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INTRODUCTION

Growth is one of the most important traits related to tness and production for any organism. Traits associated with fast growth have been one of the major breeding goals to enhance the pro tability of production for all food animals. For aquaculture species, growth rate is especially important because aquaculture

drawn using R scripts to exhibit the overall distribution of DEGs. Gene ontology (GO) enrichment analysis was conducted using the R package GOseq to study the distribution of DEGs in gene ontology in order to clarify the biological meaning as indicated in terms of gene function (Young et al., 2010). GO terms with corrected *P*-value of less than 0.05 were considered as significantly enriched with DEGs. KEGG pathway analysis was conducted to understand high-level functions and utilities of the biological system from molecular-level information (Kanehisa et al., 2007). The statistical enrichment of DEGs in KEGG pathways was tested using KOBAS (2.0) software (Mao et al., 2005), and multiple-testing-corrected *P*-value of less than 0.05 was regarded as significantly enriched in the pathway.

Quantitative Real-Time PCR Validation

To validate the results of RNA-Seq, 12 differentially expressed genes were selected for quantitative real-time PCR (qRT-PCR) analysis. The RNA samples used for the qRT-PCR assay were same as those used for RNA-Seq. The cDNA was synthesized for qRT-PCR by Prime Script TM RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). Specific primers for qRT-PCR were designed according to the reference sequences using Primer Premier 5.0 (**Supplementary Table 1**). *Eukaryotic elongation factor 1* (*eEF-1*) gene was used as an endogenous control to normalize gene expression by real-time PCR (Renault et al., 2011). The amplification was performed on the LightCycler 480 real-time PCR instrument (Roche Diagnostics, Burgess Hill, UK) using SYBR® Premix Ex Taq™ (TaKaRa). Cycling parameters were 95°C for 5 min and then 40 cycles of 95°C for 5 s, 58°C for 30 s, and 72°C for 30 s. The melting curve of PCR products was performed to ensure specific amplification. Relative gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). Data were analyzed by *t*-test using software SPSS 18.0, and *P*-value < 0.05 was considered as statistical significance.

Alternative Splicing Analysis

Alternative splicing (AS) of genes creates multiple mRNA transcripts from one gene, resulting in tremendous proteomic complexity in higher eukaryotes (Keren et al., 2010; Nilsen and Graveley, 2010). AS events were analyzed using the software rMATS (v3.2.5) (Shen et al., 2014a). The AS events were divided into five categories, including skipped exon (SE), alternative 5′ splice site (A5SS), alternative 3′ splice site (A3SS), mutually exclusive exons (MXE), and retained intron (RI). The expression of each type of AS events was then calculated. The differential alternative splicing (DAS) events were determined from two-group RNA-Seq data with replicates. False discovery rate (FDR) < 0.05 was regarded as the screening criterion for DAS events. Similar to analysis of DEGs, GO and KEGG enrichment analyses were also conducted for the DAS genes.

Transcriptome *De Novo* Assembly and Annotation

De novo assembly of transcriptome was carried out using Trinity (Grabherr et al., 2011) with parameters set as default, followed by mapping cleaned reads to the *de novo* assembled transcript sequences using RSEM software (Li and Dewey, 2011). The assembled transcripts were annotated based on seven public databases, including the NCBI non-redundant protein sequences (Nr) database, NCBI non-redundant nucleotide sequences (Nt) database, Protein family (Pfam) database, euKaryotic Ortholog Groups (KOG) database, Swiss-Prot database, KEGG Ortholog (KO) database, and Gene Ontology (GO) database. For the genes that had multiple assembled transcript sequences, the longest transcript was chosen to represent the gene that is referred to as unigene. Coding sequences (CDSs) were predicted by matching unigenes to the Nr database and Swiss-Prot database by BLASTX.

Analysis of Positively Selected Genes during Artificial Selection

Putative orthologs between two groups ("breed" vs "wild") of the Pacific oysters were identified using BLAST-based approach. The CDSs were first extracted from unigenes, and then self-to-self BLASTP was conducted for all amino acid sequences with a cut-off *E*-value of 1E−5, and finally, orthologous pairs were constructed from the BLASTP results with OrthoMCL (v2.0.3) (Li et al., 2003) with default settings. The ratio of the number of nonsynonymous substitutions per nonsynonymous site (Ka) to the number of synonymous substitutions per synonymous site (Ks) was used to test for positive selection. Ka/Ks calculation was performed with PAML (Yang, 2007) package with default settings. The orthologs with Ks > 0.1 were excluded from further analysis to avoid potential paralogs (Elmer et al., 2010). The Ka/Ks ratio greater than 1 usually indicates genes evolving under positive selection (divergent), while those orthologs with a Ka/Ks ratio less than 0.1 indicates that these genes are under heavy selection pressure (conserved).

RESULTS

Growth Comparison

Growth of the "breed" and "wild" oysters were compared at 6 months of age as shown in **Table 1**. Apparently, the "breed" oysters showed significant growth advantage to the unselected "wild" oysters in terms of all quantified growth-related traits including shell height, shell length, shell width, and body weight. In addition, the growth-related traits of "breed" oysters were relatively uniformed as indicated by smaller variations of phenotypic traits (**Table 1**).

TABLE 1 | Growth comparison between "breed" and "wild" populations of the Pacific oysters.

Populations	Shell height (mm)	Shell length (mm)	Shell width (mm)	Total weight (g)
Breed	71.74 ± 3.5**	32.93 ± 2.7**	18.41 ± 1.7**	20.73 ± 3.3**
Wild	56.44 ± 3.9**	26.17 ± 3.2**	14.93 ± 1.9**	10.99 ± 4.4**

** ... *t* ... *t t* 0.01 ... (< 0.01). ... , 100.

Transcriptome Sequencing and Mapping

A total of 300.9 million clean reads were obtained a er trimming over 307 million 150-bp paired-end raw reads, with Q20 varying from 97.2% to 97.7%. e total bases of clean reads generated from each sample ranged from 7.0 to 8.2 Gb, which is about 15× of the oyster genome size. For the six samples, 79.3–82.2% of the total clean reads were aligned to the genome, of which 71.0–73.1% had a unique alignment and 8.1–9.1% had multiple alignment positions on the genome (**Table 2**). e abundance of transcript sequences for all gene models (35,362) was normalized and calculated by FPKM method using uniquely mapped reads. Nearly half (39.2–47.7%) of the genes were considered not to be expressed or expressed at very low levels (0 < FPKM < 1), and less than 4% (3.4–3.7%) were highly expressed (FPKM > 60). e correlation of gene expression among biological replicates was reasonably high with Pearson's R^2 values greater than 0.8 for all samples (**Supplementary Figure 1**).

Analysis of Differentially Expressed Genes

A total of 1,303 di erentially expressed genes (DEGs) were identi ed between the "breed" and "wild" Paci c oysters, of which 888 genes were expressed at higher levels in "breed" oysters while 415 genes were expressed at higher levels in the unselected "wild" oysters (**Figure 1** and **Supplementary Table 2**). e number of genes expressed at higher levels in "breed" oysters is signi cantly larger than that in the "wild" oysters.

Twelve DEGs were selected for qRT-PCR validation, and the results were compared with those from RNA-Seq data analysis. e results showed that expression levels of most genes detected by qRT-PCR were consistent with the results as determined based on RNA-Seq analysis, with the exception of *MFAP4*, which showed a similar expression pattern but a signi cantly di erent degree of fold change between RNA-Seq and qRT-PCR (**Figure 2**).

To further understand the biological meanings of these DEGs, gene ontology (GO) term enrichment analysis (*P* 0.05) was performed. For the 888 genes expressed at higher levels in "breed" oysters, the most signi cantly enriched GO terms were "microtubule-based movement" in the biological process (BP), "microtubule motor activity" in the molecular function (MF), and "dynein complex" in the cellular component (CC) (**Figure 3A** and **Supplementary Table 3**). erefore, microtubule-related genes were highly enriched in top three GO categories in the DEGs expressed at higher levels in the fast-growing "breed" oysters. Besides, signi cantly enriched GO terms associated with microtubule or cell movement also include "movement of cell or subcellular component," "microtubule-associated complex," "motor activity," and "microtubule-based process" (**Figure 3A**). A total of 42 microtubule-related genes expressed at higher levels in "breed" group were identi ed. For example, *C1ql4* (LOC105334943) showed 16.7-fold, *DNAH5* (LOC105330782) showed 3.5-fold, and *KIF12* (LOC105329973) displayed a 2.8-fold higher expression in "breed" oyster than "wild" oysters (**Supplementary Table 4**). In addition, genes involved in the process of biosynthesis and metabolism of nucleotide compounds (GTP, UTP, and CTP), ribonucleotide (pyrimidine ribonucleotide), nucleoside triphosphate (pyrimidine nucleoside triphosphate and pyrimidine ribonucleoside triphosphate), and nucleoside (pyrimidine nucleoside and pyrimidine ribonucleoside) were also highly enriched in the DEGs that were expressed at higher levels in the "breed" oysters (**Figure 3A**). A total of four DEGs were involved in these pathways including *NME5* (2.2-fold), *NME7* (2.3-fold), *CiIC3* (2.8-fold), and LOC105346007 (2.4-fold), suggesting that activation of cell movement, microtubule, dynein, and nucleoside compound-related genes may be associated with growth of the Paci c oyster. In addition, a total of 258 DEGs expressed at higher levels in the "breed" oysters were enriched in "protein binding" pathway, with the number of enriched genes far more than that of other pathways (**Figure 3A**).

For the DEGs that were expressed at higher levels in "wild" oysters, the signi cantly enriched GO terms include "chitin metabolic process," "glucosamine-containing compound metabolic process," "chitin binding," "amino sugar metabolic process," and "aminoglycan metabolic process" (**Figure 3B**), of which a total of 11 genes were involved, including *Col14a1*, *CHIA*, *EXT*, *ITIH3*, and other seven uncharacterized genes (**Supplementary Table 5**).

TABLE 2 | Summary of RNA sequencing data and statistics of read mapping to the Pacific oyster genome assembly.

Sample name	DW1	DW2	DW3	ZW1	ZW2	ZW3
Raw reads	48,275,126	53,610,846	55,591,318	47,969,616	51,441,560	50,454,936
Clean reads	47,199,394	52,639,950	54,719,524	46,899,346	50,194,624	49,260,812
Clean bases	7.08G	7.9G	8.21G	7.03G	7.53G	7.39G
Q20 (%)	97.74	97.15	97.29	97.32	97.35	97.36
GC content (%)	43.88	43.27	43.18	43.16	42.80	42.83
Total mapped	38,514,796 (81.6%)	42,635,453 (80.99%)	44,986,750 (82.21%)	37,578,987 (80.13%)	39,807,025 (79.31%)	39,593,155 (80.37%)
Multiple mapped	4,210,112 (8.92%)	4,566,862 (8.68%)	4,983,688 (9.11%)	3,800,378 (8.1%)	4,147,059 (8.26%)	4,119,884 (8.36%)
Uniquely mapped	34,304,684 (72.68%)	38,068,591 (72.32%)	40,003,062 (73.11%)	33,778,609 (72.02%)	35,659,966 (71.04%)	35,473,271 (72.01%)
Exon	88.20%	89.80%	91.40%	89.80%	83.90%	88.80%
Intron	3.00%	3.30%	3.20%	3.40%	3.60%	3.70%
Intergenic	8.80%	6.80%	5.40%	6.80%	12.60%	7.50%

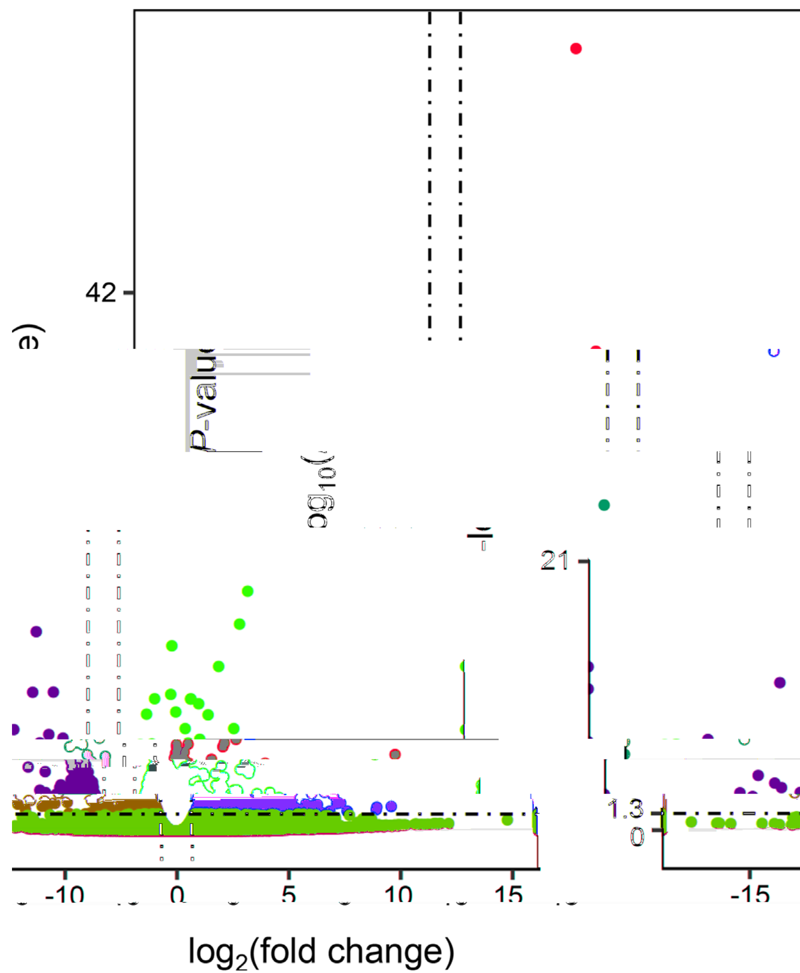

FIGURE 1 | Identification of differentially expressed genes between "breed" and "wild" Pacific oysters. The red dots denote genes that were significantly expressed at higher levels in "breed" oysters, while the green dots denote genes that were significantly expressed at higher levels in "wild" oysters.

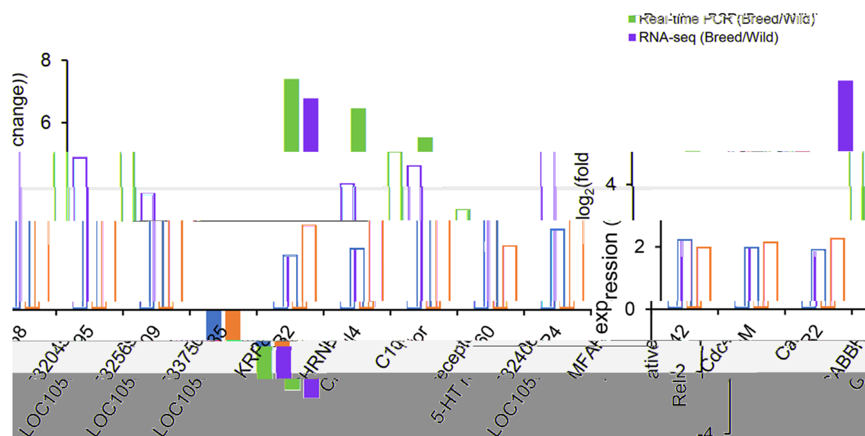

FIGURE 2 | Validation of differentially expressed genes by real-time PCR. . *-1* gene was used as internal control.

KEGG enrichment analysis of these DEGs was performed to further determine the metabolic processes and signal transduction pathways. e results revealed that the DEGs are signi cantly enriched in 25 pathways, such as “phototransduction,” “long-term potentiation,” “vascular smooth muscle contraction,” “calcium signaling pathway,” “phosphatidylinositol signaling system,” “gastric acid secretion,” “salivary secretion,” “adrenergic signaling in cardiomyocytes,” and “ABC transporters” (**Figure 4**

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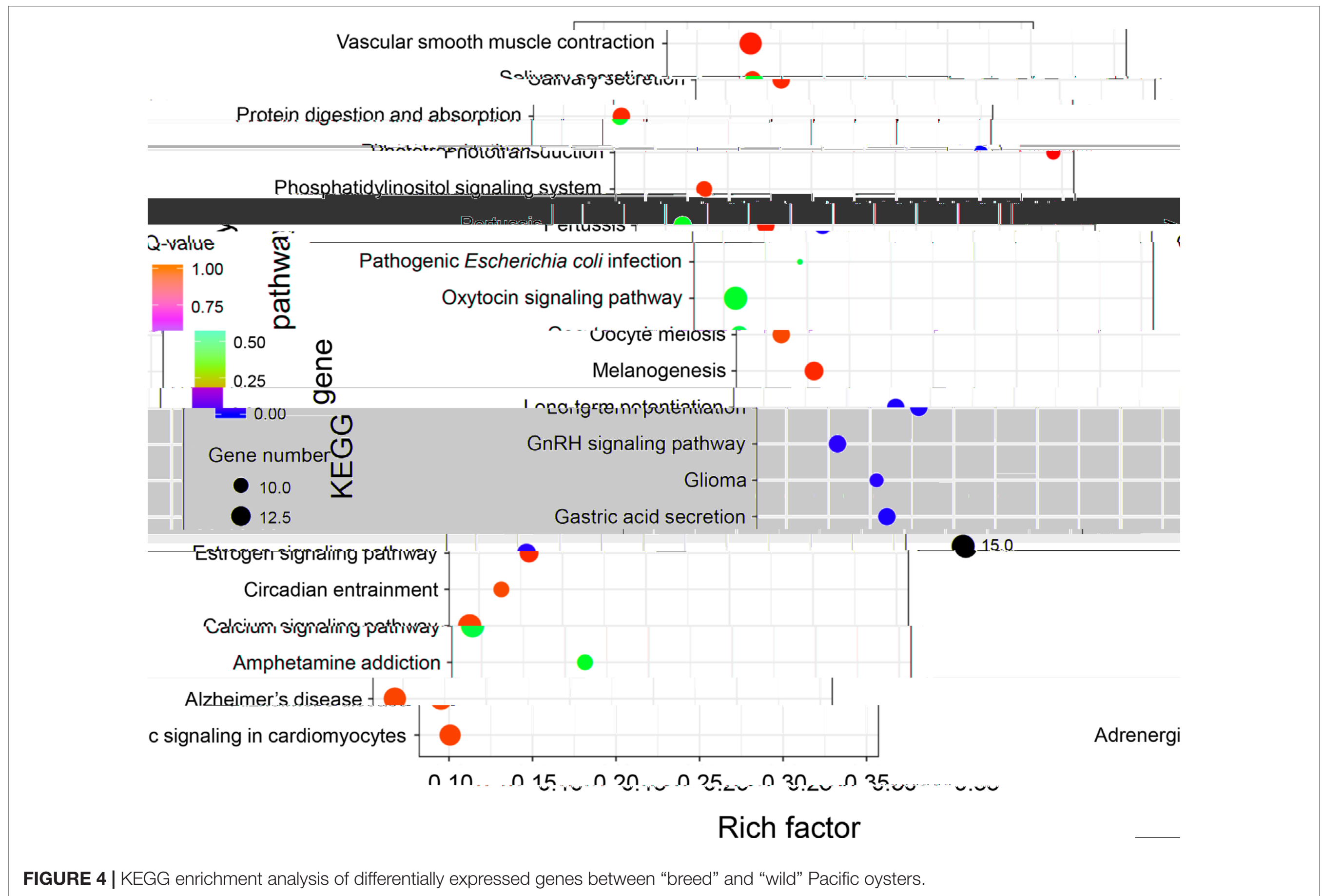

FIGURE 4 | KEGG enrichment analysis of differentially expressed genes between “breed” and “wild” Pacific oysters.

TABLE 3 | Categories of alternative splicing (AS) events in all libraries.

AS events	No. of AS events	Rate of AS events	No. of AS genes	Rate of AS genes*	Average AS per gene
SE	16,974	75.2%	7,346	89.9%	2.3
A5SS	1,156	5.1%	913	11.2%	1.3
A3SS	1,276	5.7%	995	12.2%	1.3
MXE	2,921	12.9%	1,373	16.8%	2.1
RI	246	1.1%	211	2.6%	1.2
Total	22,573	100%	8,176	132.7%	2.8

*[illegible] 100% [illegible] 5′ [illegible] 3′ [illegible]

By choosing the longest transcript to represent the gene when the genes had multiple transcript sequences assembled, the transcriptome assembly provided 194,978 unigenes for “breed” and 172,863 unigenes for “wild,” respectively (**Supplementary Table 9**). Annotation of the unigenes against the public databases including Nr, Nt, KOG, KO, Swiss-Prot, GO, and Pfam provided a total of 70,419 (36.1%) unigenes from “breed” and 67,173 (38.9%) unigenes from “wild” (**Supplementary Table 10**).

A total of 5,453 pairs of putative orthologs were identi ed between “breed” and “wild” oysters, of which 3,328 ortholog pairs with all nonsynonymous substitutions and synonymous substitutions were used for calculating Ka/Ks ratios, and the results revealed that 1,198 pairs had Ks > 0.1 that were determined as potential paralogs. A er removal of potential paralogs, 2,130 pairs of orthologs were nalized with mean Ka of 0.0097, mean Ks of 0.0304, and mean Ka/Ks ratio of 0.359. A total of 589 ortholog pairs with a Ka/Ks ratio > 1 were identi ed (**Figure 6**), which might have experienced or be experiencing positive selection during arti cial selection.

KEGG pathway analysis of the 589 positively selected genes showed that genes related to ribosomal proteins were greatly divergent between the “breed” and “wild” Paci c oysters. ese ribosomal protein-related genes include *RP-L24e*, *RPL24*, *RPS18*,

FIGURE 5 | KEGG enrichment analysis of differential alternative splicing genes between “breed” and “wild” Pacific oysters.

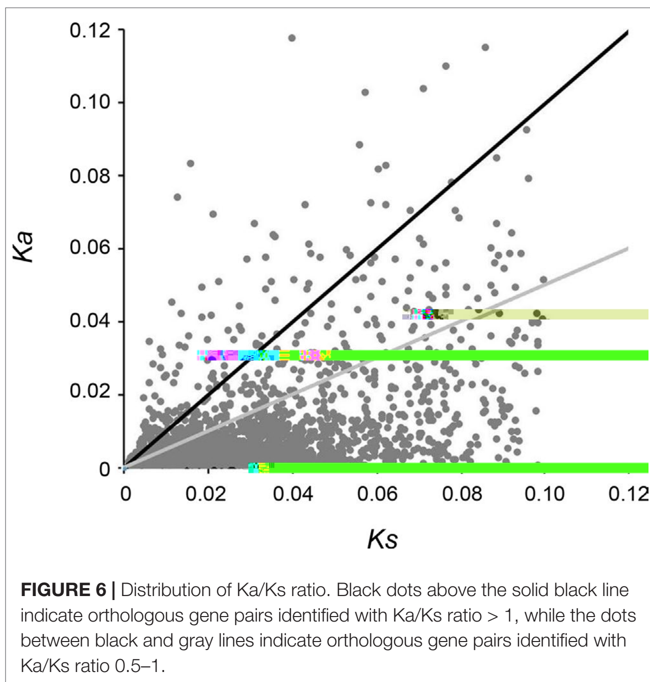

FIGURE 6 | Distribution of Ka/Ks ratio. Black dots above the solid black line indicate orthologous gene pairs identified with Ka/Ks ratio > 1, while the dots between black and gray lines indicate orthologous gene pairs identified with Ka/Ks ratio 0.5–1.

RP-S3Ae, *RPS3A*, *RP-L21e*, *RPL21*, *RP-L30*, *MRPL30*, *rpmD*, *RP-L36E*, *RPL36*, *RP-S28e*, and *RPS28* (**Figure 7**).

DISCUSSION

Growth trait is implicated in a variety of cellular processes and is subject to regulation by multiple complex biological processes. Growth rate is heavily a ected by environment variables, especially in aquatic animals inhabiting in highly variable water environments. Generation of fast-growing varieties of animals by selection breeding approach would provide good materials with similar genetic background but contrast phenotype for genetic dissection of growth trait. We initiated a selective breeding program of the Paci c oyster back in 2006. Up to 2017, the selectively bred lines have undergone 10 successive generations of intensive arti cial selection for fast growth. Great enhancement of growth has been achieved as indicated by growth trial experiments, while the e ects of arti cial selection on the Paci c oyster genome remain unexplored. In this study, we used the selectively bred fast-growing oysters as research material to investigate the molecular basis of growth in the Paci c oyster.

We performed transcriptome comparative analysis of the fast-growing selectively bred oysters with the unselected wild oysters. We identi ed a total of 1,303 protein-coding genes that were di erentially expressed (DEGs) between fast-growing oysters and wild controls. Functional analysis of the DEGs showed that microtubule, cell movement, and nucleotide compound-related genes were signi cantly enhanced for expression in the fast-growing oysters. Microtubules are reported to be essential for proper cell division and cell expansion (Maiato and Sunkel, 2004; Bichet et al., 2008; Jiang et al., 2015). e microtubule-associated proteins (*dynein* and *kinesin*), as well as many other microtubule-related proteins (*C1ql4*, *Cas8*, and *I 46*), were found to be expressed at higher levels in the selectively bred Paci c oysters. e higher expressions of kinesin genes such as *Kif9* and *Kif12* in the fast-growing oysters are consistent with observations in previous studies that the expressions of *Kif9* and *Kif12* were positively correlated with cell division and cell growth (Gong et al., 2009; Andrieu et al., 2012). e results indicated that microtubule- and cell-movement-related genes could probably play critical roles in growth regulation in the Paci c oysters.

Cell movement plays an important role in the growth and development of organisms, participating in embryonic development and wound healing. e cell movement process needs to be driven by the physical forces generated by cytoskeleton (composed of microfilaments, intermediate filaments, and microtubules) and the participation of many other proteins (

the growth regulation. Nucleotides carry packets of chemical energy in the form of the nucleoside triphosphates (ATP, GTP, CTP, and UTP) and plays an important role in metabolism at the cellular level, such as synthesis of amino acids and proteins, movement of the cell and cell parts, and division of the cell (Pedley and Benkovic, 2017). The enhanced expression of nucleotide metabolism-related genes, therefore, may contribute to increase the efficiency of protein synthesis and cell division for enhanced growth performance.

Gene functional annotation analysis showed that both differentially expressed genes and alternatively spliced genes were significantly enriched in long-term potentiation, phosphatidylinositol signaling system, ABC transporters, and salivary secretion pathways. In long-term potentiation pathway, differentially expressed genes are mainly calmodulin kinase and its regulators. When long-term potentiation increased, the binding efficiency of Ca^{2+} to calmodulin is increased, causing the increased level of CaMK II and CaMK IV contents. Then, EPK is activated, which promotes increased synthesis of synapse growth protein (Silva et al., 1992; Strack et al., 1997). In this process, calcium ions and calmodulin play critical roles in regulation. Calcium ion carries out its functions by binding to specific calcium receptors or calcium-binding proteins (CaBPs). Genes associated with calcium ion regulation were found to be differentially expressed between "breed" and "wild" groups of the Pacific oysters (**Supplementary Table 7**). For example, the calmodulin-related genes such as *CaM* (LOC105328007), *CML12* (LOC105319978), *CAMK* (LOC105335050), and *SLC8A* (LOC105340116) were expressed 4.5-, 3.2-, 3.0-, and 3.1-fold higher in the "breed" than "wild" oysters, respectively. Exceptionally, the expression of *CALCRL* (LOC105320473) gene was expressed 3.3-fold higher in the unselected "wild" oysters (**Supplementary Table 7**). In the phosphatidylinositol signaling pathway, external signaling molecules bind to G protein-coupled receptors (GPCRs) to activate phospholipase C (PLC), decomposing PIP2 into IP3 and DG and finally activating protein kinase C (PKC) to generate cellular responses, including cell secretion, cell proliferation, and differentiation.

Besides the altered expression patterns of genes between "breed" and "wild" oysters, the effects of artificial selection on these protein coding genes are also of importance. Enrichment analysis of positively selected genes between the "breed" and "wild" groups of the Pacific oysters showed that genes related to the biosynthesis of ribosomal proteins were significantly divergent during the artificial selection process. Ribosomal proteins are crucial for the growth and development of the organisms (Xie et al., 2009; Baloglu et al., 2015). In the larval Pacific oysters, a previous study reported that ribosomal protein-related genes were essentially involved in growth heterosis (Hedgecock et al., 2007b). The divergence of ribosomal protein genes may be associated with differential efficiency of transcription and protein biosynthesis, eventually resulting in growth phenotypic difference between "breed" and "wild" oysters. However, this observation requires future investigation.

CONCLUSION

To unravel the molecular basis for fast growth of the selectively bred Pacific oyster, we performed comparative transcriptome analysis of the fast-growing "breed" with the unselected "wild" Pacific oysters in terms of gene expression, alternative splicing, and molecular evolution. The most significant outcome is the identification of potential growth-related genes in the Pacific oysters. Further functional analysis revealed that genes involved in microtubule motor activity, and biosynthesis of nucleotides and proteins would be important for oyster growth. Transcriptome-wide analysis of positively selected genes revealed the important roles of ribosomal protein genes, which further suggested that the process of protein biosynthesis may be a key biological process related to the growth difference between selectively bred oysters and unselected wild oysters. This study provides valuable resources for further investigations on the growth regulation mechanisms and will be useful to support the breeding application to integrate fast growth with other superior traits in the Pacific oysters.

DATA AVAILABILITY STATEMENT

The Pacific oyster reference genome and gene model annotation files in this study were downloaded from the NCBI (ftp://ftp.ncbi.nlm.gov/genomes/Crassostrea_gigas). All raw RNA-Seq data have been deposited in the NCBI Sequence Read Archive with BioProject accession no. PRJNA524442 (sequence accessions: SRR9089186-SRR9089191).

AUTHOR CONTRIBUTIONS

SL conceived and designed the study. FZ, BH, and HF collected the samples and executed the experiments. FZ, BH, HF, ZJ, and SL analyzed the data. FZ drafted the manuscript, and SL revised the manuscript. QL provided reagents and materials and supervised the study. All authors have read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.00610/full#supplementary-material

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Con ict of Interest Statement: e authors declare that the research was conducted in the absence of any commercial or nancial relationships that could be construed as a potential con ict of interest.

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