

Perspective

Comprehensive Genome-Wide Analysis of *Wnt* Gene Family and Expression Profiling during Limb Regeneration in *Portunus trituberculatus*

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Abstract: *Wnt* genes encode a family of secretory glycoproteins that are involved in various stages of organ development through regulation of cell proliferation, differentiation, and growth. Recently, *Wnt* genes have been shown to play an important role in regeneration processes. However, there have been no previous genome-wide analyses of the *Wnt* gene family members in crab species. In this study, a total of 13 *Wnt* family genes were identified from the genome of the swimming crab *Portunus trituberculatus* and classified into three main groups based on the conserved domain, protein sequence, and motifs. Chromosome location analysis showed that tandem duplication may have resulted in the expansion of the *PtWnt* gene family. RNA-seq results indicated that most *PtWnt* genes had a higher Fragments Per Kilobase of transcript sequences per Million base pairs (FPKM) value in the regenerating limb bud muscle than in the normal limb muscle, and the genes were enriched in a number of pathways that had biological functions underlying limb regeneration such as the cell surface receptor signaling pathway, the hippo signaling pathway, receptor binding, and basal cell carcinoma. Moreover, quantitative real-time PCR (RT-qPCR) analysis confirmed that the expression levels of nine *PtWnts* except for *PtWnt1*, *PtWnt2*, and *PtWnt6* had a consistently increasing trend during limb regeneration. *PtWnt1*, *PtWnt2*, and *PtWnt6* exhibited significantly up- or down-regulated expression at different limb regeneration stages. These results provide valuable information for further evolutionary and functional characterization of *PtWnt* genes and indicate the complexity and specialized mechanism of the *Wnt* signaling pathway regulating regeneration in crustaceans.

Keywords: *Portunus trituberculatus*; *Wnt* gene family; limb regeneration; *Wnt* signaling pathway



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1. Introduction

Regeneration is a complex biological process by which animals repair damaged or lost body parts [1]. In this regard, limb regeneration in crustaceans has been one of the most extensively studied model systems, as crustaceans, from larvae to adults, have the ability to regenerate an entire injured limb. Previous studies in crustacean limb regeneration have focused on the mechanism of regenerative wound repair [2], descriptions of morphological changes [3,4], neuroendocrine regulation [5–7], and expression and functional analysis of development-related genes [8,9]. However, the molecular signaling pathways that coordinate the morphogenesis and cellular events of regenerated limbs have not been reported in crustaceans.

The *Wnt* signaling pathway is a highly conserved signaling pathway that plays an important role in regulating organ morphogenesis during animal development and regeneration [10,11]. A total of 19 different *Wnt* gene subfamilies have been identified in humans, and the *Wnt* ligands encode a set of conserved glycoproteins regulating early

development, cell differentiation and proliferation, and organ regeneration [12,13]. Previous studies in arthropods indicated that different species have different numbers of *Wnt* family members. For example, 12 subfamilies have been identified in *Daphnia pulex*, while there are only seven *Wnt* family members in *Apis mellifera* and *Drosophila melanogaster* and nine in *Tribolium castaneum* [14,15]. Moreover, different *Wnt* ligands have different signal transduction properties in animal regeneration [12,16]. It has been reported that *Wnt1*, *Wnt3a*, and *Wnt8* play roles in the transformation and axial growth of mouse and *Xenopus* embryos through the classic *Wnt*/ β -Catenin pathway [17,18], while *Wnt5A*, *Wnt4*, and *Wnt11* can activate the non-canonical β -catenin-independent *Wnt* pathway and affect morphogenesis by inducing the extension of the epithelial layer [19,20]. At present, there is limited understanding of the *Wnt* gene family in crustaceans due to a long-standing paucity of genomic and transcriptomic resources. The latest research has initially revealed the role of six *Wnt* family members in the growth and development of *Litopenaeus vannamei* as ecdysone-responsive genes [21]. However, *Wnt* information concerning gene family identification and the function in regeneration is still limited in other crustaceans, especially in crab species.

Portunus trituberculatus is one of the most economically important aquaculture species in China due to its high yield, high nutritive value, and pleasant taste. The recent accomplishment of genome sequencing in *P. trituberculatus* provides valuable genomic resources to enable systematic characterization of important development-related gene families in this species [22,23]. Moreover, our previous transcriptome and gene function exploratory studies have indicated that the *Wnt4* gene and *Wnt* signaling play critical roles in limb regeneration of *P. trituberculatus* [9,24,25]. However, there has been no investigation of genome-wide identification and characterization of the *Wnt* family in *P. trituberculatus*. Herein, we carried out a genome-wide identification and evolutionary analysis of the *Wnt* gene family in *P. trituberculatus* and further analyzed the expression profiles of the *Wnt* genes during limb regeneration stages based on transcriptome and quantitative real-time PCR (qRT-PCR) analyses. Additionally, the characterization of genomic structures and conserved motifs, phylogenetic analysis, and chromosomal location analyses were also performed in this study.

2. Materials and Methods

2.1. Identification of the *P. trituberculatus* and *L. vannamei* *Wnt* Families

We collected the whole-genome data of *P. trituberculatus* with National Center for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov/>, accessed on 13 January 2021) accession number of PRJNA555262 and *L. vannamei* with NCBI accession number of PRJNA508983 from the NCBI database. The transcriptome data for *P. trituberculatus* with NCBI accession number PRJNA422610 were collected and used for the expression annotation as described in our previous study [24]. The Hidden Markov Model (HMM) domain profile (PF00110) was obtained from the Pfam database (<http://pfam.janelia.org>, accessed on 15 January 2021) and was employed to match each potential protein encoded in the genome using HMMER 3.0 [26] with E-value $\leq 10^{-5}$. All candidate *Wnt* protein sequences were further analyzed on the Conserved Domain Database (CDD) (<https://www.ncbi.nlm.nih.gov/cdd>, accessed on 20 January 2021) and Pfam (<https://pfam.xfam.org/>, accessed on 20 January 2021) websites. The ExPASy Proteomics Server (<https://web.expasy.org/protparam/>, accessed on 20 January 2021) was used to analyze the molecular weights, amino acid sequence lengths, and isoelectric points of *Wnt* proteins of *P. trituberculatus*.

2.2. Phylogenetic Analysis, Gene Structure, and Motif Identification Analysis of the *Wnt* Family in *P. trituberculatus*

Multiple alignment of full-length amino acid sequences of *Wnt* family proteins of *P. trituberculatus* and *L. vannamei* was performed using the Clustal W [27] program. The neighbor-joining phylogenetic trees were constructed based on the amino acid sequences

using the MEGA 7.0 [28] software, applying the amino acid substitution type and Poisson model and boot strapping procedure with a minimum of 1000 bootstraps. The exon structures of *PtWnt* genes were determined through the comparison of the coding sequence of each gene with its genomic sequence using the Gene Structure Display Server [29]. Conserved motifs in the Wnt proteins were analyzed using MEME [30], and the parameters were optimized as follows: Any number of repetitions, maximum number of motifs = 10, and the optimum width of each motif set to between 6 and 100 residues.

2.3. Chromosome Localization and Gene Duplication

The chromosomal locations of the *PtWnt* genes were determined using TBtools software [31], and chromosomal localization mapping was performed with MapChart version 2.32 (<https://www.wur.nl/en/show/Mapchart.htm>, accessed on 10 February 2021) [32]. The parameters were optimized as follows: Users were required to prepare the input data according to an excel template provided on the website. The input data were divided into two parts. One was gene location (input1), and the other was chromosome length (input2). The former contained 5 fields: gene_ID, gene_start, gene_end, chr_ID, and gene_color. Gene_color is an optional field used to customize color of the gene ID, with the default in black. The latter contains two fields: chr_ID and chr_length. The delimiter between the fields is "TAB". Users copy and paste the input data into the corresponding textbox, left-click the "DRAW" button and a genetic map is generated. The synonymous substitution rate (Ks), the non-synonymous substitution rate (Ka), and the Ka/Ks ratio were calculated by codeml in the PAML package [33].

2.4. Transcriptome-Based Expression Profiling and Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment Analysis of *PtWnt* Genes

To analyze the expression profiles of *PtWnt* genes, the NCBI protein NR annotated information (Table S1) and Fragments Per Kilobase of transcript sequences per Million base pairs (FPKM) values (Table S2) of *PtWnt* were obtained from our published RNA-Seq datasets [24] of regenerative limb bud muscle (BM) and normal muscle (NM) of *P. trituberculatus* with NCBI accession number PRJNA422610. The expressional heatmaps of *PtWnt* genes were constructed using the edgeR R package (3.18.1). Unless specifically stated, default parameters were adopted for the tools used in this study.

GO enrichment analysis of *PtWnt* genes was performed using Goseq R packages based on the Wallenius non-central hypergeometric distribution. KEGG pathway enrichment analysis of *PtWnt* genes was performed using KOBAS software [34].

2.5. Expression Patterns of the *Wnt* Family by qRT-PCR during Limb Regeneration in *P. trituberculatus*

For analyzing the expression patterns of the *Wnt* gene family during different limb regeneration stages, *P. trituberculatus* individuals (49.1 ± 3.4 g) were collected from a commercial farm in Fenghua, Zhejiang Province, China ($121^{\circ}44'55''$ E, $29^{\circ}36'35''$ N). All crabs were fed for a week and acclimated to the laboratory conditions (23 ppt, 28 °C) before the experimental treatment. Then, the autotomy treatments for removal of the first claw were carried out according to the method used in our previous studies [9,24,25] by gently applying pressure to the limbs. As shown in Figure 1, four landmark developmental stages for the regeneration of the limb were selected for analyzing the expression patterns of *Wnt* gene family members based on growth of the limb bud in *P. trituberculatus*. We collected limb bud tissue from the first claw during these four developmental stages from six crabs and combined them to generate a single sample. In total, 36 crabs were randomly sampled, with three biological replicates and three crabs per biological replicate for each developmental stage. All procedures involving animals throughout the experiments were conducted in strict accordance with the National Institute of Health guidelines for the care and use of laboratory animals.

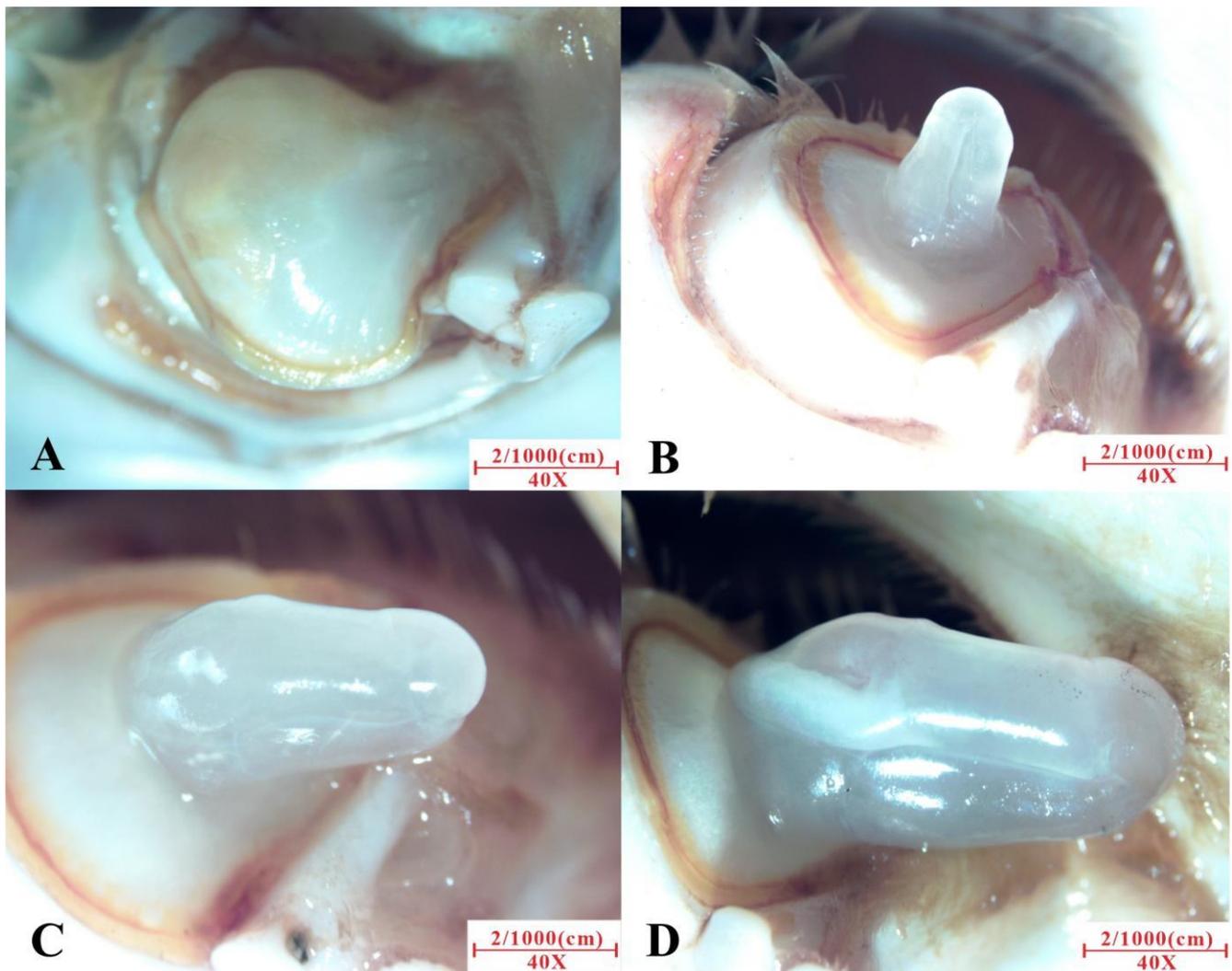


Figure 1. Four landmark developmental stages for the regeneration of the limb in *P. trituberculatus*. (A) Stage I of regenerative limb with wound healing; (B) stage II of regenerative limb with regeneration limb bud appear; (C) stage III of regenerative limb with segmentation of regeneration limb bud; (D) stage IV of regenerative limb with mature regeneration limb bud.

Total RNAs were isolated using Trizol reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions. Gene-specific primers for the qRT-PCR were designed using Primer Premier 5.0 software [35] (Table S3). The β -actin gene was used as the internal control. The qRT-PCR was performed using a reaction procedure as follows: 3 min at 95 °C for pre-denaturation, 40 cycles of 95 °C for 5 s, 58 °C for 30 s, 72 °C for 30 s. Then, a melting curve analysis was added to check the specificity of the PCR products. All PCR amplifications were repeated with three biological replicates. The relative expression of *PtWnt* genes was calculated using the $2^{-\Delta\Delta Ct}$ method [36].

2.6. Statistical Analysis

All data are presented as the mean values \pm SEM. Significant differences in the qRT-PCR were examined using one-way ANOVA, followed by a Tukey test in SPSS software version 20 (Portsmouth, Hampshire, UK). Values were considered statistically significant if $p < 0.05$.

3. Results

3.1. Identification of Wnt Genes in *P. trituberculatus* and *L. vannamei*

The detailed information on *PtWnt* genes is presented in Table 1, including the genomic position, chromosome location, gene strand, protein length (aa), theoretical pI, and molecular weight. In total, we identified 13 *Wnt* genes in *P. trituberculatus* (Table 1). *PtWnt4* was isolated and characterized concerning its function during limb regeneration in our previous study [25]. The remaining 12 genes, to our knowledge, were identified for the first time in crabs in this study, and we named these genes *PtWnt1*, *PtWnt2*, *PtWnt3*, *PtWnt5*, *PtWnt6*, *PtWnt7*, *PtWnt8*, *PtWnt9*, *PtWnt10*, *PtWnt11*, *PtWnt12*, and *PtWnt13* depending on their position on the chromosome. All 13 *PtWnt* genes had a complete open reading frame (ORF) (Table S4). The length of the *PtWnt* proteins varied extensively, ranging from 72 to 666 amino acids, and the average length was 334 amino acids. However, the genomic position, chromosome location, and gene strand information were absent for *PtWnt10*, *PtWnt11*, *PtWnt12*, and *PtWnt13*. The predicted molecular weights of the 13 *PtWnt* proteins varied from 8.03 to 73.68 kDa. The results revealed that the pI of *PtWnt* proteins ranged from 5.39 to 10.54. In *L. vannamei*, 17 *Wnt* genes were identified, 12 of which had previously been reported [21,37], while *LvWnt3*, *LvWnt12*, *LvWnt13*, *LvWnt14*, and *LvWnt15* were newly identified.

Table 1. The identification of *Wnt* gene family members in *P. trituberculatus*.

Gene Name	Genomic Position	Chr	Chromosome Length	ORF	Gene Stand	Protein Length (aa)	Theoretical pI	Molecular Weight (kDa)
<i>PtWnt1</i>	5,505,983–5,542,897	3	10,158,951	1710	reverse	569	5.39	58.91
<i>PtWnt2</i>	5,544,234–5,546,536	3	10,158,951	369	reverse	122	10.04	13.65
<i>PtWnt3</i>	28,210,259–28,217,084	40	29,382,946	1056	reverse	351	9.09	39.62
<i>PtWnt4</i>	6,906,935–6,911,930	28	12,784,577	219	forward	72	10.54	8.03
<i>PtWnt5</i>	4,822,813–4,826,764	30	12,291,475	1353	reverse	450	9.34	50.85
<i>PtWnt6</i>	4,925,695–4,935,753	30	12,291,475	477	forward	158	9.67	17.95
<i>PtWnt7</i>	4,941,929–4,949,590	30	12,291,475	1272	forward	423	9.87	47.80
<i>PtWnt8</i>	18,612,647–18,621,734	24	20,371,438	1035	forward	344	9.37	38.59
<i>PtWnt9</i>	18,843,485–18,849,022	24	20,371,438	780	reverse	259	8.97	28.59
<i>PtWnt10</i>				987		328	9.86	36.29
<i>PtWnt11</i>				639		212	9.68	24.57
<i>PtWnt12</i>				1176		391	9.85	43.25
<i>PtWnt13</i>				2001		666	9.49	73.68

ORF, open reading frame.

3.2. Phylogenetic Analysis

To analyze the evolutionary relationships of *Wnt* proteins in crustaceans, 11 *PtWnts* and 16 *LvWnts* were used to construct the phylogenetic tree (Figure 2). The results show that the *Wnt* proteins were split into eight clusters, which are significantly different on the phylogenetic tree (Figure 2). Phylogenetic tree display that *PtWnt1*, *PtWnt2*, *PtWnt3*, *LvWnt2*, *LvWnt3*, *LvWnt17*, and *LvWnt10* were clustered into a clade. *PtWnt13*, *PtWnt12*, *LvWnt2*, *LvWnt7*, and *LvWnt14* were clustered into a clade. *PtWnt2*, *PtWnt3*, and *LvWnt16* were clustered into a clade. *PtWnt3*, *PtWnt11*, *LvWnt4*, and *LvWnt6* were clustered into a clade. *PtWnt9*, *LvWnt5*, and *LvWnt9* were clustered into a clade. *PtWnt8*, *LvWnt8*, and *LvWnt1* were clustered into a clade. *PtWnt10*, *LvWnt13*, and *LvWnt15* were clustered into a clade.

No. XP_045133204.1); *Homo sapiens Wnt4* (Accession No. BAC23080); *Mus musculus Wnt4* (Accession No. EDL29922); *Danio rerio Wnt4* (Accession No. AAI63457); *Xenopus laevis Wnt4* (Accession No. P49338); *Gallus gallus Wnt4* (Accession No. NP_990114); *H. sapiens Wnt5* (Accession No. BAB62039); *M. musculus Wnt5* (Accession No. AAH51406); *D. rerio Wnt5* (Accession No. AAI62981); *X. laevis Wnt5* (Accession No. P33945); *H. sapiens Wnt6* (Accession No. BAB55603); *M. musculus Wnt6* (Accession No. AAH48700); *Takifugu rubripes Wnt6* (Accession No. XP_011609805.1); *X. laevis Wnt6* (Accession No. ABY53107); *G. gallus Wnt6* (Accession No. NP_001007595); *H. sapiens Wnt7* (Accession No. BAA82509); *M. musculus Wnt7* (Accession No. EDK99298); *G. gallus Wnt7* (Accession No. NP_989623); *D. rerio Wnt7* (Accession No. NP_001020711); *X. laevis Wnt7* (Accession No. AAI69513); *H. sapiens Wnt10* (Accession No. AAG45153); *M. musculus Wnt10* (Accession No. AAB08085); *D. rerio Wnt10* (Accession No. AAA03431); *G. gallus Wnt10* (Accession No. BAD61009); *X. laevis Wnt10* (Accession No. ABG49498); *H. sapiens Wnt16* (Accession No. AAD38052); *M. musculus Wnt16* (Accession No. AAD49352); *D. rerio Wnt16* (Accession No. NP_001093516).

3.3. Characteristics of PtWnt Domains

The phylogenetic tree result revealed that the members of the *Wnt* family in *P. trituberculatus* were classified into three main groups and nine subgroups (Figure 3A). Meanwhile, the members of subgroup 3 (*PtWnt1*, *PtWnt2*, *PtWnt4*, and *PtWnt8*) were clustered in a clade. The Multiple Expectation maximization for Motif Elicitation (MEME) online software was used for conserved motif analysis among different PtWnt proteins, and a total of 10 conserved motifs were predicted (Table S5, Figure 3B). The types and numbers of motifs in different *PtWnt* groups had several differences. Among these, motif 1 was conserved in all 10 *PtWnts* of group 1, while motif 7 was observed in nine *PtWnts* of group 1. Motifs 1, 2, 3, 4, 5, 6, and 7 were shared in four *PtWnts*. Motifs 9 and 10 were only found in two *PtWnts*. Motif 4 was present in all three groups. Moreover, we observed that the proteins of group I contained more than three motifs. As an exception, *PtWnt6* had only three motifs. In addition, *PtWnt4* in group II and *PtWnt2* in group III only contained one motif (motif 4 or motif 5). The Coding sequence (CDS) structure of *PtWnt* genes was further characterized. The results showed that the numbers of CDSs in each *PtWnt* gene varied from 3 to 13 (Figure 3C). The CDSs of *PtWnts* were disrupted by introns. However, the exact exon-intron structure of *PtWnt* genes was not further characterized.

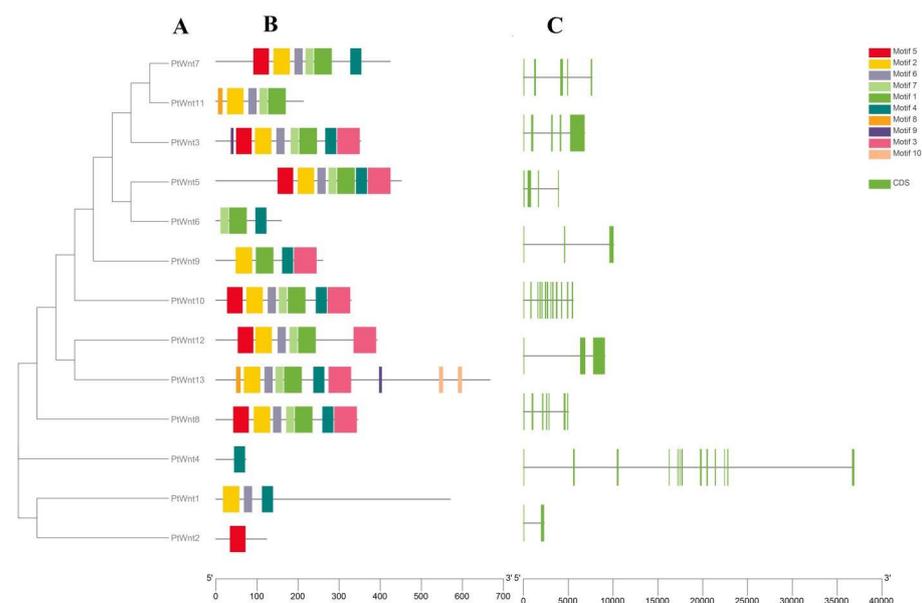


Figure 3. Unrooted neighbor-joining phylogenetic tree, architecture of conserved motifs, and gene structure of the *PtWnt* gene family. (A) The phylogenetic tree was generated based on the sequences of PtWnt proteins; (B) conserved motif analysis of PtWnt proteins. Different colored boxes represent different types of motifs; (C) coding sequence (CDS) structure of *PtWnt* gene family.

The results of the analysis of the structures of PtWnt proteins indicated that all the deduced PtWnt proteins contained a conserved cysteine-rich Wnt1 domain. The signal peptides of 20–31 amino acid residues were only identified in *PtWnt2*, *PtWnt10*, and *PtWnt13* (Figure 4).

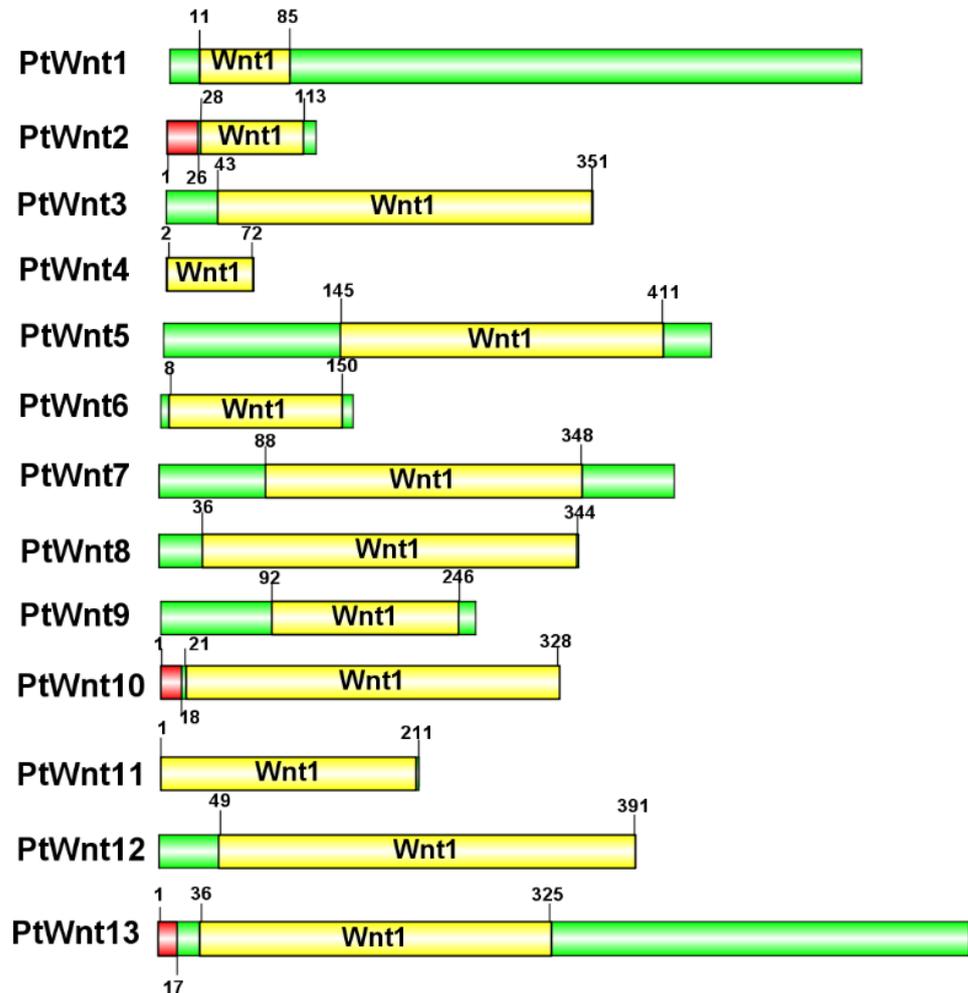


Figure 4. Structure of PtWnt proteins. The yellow boxes indicate the conserved Wnt1 domains. The red boxes show signal peptides. The green boxes indicate non-specific domains.

3.4. Chromosomal Distribution

P. trituberculatus have 53 pairs of chromosomes, the chromosomal distribution showed that nine *PtWnts* were located in five chromosomes of *P. trituberculatus* (Figure 5). The distribution of genes among different chromosomes was uneven, ranging from 1 to 3 genes per chromosome. In detail, Chromosome 28 and Chromosome 40 harbored only one *PtWnt*, whereas Chromosome 3 and Chromosome 24 harbored two *PtWnts*. A maximum of three genes were clustered on chromosome 30. To better understand the evolutionary constraints acting on *Wnt* gene family, the K_a/K_s ratios of the *Wnt* gene pairs were calculated. The ratio of K_a/K_s of tandem duplication gene pairs of *PtWnt6* and *PtWnt7* was 0.3606.

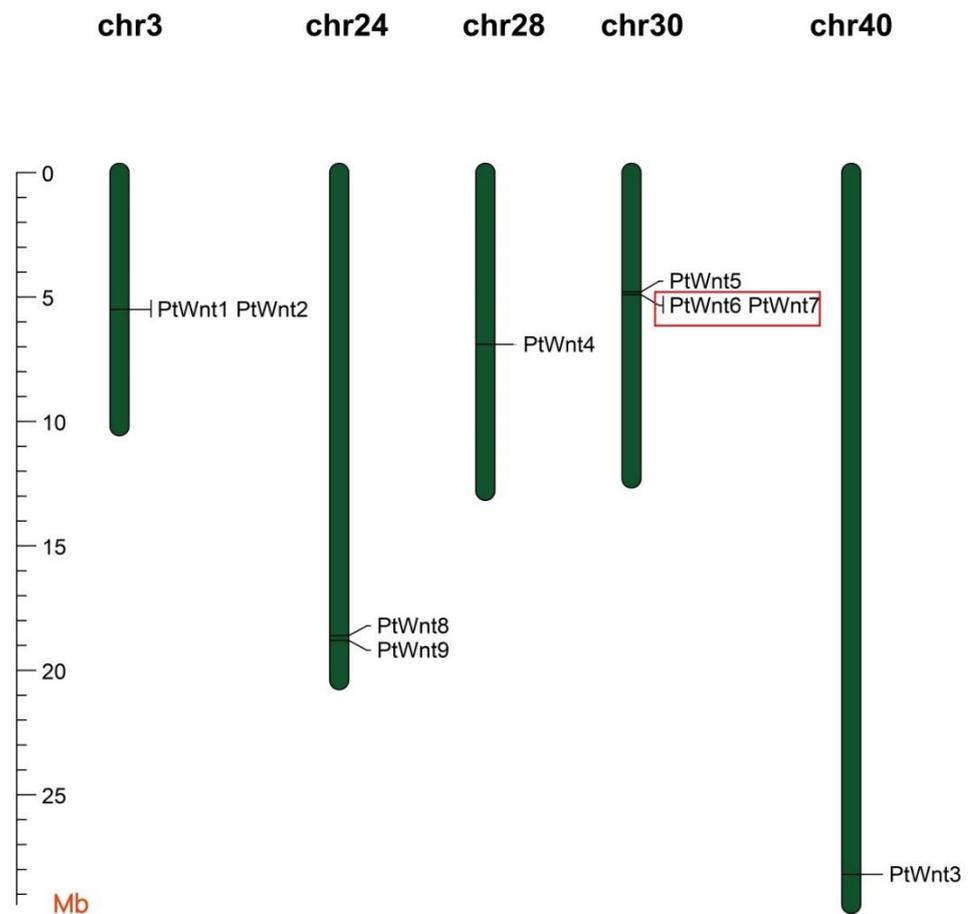


Figure 5. Chromosomal location and duplication events of nine *Wnt* genes on five different chromosomes of *P. trituberculatus*. Each chromosome number is indicated on the top of the chromosome bar. The scale of the chromosome is in millions of bases (Mb) (The length of green represents the number of bases). Each *PtWnt* gene is indicated on the exact position of a particular chromosome that can be calculated by using the scale provided on the left. Tandem duplicated genes are indicated by red boxes.

3.5. Regeneration Limb Transcriptome-Based Expression Profiling and GO and KEGG Enrichment Analysis of *PtWnt* Genes

In order to discover genes underlying the molecular mechanisms governing regenerated limb development in *P. trituberculatus*, we applied next-generation sequencing technology to identify functional genes potentially associated with limb regeneration in our previous study [24] and found differentially expressed *PtWnt* genes in the comparison of regenerating limb BM and NM. In this study, the available mRNA-seq data of relative expression in regenerating limb BM and NM for the *Wnt* gene family were obtained and analyzed for gene expression pattern, followed by GO and KEGG enrichment analyses.

Ten of 13 *PtWnt* genes (*PtWnt1*, *PtWnt2*, *PtWnt4*, *PtWnt5*, *PtWnt6*, *PtWnt7*, *PtWnt9*, *PtWnt10*, *PtWnt11*, and *PtWnt16*) were differentially expressed in the comparison of regenerating limb BM and NM (Figure 6 and Table S6). According to the expression patterns in regenerating limb BM and NM, the 10 *PtWnt* genes were clustered into three main groups, and the genes clustered in the same group shared a similar expression pattern. As shown in Figure 6, *PtWnt6*, *PtWnt10*, and *PtWnt11* clustered together into the first group, as they appeared to have higher expression levels in NM than in BM. In the second group, *PtWnt2*, *PtWnt9*, *PtWnt4*, *PtWnt1*, and *PtWnt5* were clustered together as they showed higher expression levels in BM than in NM. Finally, *PtWnt7* and *PtWnt16* were clustered into a single branch, as their expression levels in the two compared groups were randomly distributed.

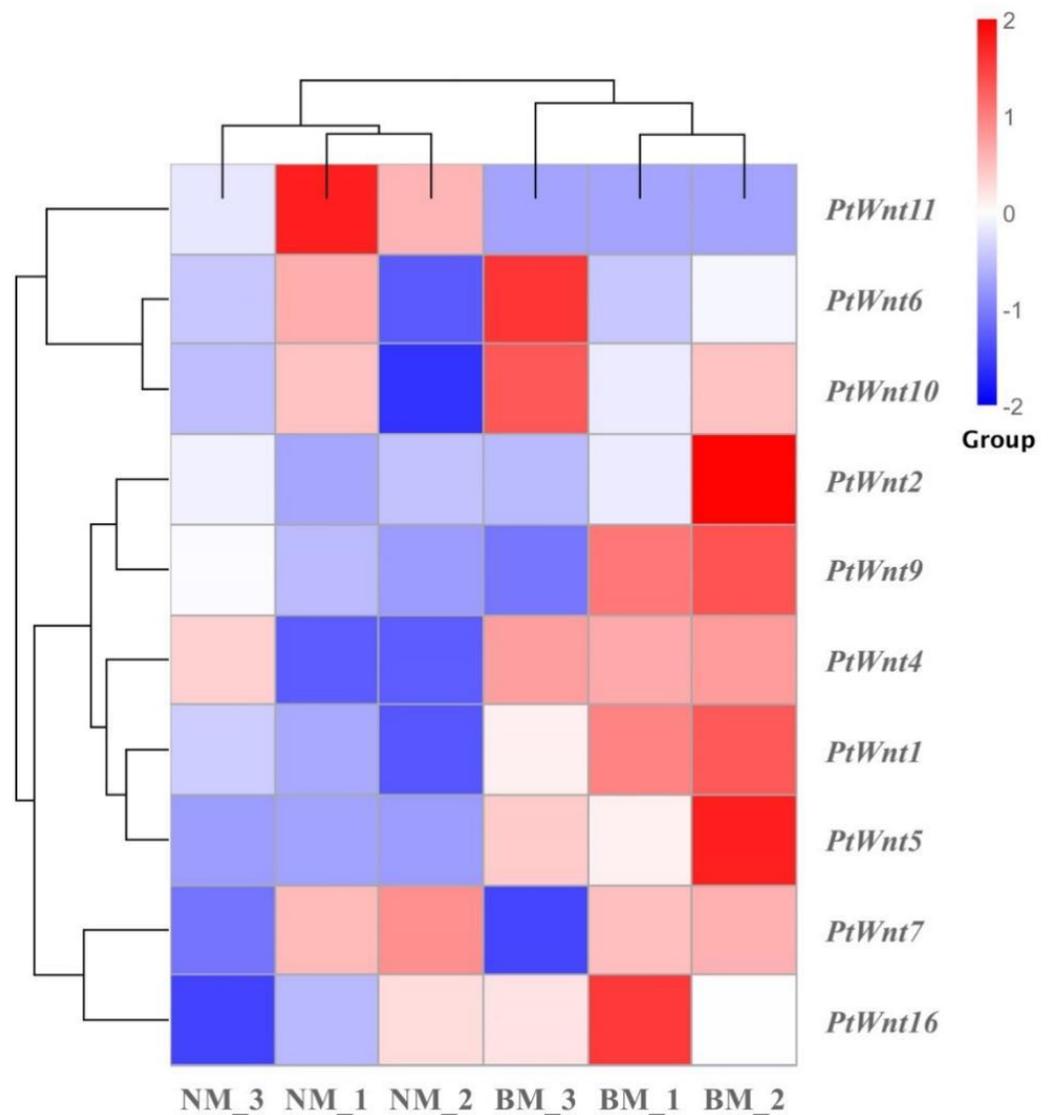


Figure 6. Heatmap of *PtWnt* gene expression profiles in regenerating limb bud muscle (BM) and normal limb muscle (NM).

To associate *PtWnt* genes exhibiting different expression patterns with physiological and morphological changes during limb regeneration, we performed GO enrichment analysis using the GOrseq R package. The results suggested that all the *PtWnts* were sorted into 22 functional groups that belonged to three main GO categories: Biological processes, cellular components, and molecular functions (Figure 7A and Table S7). In the biological process category, the cell surface receptor signaling pathway (GO: 0007166), *Wnt* signaling pathway (GO: 0016055), single organism signaling (GO: 0044700), signaling (GO: 0023052), and signal transduction (GO: 0007165) were prominently represented. Receptor binding (GO: 0005102), binding (GO: 0005488), and protein binding (GO: 0005515) were included in the molecular function category, while extracellular region (GO: 0005576) was only included in the cellular component category.

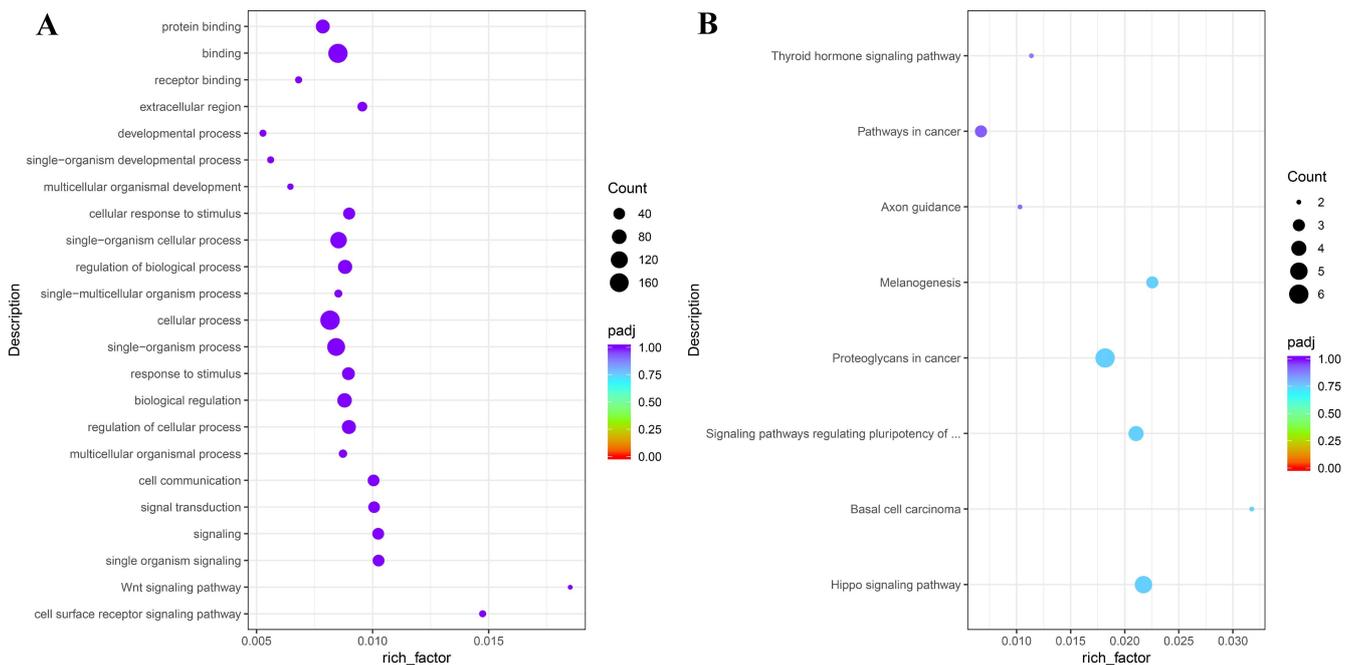


Figure 7. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of *PtWnt* gene family members between comparisons of regenerating limb bud muscle versus normal limb muscle. (A) GO enrichment results; (B) statistics of KEGG pathway enrichment.

To understand potential gene interactions and the biological functions underlying limb regeneration, all *PtWnts* were searched against the KEGG database and subsequently assigned to eight pathways (Figure 7B and Table S8). The most dramatically enriched pathway was the hippo signaling pathway (ko04390), followed by basal cell carcinoma (ko05217), signaling pathways regulating pluripotency of stem cells (ko04550), proteoglycans in cancer (ko05205), melanogenesis (ko04916), axon guidance (ko04360), pathways in cancer (ko05200), and thyroid hormone signaling pathway (ko04919).

3.6. Expression Profiling of *PtWnt* Genes at Different Developmental Stages of Limb Regeneration

To study the expression patterns of *PtWnts* during limb regeneration, 12 *PtWnts* were selected for qRT-PCR. The results showed that the expression levels of nine *PtWnts* (except *PtWnt1*, *PtWnt2*, and *PtWnt6*) had a similar trend during limb regeneration. Among these, *PtWnt4*, *PtWnt5*, and *PtWnt13* began their expression at stage I, increased significantly ($p < 0.05$) at stage II, and increased most significantly ($p < 0.01$) at stage III and stage IV (Figure 8). In addition, the expression levels of *PtWnt7*, *PtWnt11*, and *PtWnt16* had a similar trend of increasing slightly at stage I and then clearly increasing ($p < 0.01$) in stage III and stage IV, throughout the process of limb regeneration. Moreover, *PtWnt8*, *PtWnt9*, and *PtWnt12* were most significantly up-regulated ($p < 0.01$) from stage II to stage IV compared to the expression level at stage I. In contrast, *PtWnt2* had the highest expression level at stage I and showed a downward trend from stage II to stage IV. Interestingly, the expression of *PtWnt1* declined significantly ($p < 0.05$) at stage II and stage III compared to stage I and increased from stage III to stage IV but was still significantly lower ($p < 0.05$) than at stage I. Conversely, *PtWnt6* continued to increase from stage I to stage III, with no significant difference between stage I and stage II, and then declined in stage IV ($p < 0.05$).

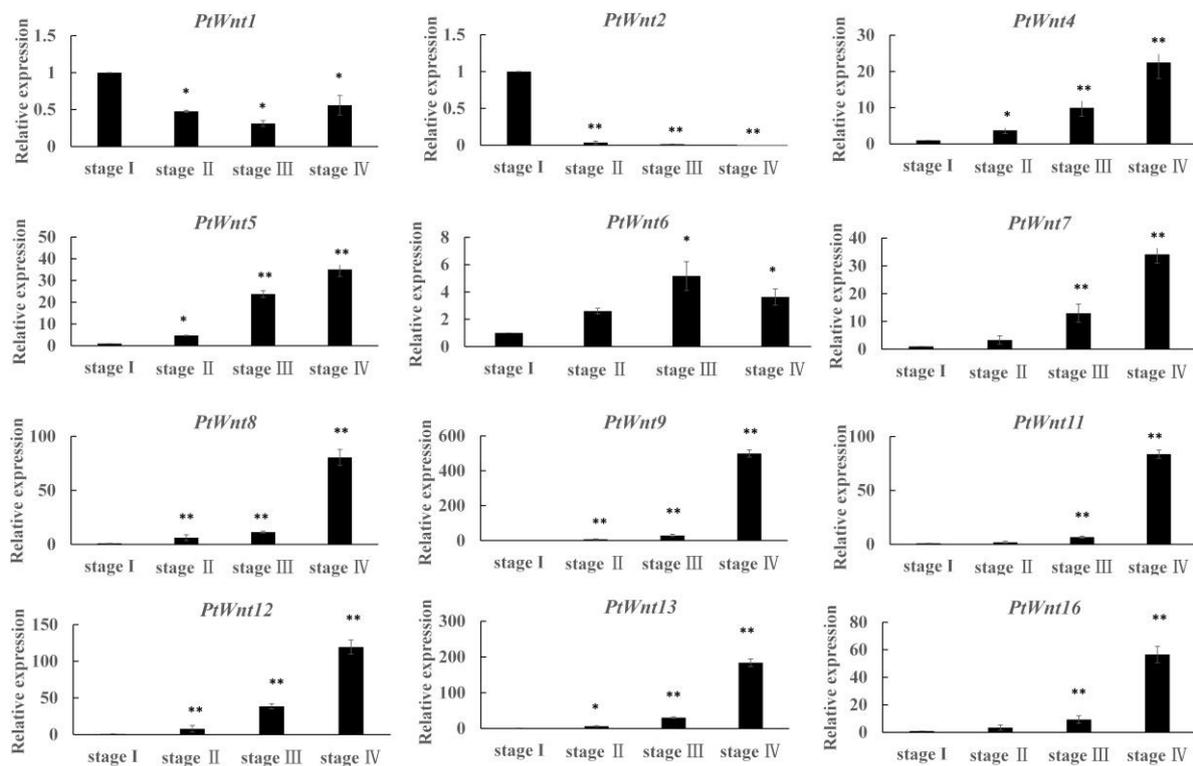


Figure 8. Expression patterns of 12 *PtWnts* at four developmental stages of the regenerating limb. The expression of target genes was detected by qRT-PCR and normalized to β -actin gene as the internal reference. The results were based on three independent biological replications and show mean values \pm SD. (* $p < 0.05$, ** $p < 0.01$).

4. Discussion

Regulation of *Wnt* signaling and *Wnt* ligands during regeneration has been widely studied during various regeneration processes of vertebrate and invertebrate animals such as *Xenopus* limb regeneration [10,38], zebrafish retina regeneration [39,40], and insect limb regeneration [41,42]. Different species of arthropods have different numbers of *Wnt* family members. In this study, we identified 13 *Wnt* genes from the newly assembled genome of *P. trituberculatus* [22], a number that was greater than that in *L. vannamei* [21] and other arthropods. The higher number of *Wnt* family members in *P. trituberculatus* and *L. vannamei* than in other arthropods is the ultimate result of their higher gene abundance and repetitive sequences of their genome [22]. In contrast, our results suggest that the number of *Wnt* subfamilies in crustaceans is evolutionarily conserved.

To delineate the evolutionary history and more accurately predict the function of *PtWnts*, a phylogenetic tree was constructed using the amino acid sequences of *P. trituberculatus* and *L. vannamei*. As shown in Figure 2, *PtWnt* genes were clustered with respective counterparts as expected, and 8 clades were generated. Most clades of orthologues (except for the *Wnt4* and *Wnt6* clades) were divided into two branches, a *P. trituberculatus* branch and a *L. vannamei* branch, suggesting the distant evolutionary relationships among *Wnts* between *P. trituberculatus* and *L. vannamei*. The results were identical to those in previous reports concerning *Wnt* gene family function and evolution in mollusks [43]. Notably, the gene number of *P. trituberculatus* was not consistent with *L. vannamei* in the same clade, which could be attributed to their name annotation depending on their position on the chromosome. In general, *Wnt* genes have been relatively conserved during evolution.

As shown in Figure 3, only the CDS structure was present, while the main introns and exons of *PtWnts* were not obtained, which may have contributed to the genome quality. However, the *PtWnt* gene structure was conserved, as all the deduced *PtWnt* proteins

had a conserved cysteine-rich Wnt1 domain, similar to other crustacean species [21]. The motif composition showed that the protein structures of the *PtWnt* family were broadly conserved, and some motifs were prevalent in the 13 *PtWnt* proteins, such as motif 1 and motif 4 in 10 *PtWnt* proteins and motif 7 in nine proteins. These results demonstrated that the protein domain and motifs of *PtWnts* were highly conservative. Moreover, the conservative motif of *PtWnt* proteins also highlights their evolutionary proximity [44]. As a result, the motif analysis further confirmed the annotation of *PtWnt* genes.

Compared with other previous *Wnt* gene family studies in crustaceans, this is the first report to map their chromosomal locations and to indicate the occurrence of chromosome duplication events during evolution. Chromosome localization of genes is the result of both fragment replication and tandem duplication and plays an important role in promoting gene family expansion [45,46]. In this study, *PtWnt6* and *PtWnt7* evolved from tandem duplication, indicating that segment duplication may be a major driving force for *PtWnt* gene evolution [47].

GO analysis was performed to characterize the functions of *PtWnt* proteins in limb regeneration of *P. trituberculatus*. A number of *Wnt* proteins have been characterized in crustaceans, and a functional analysis of their roles in the defense against pathogenic virus infection has been performed concerning the innate immunity of *L. vannamei* [21,37]. However, there are limited reports on crab species with which to understand the biological processes associated with *Wnt* genes during limb regeneration. In this study, cell surface receptor signaling pathway (GO:0007166), single organism signaling (GO:0044700), signaling (GO:0023052), and extracellular region (GO:0005576) were the most enriched processes, besides the *Wnt* signaling pathway (GO:0016055) that has been well described in previous regeneration studies of other animals [41,48–50]. These processes are responsible for their functions during limb regeneration and development. For example, *Wnt* proteins play roles as secreted glycoproteins by binding to their respective receptors on the cell surface, a process that is pivotal in the signaling pathways. Moreover, the effects of *Wnt* signaling include a wide range of cellular responses, including cell fate determination and cytoskeletal remodeling, that belong to extracellular region processes [51]. The exact function of the *PtWnt* gene family involved in a complex network of signaling events of these enriched processes during limb regeneration needs further investigation. Pathway-based KEGG enriched results are helpful for understanding the biological functions of *PtWnt* genes during limb regeneration, which our previous transcriptome-based analysis [24].

At present, expression and functional analyses of *Wnt* genes have been reported in several Arthropoda species, including *Tribolium castaneum* [15] and *L. vannamei* [21,37]. However, most knowledge concerning the expression of *Wnt* genes comes from the analysis of tissue-specific expression, and little is known about their expression patterns during regeneration. Recent reports have provided valuable insights into *Wnt* function and evolution during organ regeneration in other invertebrates such as *Hydra* [12] and *Ruditapes philippinarum* [43]. To date, there are no reports on the characterization of the *Wnt* gene family or their functions in crustaceans. According to our published transcriptome data on regenerating limbs in *P. trituberculatus*, most *PtWnt* genes had a higher FPKM value in the regenerating limb BM than in NM (Figure 6) [24]. In that study, the expression levels of *Wnt* genes in *P. trituberculatus* were measured during different developmental stages of limb regeneration using qPCR.

An increasing number of gene family functional studies have shown that genes belonging to the same group seem to have similar expression patterns [46]. Moreover, different *Wnt* ligands have different signal transduction properties during animal regeneration process [12,16]. It has been reported that *Wnt1*, *Wnt3a*, and *Wnt8* play roles in the transformation and axial growth of mouse and *Xenopus* embryos through the classic *Wnt*/ β -Catenin pathway [17,18], while *Wnt4*, *Wnt5A*, and *Wnt11* can activate the non-canonical β -catenin-independent *Wnt* pathway and affect morphogenesis by inducing the extension of the epithelial layer [19,20]. In our study, five β -catenin-independent *Wnt* signaling members (*Wnt4*, *Wnt5*, *Wnt7*, *Wnt11*, and *Wnt16*) had a similar expression pattern, beginning their

expression at stage I, increasing the level at stage II, and increasing most significantly from stage III to stage IV (Figure 8). However, the β -catenin-dependent *Wnt* signaling members showed different expression trends at different stages. For example, *PtWnt2* had the highest expression level at stage I and showed a downward trend from stage II to stage IV. Another β -catenin-dependent signaling gene, *PtWnt1*, showed the highest expression level at stage I, which declined significantly at stage II and stage III and increased again from stage III to stage IV. Our results showed that there were similar expression patterns not only in the same subfamily but also in some family members. Moreover, it can be predicted that the canonical *Wnt*/ β -catenin signaling pathway and the non-canonical *Wnt* pathway mediated by different *Wnt* ligands may cooperate to regulate the preciseness of unknown target genes through cascaded signaling molecules and transcription factors in the process of limb regeneration of *P. trituberculatus* [17,18,38,50]. The above results indicate the complexity and special mechanism of the *Wnt* signaling pathway regulating regeneration in crustaceans. In the near future, RNAi and CRISPR/Cas9 techniques will be utilized to uncover the functions of these *Wnt* genes.

5. Conclusions

Our study represents a comprehensive overview of the *Wnt* gene family in *P. trituberculatus*. To summarize, a total of 13 *Wnt* genes were identified from *P. trituberculatus* and classified into 11 groups according to the conserved protein domains and phylogenetic analysis. Then, the chromosome locations, gene structures, and motif composition were characterized. Published transcription data and qRT-PCR for different stages of limb regeneration were analyzed to gain further understanding regarding the putative functions of *PtWnt* genes during limb regeneration. These findings will facilitate future functional characterization of *PtWnt* genes and provide a foundation for exploring the molecular mechanism during limb regeneration in *P. trituberculatus* and other crustaceans.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fishes7050258/s1>, Table S1: Details of *Wnt* unigenes annotated in NR database from limb regeneration transcriptome of *P. trituberculatus*; Table S2: Fragments Per Kilobase of transcript sequences per Million base pairs (FPKM) values of *Wnt* genes from limb regeneration transcriptome of *P. trituberculatus*; Table S3: Combination of primers used in quantitative real-time PCR (qRT-PCR) assays; Table S4: The Ka/Ks ratios results for the replicated gene pairs of *Ptwnt6* and *Ptwnt7*; Table S5: Multiple Expectation maximization for Motif Elicitation (MEME) motif analysis of *Wnt* unigenes of *P. trituberculatus*; Table S6: List of the selected *Wnt* genes for differential expression analysis; Table S7: Go enrichment of all the annotated *PtWnt* genes in the comparison regenerative limb bud muscle (BM) and normal muscle (NM); Table S8: Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment of all the annotated *PtWnt* genes in the comparison regenerative limb bud muscle (BM) and normal muscle (NM).

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