Marine Aquatic Breeding Center, Wenchang, Hainan Province, China. Isolation of total RNA and library construction were performed using the same methods as described above. Trimmmomatic v0.39 (Bolger et al., 2014) was used to filter raw data. The clean data were mapped to the leopard coral grouper genome using Tophat2 v2.1.1 (Trapnell et al., 2009). Read count statistics were determined using HTSeq v0.11.1 (Anders et al., 2015), R package, Edger v3.1.0 (Robinson et al., 2010), and Limma v3.42.2 (Ritchie et al., 2015) were applied to identify DEGs between the two groups.

RESULTS
Genome assembly and assessment
A total of 94.77 Gb of Illumina raw reads were obtained. After removal of low-quality reads, adapter sequences, and polymerase chain reaction (PCR)-duplicates, the size of the leopard coral grouper genome using the K-mer approach was
estimated to be 858 Mb, with 39.54% GC content (Figure 2A). The heterozygosity and repeats were approximately 0.42% and 23%, respectively. A total of 106.10 Gb (×145) of PacBio sequencing data were used for de novo assembly of the genome (Supplementary Table S1 and Figure S1). The mean length and N50 were 12 893 bp and 18 054 bp, respectively. The reads and base number of fragments longer than 5 Kb were 6 831 058 and 101.47 Gb, respectively. The genome assembly spanned 787.06 Mb with a contig N50 of 1.14 Mb and GC content of 39.56% (Figure 2E), and was deposited in the DDBJ database. BUSCO was used to verify the integrality of the assembled genome. BUSCO alignment showed that our final assembly contained 4 149 complete BUSCOs (90.5%), including 4 034 single-copy and 115 duplicated BUSCOs (Supplementary Table S2).

**Pseudochromosome construction**

Hi-C was applied for genome assembly. A total of 99.5 Gb of clean bases were obtained with Q20 of 98.26% and Q30 of 94.77%. The mapped ratio of Hi-C reads in the genome was 78.96%. Based on the Hi-C results, a total of 1 091 contigs were anchored into 24 pseudochromosomes (Chr) of the leopard coral grouper, and a total length of 784.57 Mb was assembled, which comprised 99.62% of assembled genome.

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**Figure 2 Statistics on genome assembly and annotation of leopard coral grouper (Plectropomus leopardus)**

A: Estimation of genome size, repeat content, and heterozygosity by survey using 19-mers. B: Genome contig contact matrix using Hi-C data, color bar indicates contact density from red (high) to white (low). C: Functional annotated protein-coding genes. D: Summary statistics of non-coding RNA. E: Statistics of leopard coral grouper genome assembly.
sequences (Supplementary Table S3 and Figure 2B). The longest and shortest pseudochromosome lengths were 42 685 566 bp and 18 735 579 bp, respectively.

Repetitive element annotation
We used de novo and homologue prediction to detect repeat sequences (Supplementary Table S4). In the leopard coral grouper, a total of 1 705 874 repeat sequences with a length of 272 473 600 bp were detected, accounting for 34.596% of the whole genome. Among them, we found 808 677 DNA repeat elements (DNA, 111 693 098 bp, 14.181%); 135 729 long interspersed nuclear elements (LINE, 26 212 322 bp, 3.329%); 57 382 long terminal repeat elements (LTR, 9 810 107 bp, 1.245%); and 16 333 short interspersed nuclear elements (SINE, 2 350 545 bp, 0.298%).

ncRNA and mRNA annotation
A total of 2 483 ncRNAs were detected, including 1 042 tRNAs, 623 snRNAs, 545 miRNAs, and 246 rRNAs (Figure 2D and Supplementary Table S5).

Based on the repeat-masked genome of the leopard coral grouper, a total of 22 317 genes were detected using de novo prediction and homology searching. Based on the homologous protein sequences of Oreochromis niloticus, Cyprinus carpio, Salmo salar, Larichthys crocea, and Oryzias latipes, a total of 10 600–21 861 proteins were obtained. Based on RNA-seq data, a total of 28 329 genes were detected. Based on de novo methods, a total of 28 713–51 359 genes were detected (Supplementary Table S6). After removing redundant genes and merging, a total of 22 317 genes were found with a total length of 463 272 851 bp and mean length of 20 758 bp; a total of 25 558 transcripts were obtained with a total length of 79 212 336 bp and mean length of 3 099 bp. There were 22 000 complete ORFs (98.8%), 25 172 complete transcripts (98.4%), and 286 860 exons and 261 302 introns detected in the leopard coral grouper genome. The mean lengths of exons and introns were 276 bp and 1 890 bp, respectively. The average exon number for each gene was 11.2 and the average length of coding sequence (CDS) was 1 836 bp (Supplementary Table S6).

To understand gene function, the predicted genes were mapped against several public databases. A total of 20 921 predicted genes were mapped to these databases, including 20 107, 20 907, 13 540, 13 609, and 19 510 genes mapped to the eggNOG, NR, GO, KEGG, and SwissProt databases, respectively. A total of 10 323 genes were mapped to all these databases (Figure 2C). For KEGG, a total of 41 terms were enriched, with the top two terms being signal transduction and infectious diseases (Supplementary Figure S2). For GO, a total of 60 terms were enriched, with the top two terms being integral component of membrane and ATP binding (Supplementary Figure S3). For eggNOG, a total of 24 terms were enriched, with the top two terms being cell wall/membrane/envelope biogenesis and intracellular trafficking, secretion, and vesicular transport (Supplementary Figure S4).

Genome evolution analysis
Orthologs of the leopard coral grouper and 12 other fish species (mentioned above) were predicted. A total of 16 974 gene families were obtained (Figure 3A), including 1 211 (9.7%) common single-copy gene families. In the leopard coral grouper, the number of single-copy, multi-copy, and unique genes were 4 200, 4 040, and 384, respectively (Figure 3B). The common single-copy genes were used to construct the phylogenetic tree. Brown-marbled grouper was most closely related to the leopard coral grouper, followed by the large yellow croaker. Two other Perciformes species, tilapia and L. calcarifer, were relatively distant from the leopard coral grouper. Tilapia and medaka were clustered into one clade, whereas L. calcarifer and Asian swamp eel were clustered into another clade (Figure 3D). The genomes of brown-marbled groper and leopard coral grouper were compared (Figure 3C) and showed relatively high genomic synteny. Divergence times were predicted using single-copy orthologous genes. The divergence time of leopard coral grouper and brown-marbled groper was about 49.3 (32.5–65.9) million years ago. The ancestor of the grouper separated from the large yellow croaker about 75.7 (62.9–96.9) million years ago (Figure 4A). Changes in gene families were also predicted. A total of 39 expanded and 86 contracted gene families were observed in the leopard coral grouper (Figure 4A). The expanded gene families were mainly enriched in acetylcholine biosynthetic process, chitin catabolic process, and immune response-related terms based on the GO database (Figure 4B and Supplementary Table S7). The gene families enriched in acetylcholine biosynthetic process were high affinity choline transporters. The gene families associated with chitin catabolic process were acidic mammalian chitinases (AMCase). The gene families enriched in immune response were immunoglobulin heavy chain (IGH) and beta-2-microglobulin (B2M), PYPAF1, NACHT, LRR, and PYD domain-containing protein (NLRP) (Figure 4C).

Detection of DEGs associated with carotenoid between fish with different body colors using RNA-seq
To analyze the differences in transcriptome profiles among leopard coral groupers with different body colors, we obtained the skin transcriptome profiles of three bright red and three brown leopard coral groupers using Illumina sequencing. A total of 47.42 Gb of raw data were obtained with an average Q20 of 96.7% and Q30 of 96.2% (Table S8). Based on the edgeR and Limma methods, a total of 323 DEGs were discovered, including 31 and 302 up-regulated DEGs in brown and bright red fish, respectively (Supplementary Table S5). Potential genes associated with skin color and carotenoid metabolism were collected (Table 1). In the up-regulated DEGs of brown fish, an important gene associated with oxidoreduction of carotenoid, beta-carotene 9,10'-oxygenase (BCO2), was discovered. In red fish, several genes associated with carotenoid and liquid transportation were upregulated, such as low-density lipoprotein receptor-related protein 11 (LRP11) and angiopeitin-related proteins (ANGPTLs).
DISCUSSION

Leopard coral groupers are an important marine fish species in coral reef ecosystems and have huge commercial value. This species is an excellent research model due to its special biological characteristics and evolutionary status. In this study, we first assembled a high-quality chromosome-level leopard coral grouper genome, thus providing pivotal genomic resources for further studies on the evolution, molecular-assisted selection, and genetic mechanisms of the special biological characteristics of groupers.

Genome assembly and assessment

Estimation of basic genome characteristics is important for adoption of appropriate assembly strategies. The K-mer method is an efficient tool for estimating genome properties (Liu et al., 2013). The estimated genome size of the leopard coral grouper was 858 Mb, higher than the final genome size (784.57 Mb) based on PacBio sequencing data. This difference may be due to collapse of repetitive elements and statistical differences in the methods. Similar results have also been reported in previous research, e.g., scallop (*Patinopseten yessoensis*) (Wang et al., 2017), Murray cod (Austin et al., 2017), and pike-perch (Ngunkal et al., 2019). In addition, the leopard coral grouper genome was smaller than that of other grouper genomes, including 1.135 Gb for red-spotted grouper (Ge et al., 2019), 1.086 Gb for giant grouper

Figure 3 Prediction of gene families and analysis of genetic relationship

A: Flower chart of gene family numbers in 13 fish species. Ple: P. leopardus, Efu: E. fuscoguttatus, Dre: D. rerto, Lcr: L. crocea, Ssa: S. Salar, Mal: M. albus, Gmo: G. morhua, Omy: O. mykiss, Lca: L. calcarifer Oni: O. niloticus, Lch: L. chalumnae, Ola: O. latipes, Bbe: B. belcheri. B: Gene family clustering for leopard coral grouper and other 12 fish species. C: Collinearity analysis of leopard coral grouper and brown-marbled grouper, orange arrows indicate genome of leopard coral grouper and blue arrows indicate genome of brown-marbled grouper. D: Phylogenetic tree of leopard coral grouper and other 12 fish species, with Branchiostoma belcheri set as outgroup.
The genome sizes of most Perciformes species were smaller than that of the leopard coral grouper (Supplementary Table S9), including two closely related species, i.e., large yellow croaker and three-spine stickleback (Gasterosteus aculeatus). Therefore, our study suggests that the leopard coral grouper is in a basal evolutionary status in the grouper lineage, which agrees with previous studies on molecular and morphological phylogeny (Craig & Hastings, 2007). Moreover, we also observed lower repeat content in the leopard coral grouper (34.6%) than in the red-spotted grouper (43.02%) and giant grouper (41.01%) (Ge et al., 2019, Zhou et al., 2019a). Strong correlations between repeat content and the genome have been observed in Perciformes (Nguiinkal et al., 2019). Heterozygosity was approximately 0.42% in the leopard coral grouper, higher than that in the red-spotted grouper (Epinephelus akaara, 0.375%) (Ge et al., 2019), Murray cod (Maccullochella peeli, 0.113%) (Austin et al., 2017), pike-perch (Sander lucioperca, 0.14%) (Nguiinkal et al., 2019), Triplophysa tibetana (0.1%) (Yang et al., 2019a), and male (Ctenopharyngodon idellus, 0.25%) and

Figure 4 Analysis of divergence time and gene family of leopard coral grouper with other fish species
A: Phylogenetic tree with dynamic evolution of gene families and divergence time. MRCA: Most recent common ancestor. B: GO enrichment of expanded gene families in leopard coral grouper. C: Sketch map of expanded gene families related to immune response. Red font indicates expanded gene families.

Zhou et al., 2019a), 1.047 Gb for brown-marbled grouper (not published), and 1.02 Gb for orange-spotted grouper (Epinephelus coioides, unpublished data). The genome sizes of most Perciformes species were smaller than that of the leopard coral grouper (Supplementary Table S9), including two closely related species, i.e., large yellow croaker and three-spine stickleback (Gasterosteus aculeatus). Therefore, our study suggests that the leopard coral grouper is in a basal evolutionary status in the grouper lineage, which agrees with previous studies on molecular and morphological phylogeny (Craig & Hastings, 2007). Moreover, we also observed lower repeat content in the leopard coral grouper (34.6%) than in the red-spotted grouper (43.02%) and giant grouper (41.01%) (Ge et al., 2019, Zhou et al., 2019a). Strong correlations between repeat content and the genome have been observed in Perciformes (Nguiinkal et al., 2019). Heterozygosity was approximately 0.42% in the leopard coral grouper, higher than that in the red-spotted grouper (Epinephelus akaara, 0.375%) (Ge et al., 2019), Murray cod (Maccullochella peeli, 0.113%) (Austin et al., 2017), pike-perch (Sander lucioperca, 0.14%) (Nguiinkal et al., 2019), Triplophysa tibetana (0.1%) (Yang et al., 2019a), and male (Ctenopharyngodon idellus, 0.25%) and
found to be most closely related to the brown-marbled grouper, suggesting that the brown-marbled grouper may be more adaptable to benthic life in coral reef ecosystems than the leopard coral grouper.

Table 1 Differentially expressed genes (DEGs) between brown and red leopard coral grouper transcriptomes

| ID            | log2 (Foldchange) | q-value | Description                                      | Gene symbol |
|---------------|-------------------|---------|-------------------------------------------------|-------------|
| Chr04.g0131.m1 | −2.64             | 2.12E−04| Beta-carotene 9’,10’-oxygenase                    | BCO2        |
| Chr12.g11897.m1| −2.41             | 1.14E−03| Eosinophil peroxidase                            | EPX         |

Up-regulated candidate DEGs associated with skin color in brown fish

| ID            | log2 (Foldchange) | q-value | Description                                      | Gene symbol |
|---------------|-------------------|---------|-------------------------------------------------|-------------|
| Chr16.g1572.m1| 2.60              | 2.08E−04| Low-density lipoprotein receptor-related protein 11 | LRP11       |
| Chr05.g04888.m1| 2.13             | 7.79E−04| Angiopoietin-related protein 4                   | ANGPTL4     |
| Chr15.g14859.m1| 3.80             | 8.48E−05| High affinity cationic amino acid transporter 1  | SLC7A1      |
| Chr10.g09889.m1| 2.68             | 7.40E−04| Protein Wnt-2b                                   | WNT2B       |

female grass carp (0.09%) (Wang et al., 2015b). The relatively high heterozygosity implies high population genetic diversity for the leopard coral grouper and a lack of selective breeding in aquaculture.

Though the relatively high heterozygosity increased the difficulty of genome sequencing and assembly in the leopard coral grouper, we obtained the high-quality chromosome-level genome based on PacBio sequencing and Hi-C technology. The mean length and N50 of PacBio reads were 12 893 bp and 18 054 bp, respectively. The contig N50 was 1.14 Mb, higher than that of most published aquaculture species, e.g., arapaima (Arapaima gigas, female: 325 Kb and male: 285 Kb) (Du et al., 2019); Glyptosternon maculatum (993 Kb) (Shao et al., 2018), sea bass (Lateolabrax maculatus, 31K) (Shao et al., 2018), northern snakehead (Channa argus, 81.4K) (Liu et al., 2017), and blunt snout bream (Megalobrama amblycephala, 49.4K) (Liu et al., 2017). Lastly, a total of 1 091 contigs were anchored to 24 pseudochromosomes of the leopard coral grouper, consistent with the number of chromosomes in other groupers (Ge et al., 2019; Zhou et al., 2019a).

Genome annotation

Through de novo prediction and homologue annotation, a total of 22 317 protein-coding genes were detected in the leopard coral grouper genome. The number of predicted genes was slightly less than that in other groupers, such as red-spotted grouper (23 808) (Ge et al., 2019), giant grouper (24 718) (Zhou et al., 2019b), and orange grouper (24 593). These differences may result from the different statistical methods or may be induced by gene family expansion in Epinephelus species after dividing from a common ancestor. The number of protein-coding genes was similar to other Perciformes fish such as pike-perch (21 249) (Nguinkal et al., 2019), Chinese sillago (Sillago sinica, 22 122) (Zhou et al., 2018), spotted sea bass (Lateolabrax maculatus, 22 015), northern snakehead (19 877) (Liu et al., 2017), and Eurasian perch (Perca fluviatilis, 23 397) (Ozerov et al., 2018). The comprehensive annotated genes provide essential information for multi-omics analysis of the leopard coral grouper.

Genome evolution analysis

Based on the phylogenetic tree, the leopard coral grouper was found to be most closely related to the brown-marbled grouper, followed by the large yellow croaker. There was significant genomic synteny between the leopard coral grouper and brown-marbled grouper. Several genes in the brown-marbled grouper did not match the leopard coral grouper genome, which may be due to gene family expansion in the brown-marbled grouper. Compared to the leopard coral grouper genome, we observed 129 gene families were expanded after divergence of the two fish species, and just 23 gene families were contracted in the brown-marbled grouper. In the leopard coral grouper, we identified 39 and 86 expanded and contracted gene families, respectively.

The expanded gene families were mainly associated with immune response. Chitin is an important structural component of parasites, bacteria, and fungi, and can fix a multitude of antigenic glycoproteins, and thus its degradation plays a crucial role in determining subsequent immune responses (Van Dyken & Locksley, 2018). Structural chitin presented in microorganisms can be degraded using acidic mammalian chitinases (Elieh Ali Komi et al., 2018). NLRP3s are important regulatory factors in inflammatory responses, which increase cleavage of caspase-1 and levels of ASC and mature IL-1 (Ising et al., 2019). Beta-2-microglobulin is an important component of the class I major histocompatibility complex (MHC) and is involved in the presentation of peptide antigens to the immune system (Sreejit et al., 2014). Moreover, CD22 is an inhibitory receptor in B cell-specific transmembrane and a negative regulator of B cell antigen receptor signaling (Kelm et al., 2002). Immunoglobulins, also called antibodies, are membrane-bound or secreted glycoproteins produced by B lymphocytes with important functions in humoral immunity. Immunoglobulin heavy chains participate in antigen recognition and presentation (McHeyzer-Williams et al., 2012). These expanded gene families suggest that evolutionary changes in immune response and movement ability may have contributed to the adaptation of leopard coral groupers to their habitat. In addition, high-affinity choline transporters were also expanded, which are important proteins in regulation of movement (Banerjee et al., 2019). The mutant of this protein can induce severe dyskinesia in humans (Banerjee et al., 2019, Pardal-Fernández et al., 2018). A greater number of these genes were found in the leopard coral grouper than in the brown-marbled grouper, suggesting that the brown-marbled grouper may be more adaptable to benthic life in coral reef ecosystems than the leopard coral grouper.
Detection of DEGs between leopard coral groupers with different body colors using RNA-seq

Skin color in leopard coral groupers is an important commercial trait, which can substantially affect market price. Carotenoids, including astaxanthin, tunaxanthin, α-cryptoxanthin, and adonirubin, are the direct molecules for regulation of body color in leopard coral groupers (Maoka et al., 2017). Individuals with more astaxanthin tend to possess red skin color.

To explore the molecular mechanism of body color differences in leopard coral groupers, three red and three brown fish were used for comparative transcriptome analysis. Among the highly expressed DEGs in brown fish, BCO2 is an important gene in the regulation of carotenoid metabolism, which catalyzes the degradation of carotenoids to induce differences in skin color (Strychalski et al., 2015). This gene may influence the proportion of carotenoids, resulting in the diversity of skin color observed in leopard coral groupers. Several studies have demonstrated that BCO2 is associated with body color. For example, mutation in BCO2 can change skin color in chickens (Eriksson et al., 2008); and nonsense mutation in BCO2 can increase the concentration of carotenoids and change white fat to yellow in sheep (Våge & Boman, 2010). Here, in the red fish, several genes related to carotenoid transport, including LRP11, ANGPTL4, and ANGPTL2, were up-regulated. De novo synthesis of carotenoid is difficult in fish (Ho et al., 2013), and thus uptake efficiency can affect accumulation of carotenoids. Carotenoid transport in organisms is important for the digestion of carotenoid. Genes associated with lipid transport are major regulators of skin color and can affect the deposition of pigment (Chintala et al., 2005). Low-density lipoprotein (LDL) and chylomicron are important carriers of carotenoid in plasma (Harrison, 2019). LDL receptor-related protein (LRP) can bind with LDL and chylomicron (Lillis et al., 2015); in addition, ANGPTLs are associated with lipid utilization and storage via regulation of hydrolysis of lipids from chylomicrons and very low-density lipoproteins (VLDL) (Lei et al., 2011; Lutz et al., 2001). Therefore, changes in these genes may influence the accumulation of carotenoids and differences of skin color in leopard coral groupers.

CONCLUSIONS

In the present study, we assembled a high-quality chromosome-level genome of the leopard coral grouper using PacBio sequencing and Hi-C technology. A 787.06 Mb genome with contig N50 of 1.14 Mb and GC content of 39.56% was assembled, with 784.57 Mb (99.7%) of the genome anchored to 24 chromosomes with N50 length of 33.8 Mb. A total of 22 317 protein-coding genes were predicted, among which 22 238 (99.65%) were anchored to chromosomes and 20 921 (93.74%) were functionally annotated. This high-quality leopard coral grouper genome provides important genomic resources for further research on this species. Based on the high-quality genome, we also performed genome evolution analyses, which should help to improve our understanding of the adaptive evolution of this species. The comparative transcriptome showed that carotenoid metabolism may play an important role in the regulation of skin color in leopard coral groupers.

DATA AVAILABILITY

The leopard coral grouper genome was deposited in the DNA Data Bank of Japan (DDBJ) database. The accession No. is PRJDB9154. The accession No. of chromosomes are AP022700-AP022723; accession Nos. of contigs unanchored into chromosomes are AP022724-AP022809. Accession No. of transcriptome data for annotation is PRJDB9167. The PacBio sequencing data used for assembly of leopard coral grouper genome was deposited in Genome Sequence Archive (GSA) and accession No. is PRJCA002371. The raw data of RNA-seq for skin analysis of leopard coral grouper was deposited in GSA and accession No. is PRJCA002372.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS’ CONTRIBUTIONS

Y.Y., Z.N.M., and X.C.L. designed the study. Y.Y. and L.N.W. collected the samples. Y.Y., X.W., and Z.N.M. performed the laboratory work. Y.Y. and J.F.C. performed analyses. Y.Y., Z.N.M., X.C.L., J.H.X., and H.R.L. drafted and revised the paper. All authors read and approved the final version of the manuscript.

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