Hindawi
Journal of Applied Ichthyology
Volume 2023, Article ID 4135906, 9 pages
https://doi.org/10.1155/2023/4135906

Research Article

Identification of Genes and SNPs Related to Body Colors by Transcriptome Profiling in Leopard Coral Grouper (*Plectropomus leopardus* Lacépède)

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Received 24 October 2022; Revised 31 March 2023; Accepted 5 April 2023; Published 5 May 2023

Academic Editor: Hamed Ghafarifarsani

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Body colors are an important trait for the survival and reproduction of animals. In this study, transcriptomes and related single nucleotide polymorphisms (SNPs) of black- and red-colored *Plectropomus leopardus* (Lacépède) were identified to investigate important molecules and SNP markers associated with body color formation. In the comparison of red- and black-colored groups, 489 differential expressed genes (DEGs) were detected and among them, 236 up- and 253 down-regulated genes were found in the red-colored group. Gene ontology (GO) analysis of DEGs showed that pigmentation associated terms including pigment granule, pigmentation, melanin metabolic process, tyrosine metabolic process, pigment metabolic process, and pigmentation cell differentiation were enriched. Kyoto encyclopedia of genes and genomes (KEGG) analysis of DEGs presented that melanin related pathways (tyrosine metabolism and melanogenesis) were enriched. Furthermore, lipid metabolism and immune-related metabolism were also enriched. A total of 1,048,575 SNPs were detected from the transcriptome, and among them, 627,172 SNPs were located in the gene region. A total of 1323 SNPs were identified as differential SNPs in the comparison of black- and red-colored groups and were located in 1127 genes. Among the 1127 genes, 20 genes were identified as DEGs and were comprised of 9 SNPs with a potential role in body color selective breeding in fish farming.

1. Introduction

Plectropomus leopardus (Lacépède) belongs to the Serranidae family. As a leopard coral grouper, it is essential in ocean ecosystems as a carnivorous predator and inhabits tropical or subtropical waters. It is also important and popular among customers due to its bright body color with high ecological and economic value [1]. Body color of *P. leopardus* is an essential quality standard that determine its economic value, and prices of red or bright ones are higher than dark or black grouper, especially in Chinese markets because of the human preference of bright ones [2–6]. Therefore, selecting the genetically improved body color of *P. leopardus* is essential.

Body color formation was widely reported in fishes such as *Oncorhynchus tshawytscha* and *Cyprinus carpio* [7, 8]. Omics analysis of coral leopard grouper *P. leopardus* with different body color have been researched with different sequencing methods and showed multiple genes, proteins, miRNAs and pathways related to body color formation [9–11]. Tyrosinase related protein 1 (TYRP1) and dopachrome tautomerase (DCT) which participated in

melanin synthesis have been identified in *P. leopardus* [12]. However, the underlying mechanisms of pigmentation are still unclear.

Single nucleotide polymorphisms (SNPs) are important molecular markers that are located within trait-related genes and exhibit multiple superiorities for the identification of markers associated with target trait [13–16]. SNPs have been widely used in the genetic analysis of economic traits and hold multiple advantages over traditional microsatellite markers, including more accurate genotyping [17], fewer null alleles [18], greater selective efficiency, the potential for quantitative trait loci (QTL) detection [19, 20], being more adaptable to automation genotyping, and the ability to reveal hidden polymorphisms [21]. Transcriptome sequencing is an efficient method to identify SNPs located within genes because it focuses on protein-coding RNAs [22, 23]. Lu et al. [24, 25] obtained ammonia-associated SNP markers in Pacific white shrimp (*Litopenaeus vannamei*) by transcriptome analysis. Peng et al. [26] identified SNPs related to the hypermelanosis of the blind side in Japanese flounder (*Paralichthys olivaceus*) by transcriptome profiling. In the present study, transcriptome and SNP analyses of red- and black-colored*P. leopardus* were analyzed. We hope that the identified DEGs and SNP markers associated with body colors will aid in the marker-assisted selection in *P. leopardus* breeding.

2. Materials and Methods

2.1. Animals Used in the Present Study. *P. leopardus* (black- and red-colored individuals and body length (11.40 ± 0.75) cm) was obtained from Hainan Yonghe Biological Technology Co., Ltd., China and fed with Dongwan grouper diet (Guangdong Yuequn Biotechnology Co., Ltd.) twice per day (10 a.m. and 4 p.m.). Liquid nitrogen was used to freeze skin tissues immediately, and samples were kept at −80°C. In the present study, all animal related experiments were performed based on the guidelines of Animal care and approval of the Use Committee of Guangdong Ocean University, China.

2.2. Transcriptome Sequencing. Skin tissues of two individuals were mixed to make one sample for transcriptome sequencing, and three repetitions were used. Total RNA was obtained with the TRIzol reagent kit (Invitrogen, Carlsbad, CA, USA). Then, ribosomal RNAs were removed to and the enriched mRNAs were fragmented and reverse transcribed into complementary DNA (cDNA). Second-strand cDNA was ligated to Illumina sequencing adapters, and sequenced with Illumina HiSeqTM 4000 platform.

2.3. Bioinformatics Analysis of Transcriptome Data. Clean reads were gained from sequencing data through the filter of fastp (v.0.18.0) [27] and ribosome_RNA removement of Bowtie2 (v.2.2.8) [28]. HISAT2 (v.2.1.0) was utilized to map the clean reads to *P. leopardus* genome [6, 29]. StringTie software was utilized to calculate the expression abundance of genes [30, 31].

2.4. Correlation and Differentially Expressed Gene Analysis. R package gmodels (https://www.rproject.org/) were utilized to perform the principal component analysis (PCA). DEGs were analyzed through the software of DESeq2 [32] with the parameter of the false discovery rate (FDR) < 0.05 and $|\log_2$ fold change (FC)$| > 1$). FDR values were calculated with the method of Benjamini and Hochberg. Then, DEGs were mapped to GO (https://www.geneontology.org/) (p value ≤ 0.05). Pathway enrichment analyses were also carried out to identify significantly enriched pathways in DEGs.

2.5. SNP Identification and Comparison of Different Transcriptomes. All SNPs were identified through comparison with the reference genome of *P. leopardus* (https://www.ncbi.nlm.nih.gov/data-hub/genome/GCF_008729295.1/) using software GATK4 [6]. Nonsynonymous single nucleotide variant (SNV), synonymous SNV, stop-gain, and stop-loss were detected and statistically analyzed. SNP locations, including $5'$ untranslated region (UTR), $3'$UTR, exonic, intronic, intergenic, upstream, downstream, and splicing, were also annotated. SNP was identified as differential between transcriptomes on the condition that one of the transcriptomes holds the same genotype with the reference and the other transcriptome holds the homozygous mutations compared with the reference.

3. Results

3.1. Transcriptome Sequencing Data Analysis. A total of 75.56 M and 76.57 M clean reads were obtained for the comparison of black- and red-colored groups. A range of 93.82% to 94.24% reads were mapped to *P. leopardus* reference data. The transcriptomes of the two groups were compared using PCA and showed that the black- and red-colored groups can be well separated (Figure 1(a)). In the figure, the contribution rates of principal components (PC) 1 and PC2 are 60.2% and 29.6%, respectively. Differential expression analysis of genes identified 489 DEGs, and among them, 236 up-regulated and 253 down-regulated DEGs were identified in the red-colored group ($FDR < 0.05$ and $|\log_2 FC| > 1$) (Supplemental Table 1 and Figure 1(b)). Among the DEGs, melanin pigment synthesis related genes including Tyrosine (TYR), TYRP1, and TYRP2 expressed significantly higher in the black-colored group than that in the red-colored group (Supplemental Table 1).

3.2. GO Terms Analysis. GO terms analysis showed that DEGs identified in the present study were classified into three GO categories including molecular function, cellular

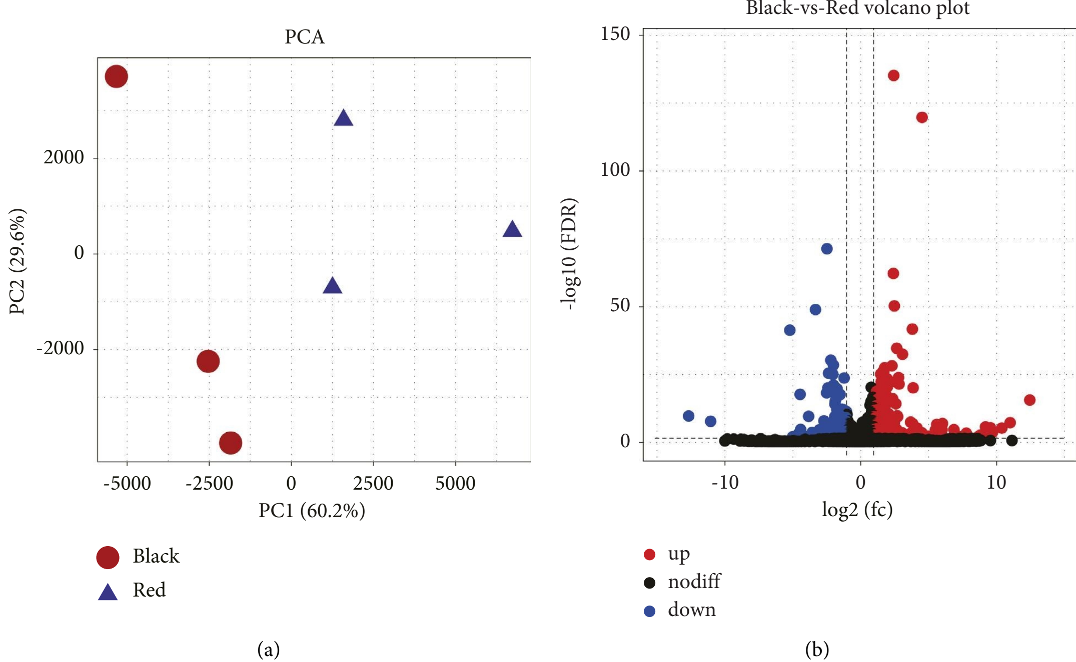

FIGURE 1: PCA and DEG analysis of black- and red-colored groups. (a) PCA of the black- and red-colored groups. Red and blue showed the black- and red-colored groups of *P. leopardus*. (b) DEGs analysis of black- and red-colored groups. Red, blue, and black dots showed the up-regulated, down-regulated, and no differentially expressed genes in the red-colored group compared with the black-colored group, respectively.

component, and biological processes which contain 11, 16, and 23 terms, respectively, (Figure 2(a) and Supplemental Table 2). GO enrichment obtained 405 GO terms ($p < 0.05$), including body color formation related pathways (pigment granule, pigmentation, melanin metabolic process, tyrosine metabolic process, and pigment cell differentiation) (Figure 2(b) and Supplemental Table 3). GO terms associated to immunity or immune response including immune system development, immune system process, and immune response were also enriched terms. Lipid mechanisms including regulation of fatty acid metabolic process, fatty acid metabolic process, unsaturated fatty acid biosynthetic process, and regulation of the unsaturated fatty acid biosynthetic process were enriched which indicated the potential of lipid related materials in the body color formation.

3.3. Pathway Enrichment Analysis. Multiple pathways were enriched through the analysis of DEGs (Supplementary Table 4). Among them, melanin formation related pathways including tyrosine metabolism, Wnt signaling pathway, melanogenesis, and mitogen-activated protein kinase (MAPK) signaling pathway were enriched (Figures 3(a) and 3(b)). Furthermore, immune response related pathways including interleukin-17 (IL-17) signaling pathway, the tumor necrosis factor (TNF) signaling pathway, toll-like receptor signaling pathway, nucleotide oligomerization domain (NOD)-like receptor signaling pathway, lipid metabolism including fatty acid elongation, arachidonic acid metabolism, and fatty acid metabolism were enriched which is consistent with the result of GO terms analysis.

3.4. Identification of SNPs from Transcriptomes. A total of 1,048,575 SNPs were identified from the transcriptomes of red- and black-colored *P. leopardus* as shown in Supplemental Table 5. SNP analysis revealed that the number of noncoding and synonymous SNPs was larger than that of coding and nonsynonymous SNPs, respectively. Among these SNPs, 639177 and 330378 SNPs were identified as transition and transversion, respectively (Figure 4(a)). Among the identical SNPs, 627,172 SNPs were located in the gene region and 62,666 SNPs were located in the coding region. Among the coding SNPs, 18859 nonsynonymous, stop-gain, and stop-loss SNPs were located in 5414 genes and 43750 synonymous SNPs were located in 7710 genes (Figure 4(b)).

3.5. Differential SNPs between Transcriptomes. According to the comparison of transcriptomes between black- and red-colored groupers, 1323 SNPs were identified as differential SNPs that may be related to body color formation (Supplemental Table 5). These SNPs were located in 1127 genes (Supplemental Table 6). Among the identical SNPs, 693 SNPs were located in the gene region and 109 SNPs were located in the coding region. Among the coding SNPs, 29 nonsynonymous, stopgain, and stoploss SNPs were located in 25 genes and 80 synonymous SNPs were located in 70 genes. Among the differential SNP-containing genes, 20 genes were identified as DEGs, including retinol dehydrogenase (Dxb_GLEAN_10013941), cytochrome P450 (Dxb_GLEAN_10001241), and transcription factor Sox (Dxb_GLEAN_10001779) (Supplemental Table 7). Analysis of the SNPs in the DEGs showed nine SNPs that were located

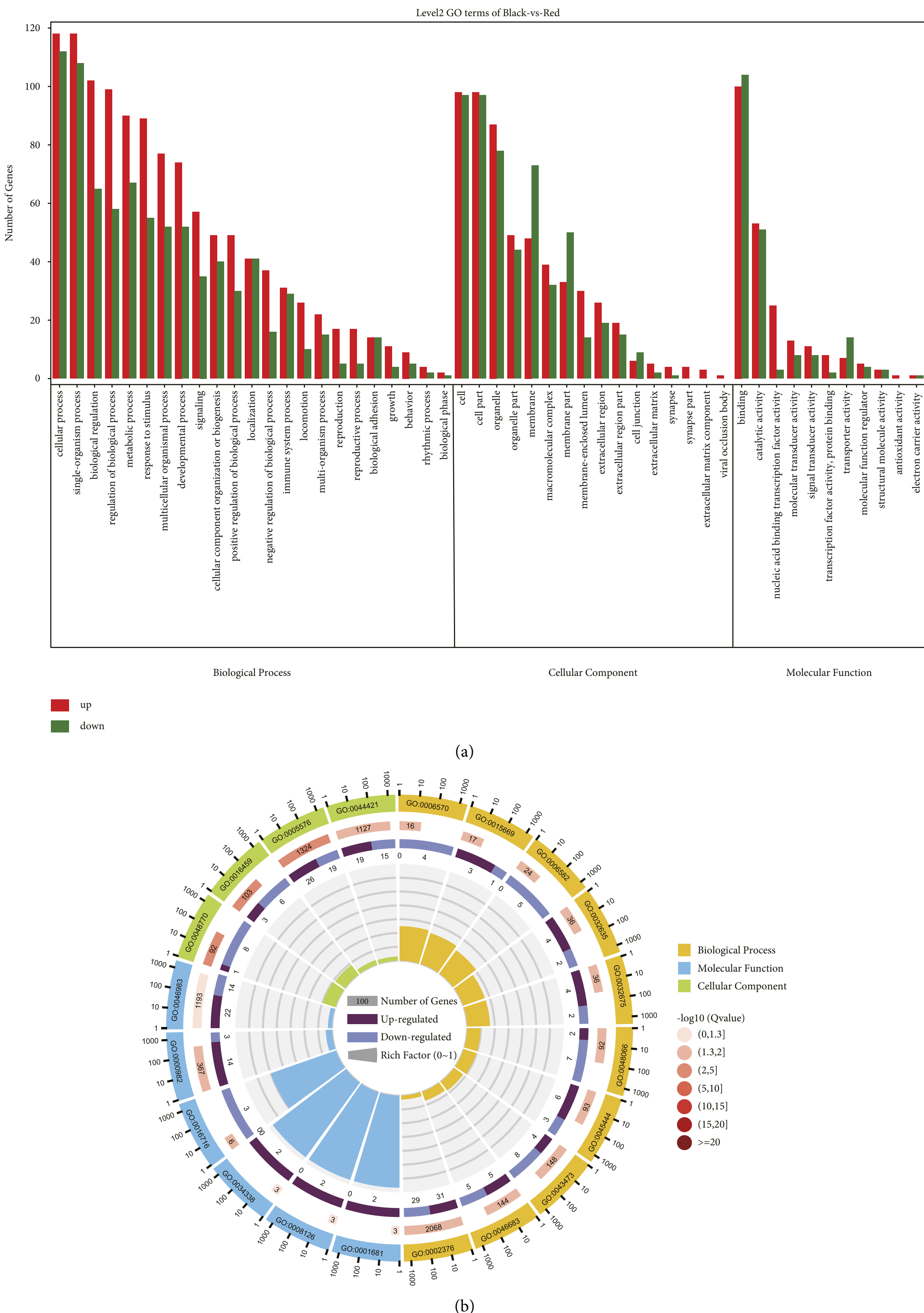

Figure 2: GO analysis of DEGs from black- and red-colored groups. (a) The histogram of GO enrichment classification. Red and green showed the number of up-regulated and down-regulated genes in GO terms. (b) The circle diagram of GO enrichment. Circle 1: The top 20 GO terms in the enrichment analysis, and the outside circle is the coordinate scale for the number of DEGs. Different colors represent different Ontologies. Circle 2: the DEG number and the Q value of the GO term in the background. The more the number of DEGs, the longer the bars, and the smaller the Q value, the redder the color. Circle 3: the number of DEGs in the GO term. Circle 4: the Rich_Factor value of each GO term.

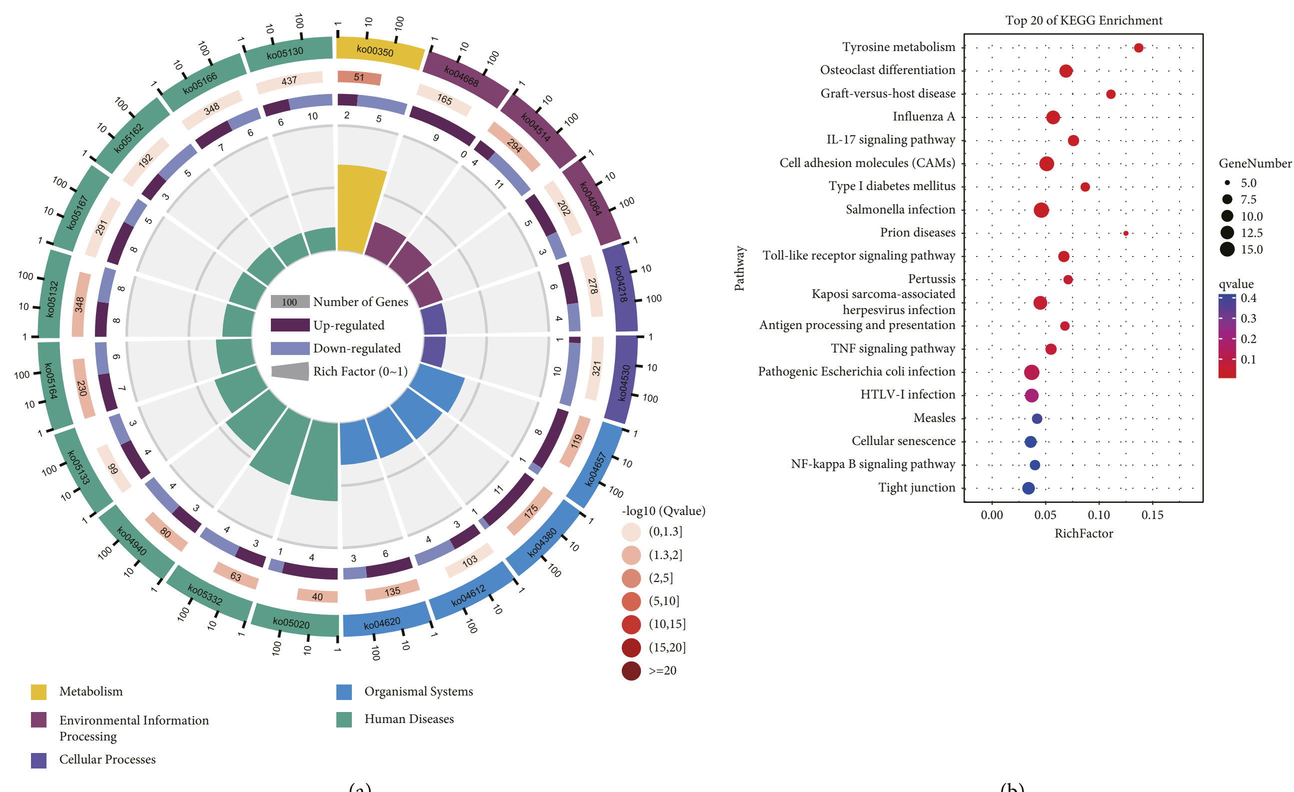

FIGURE 3: Pathway analysis of DEGs from black- and red-colored groups. (a) The circle diagram of KEGG enrichment. Analysis of the KO enrichment of DEGs. Circle 1: The top 20 pathway in the enrichment analysis, and the outside circle is the coordinate scale for the number of DEGs. Different colors represent different classes. Circle 2: The DEG number and the Q value of the pathway in the background. The more the number of DEGs, the longer the bars, and the smaller the Q value, the redder the color. Circle 3: The number of DEGs in the pathway. Circle 4: The Rich_Factor value of each pathway. (b) The bubble plot of KEGG enrichment. The more the number of DEGs, the bigger the dots, and the smaller the Q value, the redder the color.

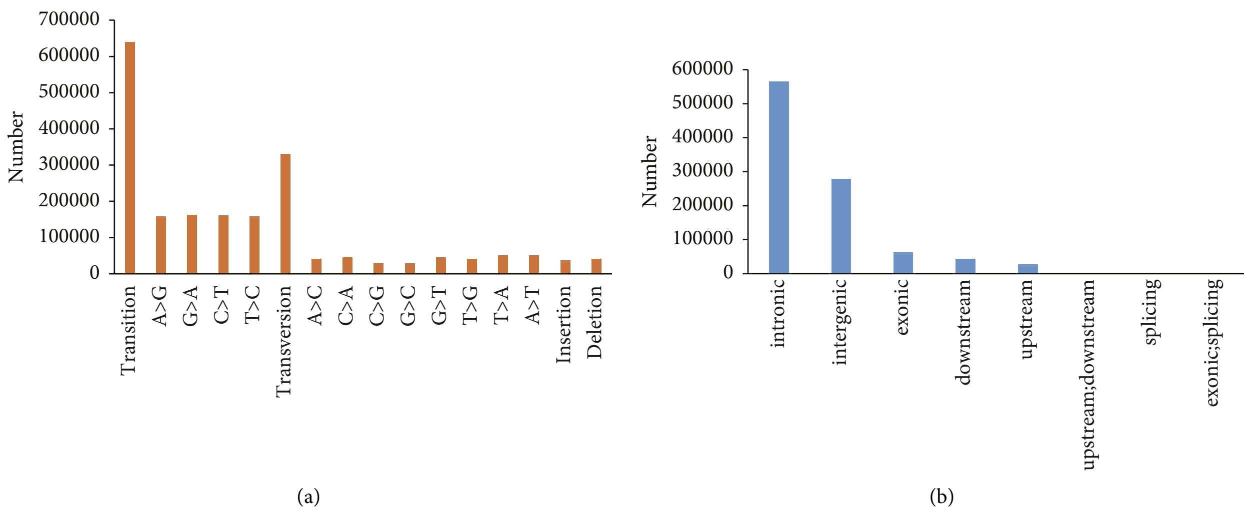

FIGURE 4: SNP analysis of black- and red-colored groups. (a) Numbers of different kinds of SNPs. The kinds include transition ($A > G$, $G > A$, $C > T$, $T > C$), transversion ($A > C$, $C > A$, $C > G$, $G > C$, $G > T$, $T > G$, $T > A$, $A > T$), insertion, and deletion. (b) Numbers of locations of SNPs. These location include intronic, intergenic, exonic, downstream, upstream, upstream; downstream, splicing and exonic; splicing.

in the gene regions. Among these nine SNPs, three were in the exonic region and 6 were in the intronic region. In addition, three SNPs exhibited synonymous mutation and no SNP showed nonsynonymous mutation. These SNPs may be used as molecular markers for color selection in the breeding of coral grouper *P. leopardus* in the future.

4. Discussion

Body coloration is an essential economic trait in fish, and individuals with bright body colors hold a high market value that affects the development of the fish aquaculture industry [26, 33]. Environmental and genetic factors mainly influence body color formation. However, genetic studies and molecular markers related to the body color formation of *P. leopardus* are insufficient. With the development of high-throughput sequencing, multiple molecular works were performed to exhibit the molecular mechanisms of fish body color formation [34, 35]. Multiple transcriptomes related to the body color of *P. leopardus* were constructed, and plenty of molecules such as genes, proteins, and metabolites participating in body color formation or pigmentation were identified with different genome data as references [3, 4, 9, 11, 12, 36]. However, the potential makers which could be used for body color breeding are very limited. Therefore, body color related SNPs were identified in the present study to promote the body color breeding process.

Melanin synthesis-related genes were found in cichlids, sticklebacks, and coral grouper for the black color formation [3, 4, 37]. Wen et al. [11] showed that DEGs and differentially abundant proteins of different skin colors in *P. leopardus* were enriched in melanin synthesis related pathways (tyrosine metabolism) which were also found in the present study. Melanin synthesis related pathways were also identified in the transcriptome, metabolome, and miRNA analysis of different colors of *P. leopardus* [4, 12]. Tyrosinase gene family members including tyrosine-related protein 1 (TYRP1), TYPR2, and TYR participated in the melanin synthesis which were detected in fish melanophores [38, 39]. Melanin related genes including microphthalmia-associated transcription factor, TYRP1, and melanocortin 1 receptor hold significantly higher expression level in dark-skinned individuals than that in red-skinned leopard coral grouper [10]. In the present study, all the tyrosinase gene family members (TYR, TYRP1, and TYRP2) of *P. leopardus* were identified as DEGs and expressed significantly higher in the black-colored group. The result was consistent with Henning et al. [40], which showed that the tyrosinase gene family exhibited a high expression level in the normal Midas cichlids with black body color compared with the gold-colored ones. However, only TYRP1 and TYRP2 were identified as DEGs in the analysis of Zhu et al. [12]. These results may result from the difference in the sequencing method and platform which showed that more RNAs can be detected and identified using the total RNA library construction method than poly(A) RNA library construction [41, 42]. Braasch et al. [43] have reported that TYR is the rate-limiting enzyme in the process of producing melanin and TYRP1 functions in the preservation of TYR catalytic activity [44, 45]. TYRP2 encoded dopachrome, tautomerase, and catalyzed the synthesis of 5,6-dihydroxyindole-2-carboxylate from dopachrome [46–48]. Our results provided more evidence for the melanin synthesis and further showed the importance of tyrosine family genes in the black body color formation process.

Lipid metabolism functioned for different body wall color formation of *Apostichopus japonicus* [49]. Lipid metabolism was enriched in the different body color of *P. leopardus* [4]. Reports have shown that arachidonic acid induces fucoxanthin formation and mediates biochemical-induced stress relevant to pigmentation [12, 50, 51]. Furthermore, fatty acids participated in the regulation of TYR ubiquitination level in melanocytes [52]. Transcriptome of red- and brown-colored *P. leopardus* presented that low-density lipoprotein receptor-related protein 11, angiopoietin-related protein 4, and high affinity cationic amino acid transporter 1 which were involved in lipid metabolism were identified as DEGs and showed their important roles in carotenoid transport [13]. In the present study, lipid metabolism was both identified in the enrichment analysis of GO terms and KEGG pathway which also displayed the importance of lipid metabolism in *P. leopardus* body color formation [4]. Furthermore, the de novo synthesis of carotenoids which is important for the bright or red color formation of aquatic animals is difficult in fish, and the carotenoid accumulation could be affected by the uptake or transport efficiency of it [53]. Multiple researches have shown the importance of lipid-related genes including fatty acid transport protein (FATP) [8, 54] and fatty acid hydroxylase [55] in carotenoid metabolism.

SNPs identified from transcriptome have been widely researched in the aquaculture industry [56]. Transcriptomic analysis of *P. olivaceus* identified 21 SNPs in normal and hypermelanotic flounders, and the SNP-containing genes participated in pigment synthesis [26, 57]. Transcriptomic analysis of whole white and red with big black spots Oujiang color common carp revealed over 52,902 SNPs [58]. Transcriptomes of red and black leopard coral trout *P. leopardus* showed 130,524 SNPs using unigenes as the reference [36], which showed the potential genomic resources for coral trout species. In the present study, 1,048,575 SNPs were identified in red- and black-colored coral groupers. Among which, 1323 SNPs were detected as differential SNPs between black- and red-colored groups that show potential use in body color selection of *P. leopardus* and provide valuable genomic information for genetic research [59, 60].

Analysis of differentially SNP-containing genes in the transcriptomes revealed 20 DEGs, including cytochrome P450 and retinol dehydrogenase that participate in the body color formation of *P. leopardus* [3, 12]. Cytochrome P450 is involved in carotenoid metabolism, which is involved in the formation of bright body colors in animals [61]. Retinoid-metabolizing enzyme, retinol dehydrogenase, converts additional carotenoid substrates and showed higher expression in orange clownfish [62–64]. Furthermore, synonymous mutations influence protein synthesis through their involvement in mRNA splicing, stability, structure, and transfer RNA translation efficiency [65]. In this study, the differential SNPs located in the DEGs were all identified as synonymous mutations. This finding showed that the

mutation may influence transcription or translation efficiency, gene expression regulation, or protein conformation changes in the protein process and further function in the body color formation [66]. These SNPs will be further validated and evaluated in future studies, identifying the specific color varieties of coral groupers with different body colors.

5. Conclusions

In this study, transcriptome of black- and red-colored groups of *P. leopardus* obtained 489 DEGs and 236 up- and 253 down-regulated genes which were found in the red-colored group compared with the black-colored group. GO terms and KEGG pathway analysis of DEGs showed that pigmentation-associated terms, lipid metabolism, and immune-related metabolism were enriched. Comparison of transcriptomes between black- and red-colored groupers identified 1323 differential SNPs in 1127 genes, and among them, 9 SNPs showed the potential use in body color selective breeding.

Data Availability

The data supporting the findings of the current study are available from the corresponding authors upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Yucong Hong performed the experiments, contributed to data analysis, and wrote the manuscript. Ruijuan Hao and Chunhua Zhu conceived and supervised the experiments. Kaihui Sun, Xiaowen Zhu, Xufeng Zhu, and Qiuxia Deng contributed to manuscript revision. Ruijuan Hao and Yang Huang gave valuable advice for the modifications of the manuscript. All authors read and approved the manuscript.

Acknowledgments

This work was financially supported by the Jieyang City Science and Technology Plan Project (grant no. 2019066), the National Nature Science Foundation of China (grant no. 32102766), and the Southern Marine Science and Engineering Guangdong Laboratory (Zhanjiang) (grant no. ZJW-2019-06).

Supplementary Materials

Supplemental Table 1 shows the DEGs identified in the transcriptomes of red-colored and black-colored groups. The GO and KEGG pathway analyses of DEGs were performed and are shown in the Supplemental Tables 2, 3, and 4. Differential SNPs detected in the transcriptomes of red-colored and black-colored groups were shown in Supplemental Table 5 and then the SNPs located in the gene region were shown in the Supplemental Table 6. The DEGs containing differential SNPs were also displayed in Supplemental Table 7. Supplemental Table 1: DEGs from blackand red-colored groups. Supplemental Table 2: GO term analysis of DEGs from black- and red-colored groups. Supplemental Table 3: GO enrichment analysis of DEGs from black- and red-colored groups. Supplemental Table 4: KEGG pathway enrichment analysis of DEGs from blackand red-colored groups. Supplemental Table 5: Differential SNPs between red- and black-colored groups. Supplemental Table 6: Differential SNPs containing genes. Supplemental Table 7: DEGs of the differential SNPs containing genes. (*Supplementary Materials*)

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