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Molecular Cloning and Polymorphism Analysis of PmFGF18 from *Pinctada fucata martensii*

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Received: 26 September 2020; Accepted: 5 November 2020; Published: 10 November 2020

Abstract: Fibroblast growth factor 18 (FGF18) plays an important functional role in skeletal growth and development. The FGF18 gene was characterized in pearl oyster *Pinctada fucata martensii* (PmFGF18) with the full-length sequence containing an open reading frame of 714 bp encoding 237 amino acids. The domain analysis of PmFGF18 showed a distinctive FGF domain, with a high similarity to FGF18 protein sequences from *Crassostrea gigas* (43.35%) and *C. virginica* (37.43%). PmFGF18 expression was revealed in all analyzed tissues with a significantly higher expression level in the fast-growing group than the slow-growing group. The analysis of PmFGF18 polymorphism demonstrated 33 SNPs (single nucleotide polymorphisms) in the CDS and promoter region of PmFGF18 sequence. Association analysis revealed 19 SNPs (2 SNPs from CDS and 17 SNPs from the promoter region) associating significantly with growth traits. Among the associated SNPs, one SNP g.50918198 A > C was verified in the other breeding line. Therefore, PmFGF18 can be utilized as a candidate gene for growth, and its related SNPs could be used in selective breeding of *P. f. martensii* for the improvement of growth traits.

Keywords: FGF18; SNP; growth traits; *Pinctada fucata martensii*

1. Introduction

The pearl oyster *Pinctada fucata martensii*, cultured for nucleated marine pearls, is naturally found in Japan, South China, and Australia [1]. The size of marine pearls is affected by the growth of pearl oysters [2,3]. This condition has prompted considerable interest in culturing fast-growing populations appropriate for producing pearls [4,5]. Growth traits, as quantitative traits regulated by multiple genes, have been reported in the construction of genetic linkage maps and SNP (single nucleotide polymorphism) identification of associated candidate genes [6–10]. Multiple genetic linkage maps have been researched for the genetic mechanism of the growth traits of bivalves, including *C. gigas*, *Hyriopsis cumingii*, *Chlamys farreri*, and *P. f. martensii* and many genes participating in the construction of growth traits have been obtained [11–13]. In order to explore the genetic mechanism of growth traits, the polymorphism of growth-associated genes, including the amylase gene, the insulin-related peptide gene from *C. gigas* [14–16], and myostatin genes from *C. farreri* [17], *Argopecten irradians* [18] and *C. nobilis* [19] have been widely researched.

The fibroblast growth factor (FGF) family regulates the development of brain patterning, branching morphogenesis, and limbs [20]. In mammals, 18 FGFs (FGF1–10 and FGF16–23) are

known and have been categorized into 6 subfamilies in accordance with the variations in sequence characters [21] and FGF18 exhibits an essential function in skeletal growth and development [22,23]. FGF18 has also been observed to stimulate the growth of costal chondrocytes of neonatal rats and the proteoglycan synthesis of mature primary porcine and human articular chondrocytes [24,25]. In a rat model of osteoarthritis, intra-articular injection of FGF18 increased cartilage formation which showed its potential in biomineralization [26]. In the invertebrate, several FGF18 genes were identified in *Mizuhopecten yessoensis* [27] and *Harpegnathos saltator* [28], but the special functions of FGF18 have not been widely researched. In the present study, one FGF18 gene of *P. f. martensii* was identified and we further analyzed its expression pattern and polymorphism to show its potential role in the growth traits' construction and pearl oyster breeding.

2. Materials and Methods

2.1. Samples Used in this Study

In the present study, *P. f. martensii* was sampled and acquired from Xuwen, Zhanjiang, Guangdong Province, China (20°25' N, 109°57' E). Tissue expression analysis required the adductor muscle (A), gonad (GO), gill (GI), marginal zone of mantle (ME), pallial zone of mantle (MP), and central zone of mantle (MC), having been sacrificed from pearl oysters from base stock. Thirty oysters from slow- (shell length: 56.81 ± 3.87 mm) (fifteen) and fast-growing groups (shell length: 72.19 ± 3.42 mm) (fifteen) (based on the shell length of pearl oysters) from base stock were also obtained for the expression level analysis of *PmFGF18* with adductor muscle as samples to explore *PmFGF18* expression levels in the two groups.

2.2. Method for the Gene Cloning and Sequence Analysis of *PmFGF18*

The FGF18 gene sequence was obtained from the genome data of *P. f. martensii* [29]. The RACE method was utilized to acquire 5' and 3' untranslated regions (UTRs) of the specified sequence with a SMART RACE cDNA amplification kit (TaKaRa, Dalian, China). The primers utilized in the present study are shown in Table 1. The analytical tools used for *PmFGF18* full-length sequence included open reading frame (ORF) Finder, SMART for conserved domain analysis, ClustalX for aligning the sequence of FGF18 genes from species including *C. virginica* (XP_022288219.1), *M. yessoensis* (OWF47747.1), *C. gigas* (XP_011453678.1), *Homo sapiens* (AAC62240.1), *Danio rerio* (AAQ22394.1), *Mus musculus* (AAC62239.1), and *Bos taurus* (AAI20281.1). MEGA 6 was utilized for the phylogenetic analysis of FGF18 from different species with neighbor joining (NJ) method and tested for reliability over 1000 bootstrap replicates.

Table 1. Primers used in the present research.

Primes	Sequence (5'–3')	Application
5'-outer	TTGTTGAAGCAGAGGTACAGGGTAGATT	Outer PCR
5'-inner	GTTCTCTGCTGCCTCCGTCTTTTCCTT	Inner PCR
3'-outer	CTACCCTGTACCTCTGCTTCAACAAGAA	Outer PCR
3'-inner	GATGTCACCTTTCCCAAAATCTGACTGC	Inner PCR
<i>PmFGF18</i> -A	GAAATGTTACCCGTCTGTGCC	qRT-PCR
<i>PmFGF18</i> -S	GGTGGGAATTGATACGTTGATC	qRT-PCR
<i>GAPDH</i> -A	CGTTGATTATCTTGGCGAGTG	qRT-PCR
<i>GAPDH</i> -S	GCAGATGGTGCCGAGTATGT	qRT-PCR

2.3. *PmFGF18* Expression Level

The isolation of total RNA from fast- and slow-growing groups and different tissues was performed with TRIzol reagent from Invitrogen (Carlsbad, CA, USA) [30]. The M-MLV First-strand cDNA Synthesis Kit (Invitrogen) was used to synthesize cDNA which was then used as a template

for qRT-PCR analysis by using the primers described in Table 1. The $2^{-\Delta Ct}$ method was utilized for the expression level analysis of *PmFGF18* in detected tissues and different groups with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a reference gene [3]. The expression level of *PmFGF18* in the development was analyzed with the data from the transcriptome of *P. f. martensii* development.

2.4. SNP Polymorphism of *PmFGF18*

In order to explore the polymorphism of *PmFGF18*, the fifth-generation selected line of *P. f. martensii* for rapid growth was used. The selected line was designated as “Haixuan NO1”, and the development of the line was described in detail by Du et al. (2015) [31]. The fifth-generation selected line of *P. f. martensii* for yellow shell color line was used for the verification of SNPs. The adductor muscle of samples was obtained for DNA extraction with the DNA Kit of Marine Animals from TIANGEN. The shell length, shell width, and shell height of each sample were measured using a digital caliper (0.02 mm accuracy) [32]. Total weight and shell weight of each sample were measured using an electronic balance (0.01 g accuracy). The exon and promoter sequences of *PmFGF18* whose mutation would influence the protein structure and gene expression [33,34] from sampled pearl oysters were genotyped by target resequencing with a filter of SNP with a minor allele frequency lower than 5% [10], and the genome of *P. f. martensii* was utilized as a reference [29]. The expected heterozygosity (H_e), observed heterozygosity (H_o), and allele frequency of the identified SNPs were calculated using PopGene 32 (version 3.2). PIC (Polymorphic information content) of SNPs was evaluated using PIC_CALC software according to its instruction. PIC can be divided into three types, namely, low ($PIC < 0.25$), medium ($0.25 < PIC < 0.5$), and high polymorphisms ($PIC > 0.5$). Comparison of the means of growth traits among different genotypes was conducted one-way ANOVA (analysis of variance) with Duncan test or T-test in both lines using SPSS version 17.0. Significance level for the analysis was specified at $P < 0.05$.

2.5. Data Analysis

ANOVA was used for tissue expression data, and t-test was used for the relative expression analysis between the fast- and slow-growing groups. Significance level for all analyses were specified at $P < 0.05$. Data analyses were accomplished on SPSS 17.0 (IBM, Chicago, IL, USA).

3. Results

3.1. Cloning and Bioinformatic Analyses of *PmFGF18*

PmFGF18 had a full-length sequence of 2534 bp with 248 bp 5'UTR and 1572 bp 3' UTR (Figure S1) and an ORF of 714bp encoding 237 amino acids. The *PmFGF18* protein sequence held an estimated molecular mass of 28.09 kDa and contained a classic FGF domain located at 45–178 amino acids.

3.2. *PmFGF18* Structure and Protein Homology Analysis

The homologous analysis of *PmFGF18* with sequences of FGF18 from *C. virginica* (XP_022288219.1), *M. yessoensis* (OWF47747.1), *C. gigas* (XP_011453678.1), *H. sapiens* (AAC62240.1), *D. rerio* (AAQ22394.1), *M. musculus* (AAC62239.1), and *B. taurus* (AAI20281.1) by using Clustal X2 exhibited the conserved functional domain of *PmFGF18* (Figure 1A). *PmFGF18* shared 43.35% identity with *C. gigas*, 37.43% identity with *C. virginica*, and 33.14% identity with *M. yessoensis*. The predicted organizational structure of *PmFGF18* and that of different species (*C. gigas*, *C. virginica*, *M. yessoensis*, *H. sapiens*, *M. musculus*, *D. rerio*, and *B. Taurus*) also showed the conservation of *PmFGF18* (Figure 1B). The phylogenetic tree analysis showed that these FGF18 proteins were divided into invertebrate and vertebrate branches which conformed to the traditional taxonomy (Figure S2).

Figure 1. Multiple sequence alignment and structural organization of PmFGF18 amino acid sequence and other species. **(A)** shows the multiple sequence alignment of PmFGF18. Dark blue background indicates conserved aa; pink background indicates aa with strong similarity; light blue indicates aa with weak similarity. The numbers on the right present the total amino acid of each protein. **(B)** represents the structural organization of FGF18 among different species. The red region represents the signal peptide. Accession number for the sequences used in this alignment and domain analysis are as follows: *C. gigas* (XP_011453678.1), *C. virginica* (XP_022288219.1), *M. yessoensis* (OWF47747.1), *H. sapiens* (AAC62240.1), *M. musculus* (AAC62239.1), *D. rerio* (AAQ22394.1), and *B. taurus* (AAI20281.1).

3.3. Expression Analysis of PmFGF18

PmFGF18 mRNA expressed in the adductor muscle, gill, marginal zone of mantle, pallial zone of mantle, gonad, and central zone of mantle of *P. f. martensii*, which presented its wide existence in *P. f. martensii* (Figure 2A). A significantly higher expression level of *PmFGF18* was recorded in the marginal zone of mantle than in other tissues. The expression pattern of *PmFGF18* during development presented a wide expression in development and a higher expression level in the late development (Figure S3). The expression level of *PmFGF18* in the fast- and slow-growing groups indicated that *PmFGF18* expressed higher in the fast-growing group than in the slow-growing group ($P < 0.05$, Figure 2B).

Figure 2. Expression pattern of *PmFGF18* from *P. f. martensii*. (A) Expression pattern of *PmFGF18* in different tissues. ME: Marginal zone of mantle; MP: Pallial zone of mantle; MC: Central zone of mantle; A: Adductor muscle; GI: Gill; GO: Gonad; Different letters indicate significant differences ($P < 0.05$) determined through one-way ANOVA, and the bar represents the standard deviation. (B) Expression pattern of *PmFGF18* in the fast- and slow-growing groups. Bars with different letters indicate significant differences ($P < 0.05$).

3.4. *PmFGF18* Polymorphism Analysis and SNP Identification

In total, 33 SNPs were detected, and the polymorphism of SNPs showed that H_e ranged from 0.0578 to 0.5004, and H_o ranged from 0.0595 to 0.5756. PIC analysis found 21 loci with a low polymorphism level and 12 loci with a medium polymorphism level. HWE analysis indicated that 8 loci significantly deviated from HWE ($P < 0.05$) (Table 2).

Table 2. Genetic diversity based on SNP polymorphisms in *PmFGF18*.

Location	Number	Locus	H_o	H_e	HWE	PIC
Promoter	SNP1	50916942 T > C	0.1023	0.0999	0.532498	0.094861
	SNP2	50916948 T > G	0.0992	0.0971	0.576594	0.092275
	SNP3	50916962 G > A	0.0885	0.0875	0.75112	0.083585
	SNP4	50916963 T > C	0.0901	0.0889	0.724239	0.084909
	SNP5	50916970 T > C	0.0947	0.093	0.647506	0.088691
	SNP6	50916971 T > A	0.0947	0.093	0.647506	0.088691
	SNP7	50916977 A > G	0.0962	0.0944	0.623226	0.089835
	SNP8	50916978 A > T	0.0977	0.0958	0.59959	0.091139
	SNP9	50916986 A > T	0.0977	0.0958	0.59959	0.091139
	SNP10	50916996 T > A	0.0977	0.0958	0.59959	0.091139
	SNP11	50917667 A > C	0.1924	0.1862	0.396673	0.168744
	SNP11	50917717 A > G	0.5756	0.4769	0	0.362973
	SNP12	50917735 A > T	0.0611	0.0592	0.426239	0.057391
	SNP13	50917780 A > T	0.5603	0.4734	0.000003	0.361182
	SNP14	50917839 A > T	0.0779	0.0805	0.396651	0.077234
	SNP15	50917897 T > C	0.1267	0.1214	0.26371	0.11401
	SNP16	50918021 T > C	0.5496	0.5	0.010962	0.37479
	SNP17	50918198 A > C	0.4763	0.4221	0.001006	0.332859
	SNP18	50918226 G > A	0.1771	0.174	0.646955	0.158772
	SNP19	50918333 C > T	0.4366	0.3989	0.01529	0.319141
	SNP20	50918342 C > A	0.5099	0.4647	0.012593	0.356514
	SNP21	50918354 T > G	0.5099	0.4663	0.016496	0.35739
	SNP22	50918365 T > G	0.2672	0.2621	0.619233	0.227641
	SNP23	50918376 C > A	0.0473	0.0462	0.541862	0.045206

Table 2. Cont.

Location	Number	Locus	Ho	He	HWE	PIC
EXON	SNP24	50918398 A > T	0.5618	0.5004	0.001655	0.374995
	SNP25	50918446 G > A	0.0611	0.0621	0.663095	0.060209
	SNP26	50918498 A > G	0.5634	0.5004	0.001261	0.37499
	SNP27	50918638 A > G	0.0641	0.0621	0.40245	0.060209
	SNP28	50918729 T > C	0.5557	0.5003	0.004568	0.374972
	SNP29	50918839 T > C	0.1405	0.1333	0.168189	0.124406
	SNP30	50926498 G > T	0.5389	0.4626	0.000024	0.355408
	SNP31	50926618 A > G	0.5252	0.4622	0.000478	0.355171
	SNP32	50926642 G > A	0.0595	0.0578	0.438406	0.056152
	SNP33	50926661 G > A	0.0595	0.0578	0.438406	0.056152

Note: Ho: the observed heterozygosity; He: the expected heterozygosity; PIC: the polymorphism information content; HWE: Hardy-Weinberg equilibrium.

3.5. Association Analysis

The association analysis between identified SNPs and the growth traits of individuals from “Haixuan NO1” indicated that 19 SNPs (2 SNPs from exon region and 17 SNPs from the promoter region) of *PmFGF18* were significantly associated with growth traits ($P < 0.05$) (Table 3). As shown in Table 3, the shell width of the genotype TT of g. 50916942 T > C was significantly larger than that of the genotype TC ($P < 0.05$). The shell width and weight of the genotype GG of g. 50916962 A > G were significantly larger than those of the genotype AG ($P < 0.05$). The shell length of the genotype AA and AC of g. 50918198 A > C were significantly larger than those of the genotype CC ($P < 0.05$) (Table 3).

Table 3. Correlation of SNPs in *PmFGF18* with growth traits in the “Haixuan NO1” (mean $\pm$ SD).

SNP	SNP Name	Genotype	Sample Number	Shell Length (mm)	Shell Width (mm)	Shell Height (mm)	Total Weight (g)	Shell Weight (g)
SNP1	50916942 T > C	TT	587	67.29 $\pm$ 6.73	24.58 $\pm$ 2.12a	68.42 $\pm$ 7.24	45.87 $\pm$ 9.18	20.75 $\pm$ 3.99
		TC	67	67.53 $\pm$ 6.46	23.80 $\pm$ 3.14b	68.99 $\pm$ 6.59	44.68 $\pm$ 8.59	19.89 $\pm$ 3.41
SNP2	50916948 T > G	TT	589	67.29 $\pm$ 6.72	24.58 $\pm$ 2.13a	68.42 $\pm$ 7.22	45.85 $\pm$ 9.19	20.74 $\pm$ 3.99
		TG	65	67.59 $\pm$ 6.53	23.83 $\pm$ 3.16b	68.94 $\pm$ 6.68	44.78 $\pm$ 8.55	19.91 $\pm$ 3.45
SNP3	50916962 A > G	GG	596	67.29 $\pm$ 6.72	24.58 $\pm$ 2.12a	68.45 $\pm$ 7.21	45.90 $\pm$ 9.23	20.76 $\pm$ 4.00a
		AG	58	67.58 $\pm$ 6.49	23.73 $\pm$ 3.30b	68.70 $\pm$ 6.77	44.21 $\pm$ 7.86	19.64 $\pm$ 3.08b
SNP4	50916963 T > C	TT	595	67.30 $\pm$ 6.73	24.58 $\pm$ 2.12a	68.45 $\pm$ 7.22	45.92 $\pm$ 9.22	20.77 $\pm$ 4.00a
		TC	59	67.51 $\pm$ 6.45	23.68 $\pm$ 3.29b	68.71 $\pm$ 6.72	44.00 $\pm$ 7.95	19.61 $\pm$ 3.06b
SNP5	50916970 T > C	TT	592	67.32 $\pm$ 6.73	24.58 $\pm$ 2.12a	68.46 $\pm$ 7.23	45.92 $\pm$ 9.25	20.77 $\pm$ 4.01a
		TC	62	67.26 $\pm$ 6.40	23.74 $\pm$ 3.19b	68.61 $\pm$ 6.62	44.13 $\pm$ 7.76	19.63 $\pm$ 3.05b
SNP6	50916971 T > A	TT	592	67.32 $\pm$ 6.73	24.58 $\pm$ 2.12a	68.46 $\pm$ 7.23	45.92 $\pm$ 9.25	20.77 $\pm$ 4.01a
		TC	62	67.26 $\pm$ 6.40	23.74 $\pm$ 3.19b	68.61 $\pm$ 6.62	44.13 $\pm$ 7.76	19.63 $\pm$ 3.05b
SNP7	50916977 A > G	AA	591	67.30 $\pm$ 6.73	24.58 $\pm$ 2.13a	68.45 $\pm$ 7.22	45.88 $\pm$ 9.21	20.75 $\pm$ 3.99
		AG	63	67.42 $\pm$ 6.48	23.77 $\pm$ 3.17b	68.76 $\pm$ 6.68	44.52 $\pm$ 8.30	19.82 $\pm$ 3.36
SNP8	50916978 A > T	AA	590	67.32 $\pm$ 6.72	24.59 $\pm$ 2.12a	68.46 $\pm$ 7.22	45.90 $\pm$ 9.20	20.76 $\pm$ 3.98a
		AT	64	67.31 $\pm$ 6.49	23.72 $\pm$ 3.17b	68.60 $\pm$ 6.74	44.32 $\pm$ 8.38	19.73 $\pm$ 3.40b
SNP9	50916986 A > T	TT	590	67.31 $\pm$ 6.73	24.59 $\pm$ 2.12a	68.44 $\pm$ 7.23	45.90 $\pm$ 9.20	20.76 $\pm$ 4.00
		AT	64	67.36 $\pm$ 6.45	23.72 $\pm$ 3.17b	68.77 $\pm$ 6.62	44.32 $\pm$ 8.37	19.78 $\pm$ 3.35
SNP10	50916996 A > T	TT	590	67.29 $\pm$ 6.74	24.58 $\pm$ 2.12a	68.43 $\pm$ 7.24	45.88 $\pm$ 9.21	20.75 $\pm$ 4.00
		AT	64	67.55 $\pm$ 6.33	23.75 $\pm$ 3.17b	68.88 $\pm$ 6.56	44.51 $\pm$ 8.29	19.85 $\pm$ 3.31
SNP12	50917717 A > G	AA	68	66.95 $\pm$ 6.49	24.32 $\pm$ 1.92	66.77 $\pm$ 9.29b	44.21 $\pm$ 8.62	20.96 $\pm$ 4.08
		GG	210	66.54 $\pm$ 6.47	24.33 $\pm$ 2.44	68.10 $\pm$ 6.86ab	45.03 $\pm$ 8.83	20.24 $\pm$ 3.68
		AG	377	67.79 $\pm$ 6.84	24.63 $\pm$ 2.21	68.97 $\pm$ 6.85a	46.39 $\pm$ 9.34	20.83 $\pm$ 4.05
SNP13	50917735 A > T	AA	615	67.21 $\pm$ 6.68	24.57 $\pm$ 2.10a	68.39 $\pm$ 7.20	45.77 $\pm$ 9.13	20.67 $\pm$ 3.95
		AT	40	68.75 $\pm$ 6.98	23.34 $\pm$ 3.82b	69.69 $\pm$ 6.71	45.13 $\pm$ 9.20	20.32 $\pm$ 3.92
SNP14	50917780 A > T	AA	68	66.70 $\pm$ 6.36	24.27 $\pm$ 1.90	66.66 $\pm$ 9.20b	44.16 $\pm$ 8.75b	20.90 $\pm$ 4.15
		TT	220	66.65 $\pm$ 6.32	24.33 $\pm$ 2.42	68.15 $\pm$ 6.79ab	44.93 $\pm$ 8.83ab	20.22 $\pm$ 3.72
SNP17	50918021 T > C	AT	367	67.80 $\pm$ 6.96	24.64 $\pm$ 2.21	68.99 $\pm$ 6.91a	46.50 $\pm$ 9.32a	20.86 $\pm$ 4.02
		TT	138	67.81 $\pm$ 6.83	24.44 $\pm$ 2.66b	68.23 $\pm$ 8.16	45.83 $\pm$ 9.65	21.05 $\pm$ 4.22a
		CC	157	66.41 $\pm$ 6.40	24.17 $\pm$ 2.60ab	68.04 $\pm$ 7.44	44.94 $\pm$ 9.11	20.04 $\pm$ 3.72b
SNP18	50918198 A > C	TC	360	67.50 $\pm$ 6.76	24.66 $\pm$ 1.89a	68.74 $\pm$ 6.63	46.03 $\pm$ 8.94	20.77 $\pm$ 3.91ab
		AA	301	68.03 $\pm$ 6.85a	24.57 $\pm$ 2.67	68.56 $\pm$ 7.99	46.33 $\pm$ 9.69	21.02 $\pm$ 4.11
		CC	42	65.01 $\pm$ 5.43b	24.70 $\pm$ 2.11	67.86 $\pm$ 4.33	45.89 $\pm$ 9.12	20.07 $\pm$ 3.67
		AC	312	66.91 $\pm$ 6.63a	24.40 $\pm$ 1.80	68.45 $\pm$ 6.63	45.13 $\pm$ 8.55	20.37 $\pm$ 3.79

Table 3. Cont.

SNP	SNP Name	Genotype	Sample Number	Shell Length (mm)	Shell Width (mm)	Shell Height (mm)	Total Weight (g)	Shell Weight (g)
SNP26	50918498 A > G	AA	145	67.96 ± 6.79a	24.62 ± 1.88	68.26 ± 8.00	45.80 ± 9.51	20.82 ± 4.11a
		GG	141	66.37 ± 6.12b	24.47 ± 1.87	67.86 ± 7.32	44.83 ± 8.39	19.96 ± 3.39b
		AG	369	67.40 ± 6.86ab	24.46 ± 2.52	68.78 ± 6.76	46.04 ± 9.25	20.85 ± 4.05a
SNP28	50918729 T > C	TT	142	68.22 ± 6.78a	24.49 ± 2.64	68.49 ± 8.04	46.15 ± 9.63	21.10 ± 4.18
		CC	149	66.48 ± 6.16b	24.48 ± 1.93	67.93 ± 7.16	45.23 ± 8.83	20.29 ± 3.65
		TC	364	67.28 ± 6.85ab	24.51 ± 2.22	68.68 ± 6.82	45.77 ± 9.07	20.62 ± 3.96
SNP32	50926642 G > A	GG	616	67.23 ± 6.68	24.58 ± 2.10a	68.40 ± 7.19	45.81 ± 9.15	20.69 ± 3.96
		AG	39	68.44 ± 7.07	23.24 ± 3.84b	69.52 ± 6.81	44.43 ± 8.86	20.00 ± 3.65
SNP33	50926661 G > A	GC	616	67.23 ± 6.68	24.58 ± 2.10a	68.40 ± 7.19	45.81 ± 9.15	20.69 ± 3.96
		AG	39	68.44 ± 7.07	23.24 ± 3.84b	69.52 ± 6.81	44.43 ± 8.86	20.00 ± 3.65

Note: Mean values with different letter within a column are significant different ($P < 0.05$).

3.6. SNP Verification in the Yellow Shell Color Line

One SNP g. 50918198 A > C associated with the growth of *P. f. martensii* in the “Haixuan NO1” was chosen for the polymorphism analysis of *PmFGF18* in the yellow shell color line for SNP verification. The association analysis of a yellow shell color line showed that shell lengths of the genotype AA of g. 50918198 A > C were significantly larger than those of the genotype CC and AC ($P < 0.05$) (Figure 3C,D). The shell heights of the genotype AA of g. 50918198 A > C were significantly larger than those of the genotype AC ($P < 0.05$) (Figure 3C,D). Therefore, the genotype AA of g. 50918198 A > C showed significantly larger shell length than genotype CC in both lines ($P < 0.05$) (Figure 3A,B).

Figure 3. SNP g.50918198A > C verification in two lines. (A,B) and (C,D) showed the result of the “Haixuan NO1” and yellow shell color line, respectively. Bars with different letters indicate significant differences among different genotypes ($P < 0.05$).

4. Discussion

Mollusca is the second largest invertebrate phylum, and its external exoskeleton or shell protects it and represents the size of individuals [35]. The success of the mollusk species can be ascribed

partially to their exoskeleton [36], which provides defense and support functions. FGF18 regulates cell proliferation and differentiation positively in osteogenesis and negatively in chondrogenesis [22] which indicates its potential in biomineralization and shell size construction. In the present study, the PmFGF18 primary sequence presented a signal peptide and an FGF domain corresponding to the result of other species. The multiple sequence alignment of FGF18 from diverse bivalves also demonstrated a conservation of their amino acid sequence and primary structure, and further indicated that the gene cloned in the present study was PmFGF18. These results showed the functional potential of PmFGF18 to the general FGF18 function.

FGF18 plays a central role in skeletal growth and development [22,23]. In invertebrates, the sequence information of some FGF18 genes is limited in their characterization and functions. This study observed the expression of PmFGF18 at various developmental stages and diverse pearl oyster tissues, showing its wide existence in the pearl oyster. PmFGF18 showed a high expression level in shell formation-associated tissues especially in the marginal zone of mantle, which indicated its potential role in the prismatic layer formation and was consequently involved in shell size construction [3,37,38].

In the research of our group, multiple genes and SNPs from growth-related genes were analyzed to obtain genes that participate in the growth of pearl oysters; we reported that some growth-related genes presented a relatively higher expression level or RPKM in the fast-growing group than in the slow-growing group [29,31,39,40]. Adductor muscles of shellfish which are the main muscular system in bivalve molluscs and connected to their shells have been widely used to research the growth genes expression pattern [39,41,42], and researchers who focused on the growth of shellfish also utilized the adductor muscle as the detected tissue for the omics analysis [29,31,43]. In the present study, adductor muscle was utilized and it was shown that PmFGF18 showed a significantly high expression level in the fast-growing group compared with the slow-growing group. Therefore, PmFGF18 hold the potential in regulating the growth traits of *P. f. martensii*.

SNP, as a molecular marker, has been widely utilized in the genetic architecture analysis of complex economic traits and promotes the selection accuracy and efficiency in economic animals and plants [44,45]. In order to analyze the potential of PmFGF18 in the genetic breeding of pearl oysters, 33 SNPs from PmFGF18 were identified. Correlation or association analysis between SNP markers and economic traits reaching a significant level may imply the existence of relation between the markers and related traits [46], which was also used to analyze the association among SNPs from PmFGF18 and growth traits. In Zhikong scallops, the SNP (c. 1815C.T) in the 3' UTRs of the TGF- β type I receptor gene was associated with the growth of scallops, and the genotype TT possessed higher growth traits than the genotype CC or CT in a full-sib family [42]. In *C. nobilis*, SNP g.-579A/C in the promoter region of an MSTN gene was significantly associated with body size, adductor muscle mass, and soft tissue [19]. Three SNPs from PmOSR1 gene showed significant association with the shell size of *P. f. martensii* [47]. 19 SNPs from PmFGF18 were significantly associated with growth traits in the present study. Among them, growth traits of genotype AA from g. 50918198 A > C showed the better traits compared with the other two genotypes in both lines. Thus, the alteration of the genetic sequence of specific genes may lead to the variation in correlated traits [48] and the selection of SNPs from these genes may promote the efficiency of breeding progress.

In the promoter region, the SNP observed had the possibility of influencing FGF18 expression. In chickens, the SNPs in E-box3 and E-box4 changed the myostatin expression [49]. In pigs, SNP interrupted a putative MEF3 binding site related to MSTN expression [50]. In PmFGF18, the capability of the identified SNPs to affect the efficiency of the gene transcription is unknown and requires further research. The current research provides key and vital molecular information to progress our knowledge on the biological roles and evolution of the PmFGF18 gene and will be valuable for future research required for the genetic enhancement of *P. f. martensii*.

5. Conclusions

PmFGF18 may exhibit a functional role in the growth of pearl oysters. The characterization of the full length of *PmFGF18* showed its conserved structures at a primary level. Expression analysis demonstrated the wide distribution of *PmFGF18* in all sampled tissues. *PmFGF18* was expressed significantly higher in the fast-growing group compared with the slow-growing group. In total, 33 SNPs were identified in the promoter and exon sequences of *PmFGF18*, and 19 SNPs were significantly associated with the growth traits of pearl oysters from “Haixuan NO1”. The growth traits of genotype AA from g. 50918198 A > C showed the better traits compared with the other two genotypes in both lines. This study demonstrated the function potential of *PmFGF18* in the body size construction of pearl oysters and provided a potential molecular marker for the selective breeding and genetic improvement of the pearl oyster *P. f. martensii*.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2077-1312/8/11/896/s1>, Figure S1: Nucleotide sequence analysis of *PmFGF18*, Figure S2: Phylogenetic tree of FGF18 among different species. Figure S3: Expression pattern of *PmFGF18* during development in the development transcriptome.

Author Contributions: Conceptualization, R.H. and C.Y.; formal analysis, R.H.; resources, C.M. and L.A.; writing—original draft preparation, R.H.; writing—review and editing, Y.D., Q.W.; supervision, C.Y.; All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Guangdong Basic and Applied Basic Research Foundation (2019A1515111026, 2020A1515010691), Young Innovative Talents Project (2019KQNCX043) and Innovation Team Project (2017KCXTD016) from the Department of Education of Guangdong Province, Modern Agricultural Industrial System (CARS-049) and Guangdong Provincial Special Fund For Modern Agriculture Industry Technology Innovation Teams, Department of Agriculture and Rural Affairs of Guangdong Province (2019KJ146).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Yang, C.; Du, X.; Hao, R.; Wang, Q.; Deng, Y.; Sun, R. Effect of vitamin D3 on immunity and antioxidant capacity of pearl oyster *Pinctada fucata martensii* after transplantation: Insights from LC-MS-based metabolomics analysis. *Fish Shellfish Immun.* **2019**, *94*, 271–279. [CrossRef] [PubMed]
2. Wada, K.T.; Komaru, A. Color and weight of pearls produced by grafting the mantle tissue from a selected population for white shell color of the Japanese pearl oyster *Pinctada fucata martensii* (Dunker). *Aquaculture* **1996**, *142*, 25–32. [CrossRef]
3. Hao, R.; Zheng, Z.; Wang, Q.; Du, X.; Deng, Y.; Huang, R. Molecular and functional analysis of *PmCHST1b* in nacre formation of *Pinctada fucata martensii*. *Comp. Biochem. Phys. B* **2018**, *225*, 13–20. [CrossRef] [PubMed]
4. Deng, Y.; Fu, S.; Lu, Y.; Du, X.; Wang, Q.; Huang, H.; Liu, D. Fertilization, Hatching, Survival, and Growth of Third-Generation Colored Pearl Oyster (*Pinctada martensii*) Stocks. *J. Appl. Aquac.* **2013**, *25*, 113–120.
5. Deng, Y.; Gao, Y.; Chen, W.; Du, X.; Lu, J. Comparison of growth performance and genetic diversity of pearl oyster (*Pinctada martensii*) families in a breeding program. *Afr. J. Agric. Res.* **2011**, *6*, 6530–6536. [CrossRef]
6. Ren, P.; Peng, W.; You, W.; Huang, Z.; Guo, Q.; Chen, N.; He, P.; Ke, J.; Gwo, J.; Ke, C. Genetic mapping and quantitative trait loci analysis of growth-related traits in the small abalone *Haliotis diversicolor* using restriction-site-associated DNA sequencing. *Aquaculture* **2016**, *454*, 163–170. [CrossRef]
7. Wang, J.; Li, L.; Zhang, G. A High-Density SNP genetic linkage map and QTL analysis of growth-related traits in a hybrid family of oysters (*Crassostrea gigas* × *Crassostrea angulata*) using genotyping-by-sequencing. *G3 Genes Genomes Genet.* **2016**, *6*, 1417–1426. [CrossRef]
8. Li, Y.; He, M. Genetic mapping and QTL analysis of growth-related traits in *Pinctada fucata* using restriction-site associated DNA Sequencing. *PLoS ONE* **2014**, *9*, e111707. [CrossRef]
9. Nie, H.; Yan, X.; Huo, Z.; Jiang, L.; Chen, P.; Liu, H.; Ding, J.; Yang, F. Construction of a high-density genetic map and quantitative trait locus mapping in the manila clam *Ruditapes philippinarum*. *Sci. Rep.* **2017**, *7*, 229. [CrossRef]
10. Li, C.; Wang, J.; Song, K.; Meng, J.; Xu, F.; Li, L.; Zhang, G. Construction of a high-density genetic map and fine QTL mapping for growth and nutritional traits of *Crassostrea gigas*. *BMC Genom.* **2018**, *19*, 626. [CrossRef]

11. Jiao, W.; Fu, X.; Dou, J.; Li, H.; Su, H.; Mao, J.; Yu, Q.; Zhang, L.; Hu, X.; Huang, X.; et al. High-resolution linkage and quantitative trait locus mapping aided by genome survey sequencing: Building up an integrative genomic framework for a bivalve mollusc. *DNA Res.* **2014**, *21*, 85–101. [[CrossRef](#)] [[PubMed](#)]
12. Liu, H.; Zhang, H.; Pan, X.; Xu, M.; Huang, J.; He, M. A high density genetic map by whole-genome resequencing for QTL fine-mapping and dissecting candidate genes for growth or sex traits in the pearl oyster (*Pinctada fucata martensii*). *Aquaculture* **2020**, *519*, 734839. [[CrossRef](#)]
13. Shi, Y.; He, M. Differential gene expression identified by RNA-Seq and qPCR in two sizes of pearl oyster (*Pinctada fucata*). *Gene* **2014**, *538*, 313–322. [[CrossRef](#)] [[PubMed](#)]
14. Prudence, M.; Moal, J.; Boudry, P.; Daniel, J.Y.; Quere, C.; Jeffroy, F.; Mingant, C.; Ropert, M.; Bedier, E.; Van Wormhoudt, A.; et al. An amylase gene polymorphism is associated with growth differences in the Pacific cupped oyster *Crassostrea gigas*. *Anim. Genet.* **2006**, *37*, 348–351. [[CrossRef](#)]
15. Cong, R.; Li, Q.; Kong, L. Polymorphism in the insulin-related peptide gene and its association with growth traits in the Pacific oyster *Crassostrea gigas*. *Biochem. Syst. Ecol.* **2013**, *46*, 36–43. [[CrossRef](#)]
16. Huvet, A.; Jeffroy, F.; Fabioux, C.; Daniel, J.Y.; Quillien, V.; Van Wormhoudt, A.; Moal, J.; Samain, J.F.; Boudry, P.; Pouvreau, S. Association among growth, food consumption-related traits and amylase gene polymorphism in the Pacific oyster *Crassostrea gigas*. *Anim. Genet.* **2008**, *39*, 662–665. [[CrossRef](#)]
17. Wang, X.; Meng, X.; Song, B.; Qiu, X.; Liu, H. SNPs in the myostatin gene of the mollusk *Chlamys farreri*: Association with growth traits. *Comp. Biochem. Physiol. B* **2010**, *155*, 327–330. [[CrossRef](#)]
18. Guo, L.; Li, L.; Zhang, S.; Guo, X.; Zhang, G. Novel polymorphisms in the myostatin gene and their association with growth traits in a variety of bay scallop, *Argopecten irradians*. *Anim. Genet.* **2011**, *42*, 339–340. [[CrossRef](#)]
19. Fan, S.; Xu, Y.; Liu, B.; He, W.; Zhang, B.; Su, J.; Yu, D. Molecular characterization and expression analysis of the myostatin gene and its association with growth traits in Noble scallop (*Chlamys nobilis*). *Comp. Biochem. Physiol. B* **2017**, *212*, 24–31. [[CrossRef](#)]
20. Beenken, A.; Mohammadi, M. The FGF family: Biology, pathophysiology and therapy. *Nat. Rev. Drug Discov.* **2009**, *8*, 235–253. [[CrossRef](#)]
21. Itoh, N.; Ornitz, D.M. Evolution of the Fgf and Fgfr gene families. *Trends Genet.* **2004**, *20*, 563–569. [[CrossRef](#)] [[PubMed](#)]
22. Ohbayashi, N.; Shibayama, M.; Kurotaki, Y.; Imanishi, M.; Fujimori, T.; Itoh, N.; Takada, S. FGF18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogenesis. *Gene Dev.* **2002**, *16*, 870–879. [[CrossRef](#)] [[PubMed](#)]
23. Liu, Z. Coordination of chondrogenesis and osteogenesis by fibroblast growth factor 18. *Gene Dev.* **2002**, *16*, 859–869. [[CrossRef](#)] [[PubMed](#)]
24. Ellsworth, J.L.; Berry, J.; Bukowski, T.; Claus, J.; Feldhaus, A.; Holderman, S.; Holdren, M.S.; Lum, K.D.; Moore, E.E.; Raymond, F.; et al. Fibroblast growth factor-18 is a trophic factor for mature chondrocytes and their progenitors. *Osteoarthr. Cartil.* **2002**, *10*, 308–320. [[CrossRef](#)] [[PubMed](#)]
25. Shimoaka, T.; Ogasawara, T.; Yonamine, A.; Chikazu, D.; Kawano, H.; Nakamura, K.; Itoh, N.; Kawaguchi, H. Regulation of Osteoblast, Chondrocyte, and Osteoclast Functions by Fibroblast Growth Factor (FGF)-18 in Comparison with FGF-2 and FGF-10. *J. Biol. Chem.* **2002**, *277*, 7493–7500. [[CrossRef](#)] [[PubMed](#)]
26. Moore, E.E.; Bendele, A.M.; Thompson, D.L.; Littau, A.; Waggle, K.S.; Reardon, B.; Ellsworth, J.L. Fibroblast growth factor-18 stimulates chondrogenesis and cartilage repair in a rat model of injury-induced osteoarthritis. *Osteoarthr. Cartil.* **2005**, *13*, 623–631. [[CrossRef](#)]
27. Wang, S.; Zhang, J.; Jiao, W.; Li, J.; Xun, X.; Sun, Y.; Guo, X.; Huan, P.; Dong, B.; Zhang, L.; et al. Scallop genome provides insights into evolution of bilaterian karyotype and development. *Nat. Ecol. Evol.* **2017**, *1*, 0120. [[CrossRef](#)]
28. Bonasio, R.; Zhang, G.; Ye, C.; Mutti, N.; Fang, X.; Qin, N.; Greg, D.; Yang, P.; Li, Q.; Li, C.; et al. Genomic comparison of the ants *Camponotus floridanus* and *Harpegnathos saltator*. *Science* **2010**, *329*, 1068. [[CrossRef](#)]
29. Hao, R.; Du, X.; Yang, C.; Deng, Y.; Zheng, Z.; Wang, Q. Integrated application of transcriptomics and metabolomics provides insights into unsynchronized growth in pearl oyster *Pinctada fucata martensii*. *Sci. Total Environ.* **2019**, *666*, 46–56. [[CrossRef](#)]
30. Du, X.; Deng, Y.; Wang, Q.; Xie, S.; Liu, D. Haixuan NO1 stock of pearl oyster *Pinctada martensii*. *China Fish.* **2015**, *10*, 53–56. (In Chinese)
31. Yang, J.; Luo, S.; Li, J.; Zheng, Z.; Du, X.; Deng, Y. Transcriptome analysis of growth heterosis in pearl oyster *Pinctada fucata martensii*. *FEBS Open Bio* **2018**, *8*, 1794–1803. [[CrossRef](#)] [[PubMed](#)]

32. Wu, Q.; Yu, H.; Wei, W.; Cheng, Y.; Huang, S.; Shi, H.; Liu, S.; Xia, J.; Jia, H.; Hao, L. Linkage disequilibrium and functional analysis of PRE1 insertion together with SNPs in the promoter region of IGFBP7 gene in different pig breeds. *J. Appl. Genet.* **2018**, *59*, 231–241. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Lei, C.; Li, J.; Zheng, Z.; Du, X.; Deng, Y. Molecular cloning, expression pattern of beta-carotene 15,15-dioxygenase gene and association analysis with total carotenoid content in pearl oyster *Pinctada fucata martensii*. *Comp. Biochem. Physiol. B* **2019**, *229*, 34–41. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Du, X.; Fan, G.; Jiao, Y.; Zhang, H.; Guo, X.; Huang, R.; Zheng, Z.; Bian, C.; Deng, Y.; Wang, Q.; et al. The pearl oyster *Pinctada fucata martensii* genome and multi-omic analyses provide insights into biomineralization. *Gigascience* **2017**, *6*, 1–12. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Kocot, K.M.; Aguilera, F.; McDougall, C.; Jackson, D.J.; Degnan, B.M. Sea shell diversity and rapidly evolving secretomes: Insights into the evolution of biomineralization. *Front. Zool.* **2016**, *13*, 23. [\[CrossRef\]](#)
36. Haszprunar, G.; Wanninger, A. Molluscs. *Curr. Biol.* **2012**, *22*, 510–514. [\[CrossRef\]](#)
37. Acosta-Salmón, H.; Southgate, P.C. Mantle regeneration in the pearl oysters *Pinctada fucata* and *Pinctada margaritifera*. *Aquaculture* **2005**, *246*, 447–453. [\[CrossRef\]](#)
38. Sato, Y.; Inoue, N.; Ishikawa, T.; Ishibashi, R.; Obata, M.; Aoki, H.; Atsumi, T.; Komaru, A. Pearl microstructure and expression of shell matrix protein genes MSI31 and MSI60 in the pearl sac epithelium of *Pinctada fucata* by In Situ Hybridization. *PLoS ONE* **2013**, *8*, e52372. [\[CrossRef\]](#)
39. Wang, Q.; Hao, R.; Zhao, X.; Huang, R.; Zheng, Z.; Deng, Y.; Chen, W.; Du, X. Identification of EGFR in pearl oyster (*Pinctada fucata martensii*) and correlation analysis of its expression and growth traits. *Biosci. Biotechnol. Biochem.* **2018**, *82*, 1073–1080. [\[CrossRef\]](#)
40. Wang, Z.; Liang, F.; Huang, R.; Deng, Y.; Li, J. Identification of the differentially expressed genes of *Pinctada maxima* individuals with different sizes through transcriptome analysis. *Reg. Stud. Mar. Sci.* **2019**, *26*, 100512. [\[CrossRef\]](#)
41. Zheng, Z.; Huang, R.; Tian, R.; Jiao, Y.; Du, X. Pm-miR-133 hosting in one potential lncRNA regulates RhoA expression in pearl oyster *Pinctada martensii*. *Gene* **2016**, *591*, 484–489. [\[CrossRef\]](#)
42. Guo, H.; Bao, Z.; Li, J.; Lian, S.; Wang, S.; He, Y.; Fu, X.; Zhang, L.; Hu, X. Molecular characterization of TGF-beta type I receptor gene (Tgfb1) in *Chlamys farreri*, and the association of allelic variants with growth traits. *PLoS ONE* **2012**, *7*, e51005. [\[CrossRef\]](#) [\[PubMed\]](#)
43. Li, Y.; He, M. Correlation between Genome Methylation Level and Growth Trait of Pearl Oyster, *Pinctada fucata*. *Pak. J. Zool.* **2017**, *49*, 833–839. [\[CrossRef\]](#)
44. Huang, X.; Wu, S.; Guan, Y.; Li, Y.; He, M. Identification of sixteen single-nucleotide polymorphism markers in the pearl oyster, *Pinctada fucata*, for population genetic structure analysis. *J. Genet.* **2015**, *941*, E1–E4. [\[CrossRef\]](#)
45. Mastrangelo, S.; Di Gerlando, R.; Tolone, M.; Tortorici, L.; Sardina, M.T.; Portolano, B. Genome wide linkage disequilibrium and genetic structure in Sicilian dairy sheep breeds. *BMC Genet.* **2014**, *15*, 108. [\[CrossRef\]](#)
46. Nazari, S.; Jafari, V.; Pourkazemi, M.; Miandare, H.K.; Abdolhay, H.A. Association between polymorphism in myostatin gene with quantitative traits in domesticated rainbow trout (*Oncorhynchus mykiss*) using PCR-SSCP method. *Iran. J. Fish. Sci.* **2016**, *25*, 109–115.
47. Yang, C.; Yang, J.; Hao, R.; Du, X.; Deng, Y. Molecular characterization of OSR1 in *Pinctada fucata martensii* and association of allelic variants with growth traits. *Aquaculture* **2020**, *516*, 734617. [\[CrossRef\]](#)
48. Liu, X.; Chang, Y.; Xiang, J.; Song, J.; Ding, J. Analysis of effects of shell size characters on live weight in Chinese scallop *Chlamys farreri*. *Oceanol. Limnol. Sin.* **2002**, *33*, 673–677. (In Chinese)
49. Hu, W.; Chen, S.; Zhang, R.; Lin, Y. Single nucleotide polymorphisms in the upstream regulatory region alter the expression of myostatin. *In Vitro Cell. Dev. Biol. Anim.* **2013**, *49*, 417–423. [\[CrossRef\]](#)
50. Stinckens, A.; Luyten, T.; Bijttebier, J.; Van den Maagdenberg, K.; Dieltiens, D.; Janssens, S.; De Smet, S.; Georges, M.; Buys, N. Characterization of the complete porcine MSTN gene and expression levels in pig breeds differing in muscularity. *Anim. Genet.* **2008**, *39*, 586–596. [\[CrossRef\]](#)

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