Hindawi
Aquaculture Research
Volume 2023, Article ID 4191343, 14 pages
https://doi.org/10.1155/2023/4191343

Research Article

An Analysis of the Cloning, Functional Analysis, and Identification of Three Cloned Digestive Enzymes Associated with Growth Traits in *Haliotis diversicolor*

Weidong Li,[1] **Huaifeng Cao**,[2] **Wenxin Xu**,[1] **Mohsin Mahmood**,[1] **Waqas Ahmed**,[1] **Sajid Mehmood**,[1] **and Weiwei You**[2]

[1]*Center for Eco-Environment Restoration Engineering of Hainan Province, School of Ecological and Environmental Sciences, Hainan University, Haikou 570228, China*
[2]*College of Ocean and Earth Sciences, Xiamen University, Xiamen 361005, China*

Correspondence should be addressed to Sajid Mehmood; drsajid@hainanu.edu.cn and Weiwei You; wwyou@xmu.edu.cn

Received 21 October 2022; Revised 23 February 2023; Accepted 7 March 2023; Published 30 March 2023

Academic Editor: Zhitao Qi

The purpose of this study was to determine the effect of different feeds such as *Porphyra, Laminaria japonica*, Compound feed, and *Gracilaria* on the activities of alginate lyase, cellulase, and amylase in the digestive juice of *H. diversicolor* and to take their genes as candidate genes related to the growth traits of *H. diversicolor*. In order to provide data for selective breeding, SNPs that were significantly associated with growth traits were screened through gene cloning, high-throughput sequencing, and association analysis. It was found that there were no significant differences in the activities of alginate lyase and cellulase in *H. diversicolor* among different feeds, but there were significant differences between males and females in the group; β-amylase activity is higher than α-amylase activity; the DNA, full-length cDNA, and upstream sequences of genes of alginate lyase, cellulase, and amylase were cloned, and we found that the expressions of three genes are similar; they have started expression in the early prostrate stage of larvae, have tissue specificity in adults, and are highly expressed in hepatopancreas; 84 SNPs were found in alginate lyase through high-throughput sequencing, and 2 which had significant differences were left after associating with growth traits, and they are ALG1016 and ALG1544.

1. Introduction

In southern China, small abalone, *Haliotis diversicolor*, is a commercially important aquaculture mollusk species [1]. Since 1986, the breeding scale of *H. diversicolor* has expanded year by year, and now, it is an important part of the aquaculture industry in the South China Sea [2]. But in recent years, the deterioration of small abalone germplasm resources caused by inbreeding reproduction and environmental deterioration led to slow growth speed and longer breeding cycles in abalone breeding. Additionally, small, cultured abalones are less able to tolerate environmental disturbances. As there is considerable evidence to suggest, there has been an increase in the number of deaths that are due to infection in developing nations in recent years [3–5]. The study of physiologically and biochemically relevant growth-related genes and molecular markers, that are associated with *Haliotis diversicolor*, would be of great importance in order to cultivate new varieties of the species and to enrich related theories of shellfish breeding.

Abalone is a herbivorous mollusk that takes seaweed as the main source of food and energy, wild abalone mainly feeds on brown algae [6], and the cell wall of brown algae is mainly composed of alginic acid, fucoidan, and cellulose [7–9]. So, seaweed polysaccharide is the main energy source, whether wild abalone or artificial breeding abalone. The most important polysaccharide degradation enzyme in abalone digestive juice was alginate lyase, cellulase, amylase, and so on [10–12]. Alginate lyase has many biological functions. For example, a microorganism producing alginate

lyase can utilize algin as a primary or secondary carbon source. Oligosaccharides, produced by the degradation of alginate lyase containing biological activity, are favored by people and are gradually used in medicine and industrial production [13]. The applications of alginate lyase also include the preparation of alginate oligosaccharide protoplasts, the auxiliary extraction of seaweed DNA, the isolation and treatment of pulmonary cystic fibrosis, and the research on the microstructure of the alginate [14–17]. Cellulose is the most abundant carbohydrate in nature, and the monosaccharide in cellulose is also glucose. They are combined through β-1,4 glycosidic bonds, and the degree of polymerization is very large, which can form polymer compounds [18, 19]. In herbivorous animals, amylase plays a crucial role in the nutrient absorption and metabolism of shellfish that feed on algae. These three digestive enzymes play an important role in the digestive juices and are closely related to the growth traits of variegated abalone [20].

There are few studies on the relationship between SNP locus and growth traits of *Haliotidae* animals, which may be related to the development of growth-related genes in abalone that are not very mature.

In this study, the alginate lyase, cellulase, and amylase of small abalone were studied from the physiological aspect, and the changes in enzyme activities were analyzed by feeding four common abalone feeds *Porphyra, Laminaria japonica*, Compound feed, and *Gracilaria*. The influence of the three enzyme activities of abalone has a certain guiding significance for practical application. Taking its gene as a candidate gene related to the growth traits of small abalone, through gene cloning, high-throughput sequencing, SNP site detection, and association analysis with growth traits, the SNP loci that were significantly associated with growth traits were screened out, which were identified as small abalone. Genetic breeding and molecular marker-assisted selection breeding provide a theoretical basis.

2. Materials and Methods

2.1. Sample Collection and Larva Culture. The entire body of *Haliotis diversicolor* was dissected at a breeding plant in Jinjiang Province, Fujian Province, to obtain different tissues, including gills, foot muscles, female and male gonads, the mantle, hemolymph, and the hepatopancreas, in addition to the gills and fat. Samples were collected during the following stages of larval development: unfertilized egg, fertilized eggs, 128 cell phase, gastrula stage, trochophore, veliger stage, precreeping larvae, and creeping larvae. Samples were washed ($1 \times$ PBS), frozen (liquid nitrogen), and stored (−80°C) until further processing.

2.2. Reagents and Vectors. LA Taq kit, 3′ and 5′ rapid amplification of complementary DNA (cDNA) ends (RACE) kit, and PrimeScript RT reagent kit were purchased from Takara (Takara, Japan), and clone vector MD-19T was purchased from Takara (Takara Biotechnology, Dalian, China). AxyPrepDNA gel purification kit from Axygen (Axygen, USA) and TRIZOL reagent were purchased from Invitrogen (Burlingame, CA, USA). SYBR Green qPCR Kit was purchased from Thermo (Thermo, USA), PhiX control kit v3, and MiSeq v2 reagent kit from Illumina (Illumina, USA). Marine animal tissue genomic DNA extraction kit (spin column type) was purchased from Tiangen (Tiangen, Beijing).

2.3. Feeding Experiment and Enzyme Activity Determination. In more than 600 families, 120 male abalones and 120 female abalones were randomly selected and divided into four groups. They were climatized for two days before the start of the experiment. Then, they were fed four different feeds: *Gracilaria, Porphyra, laminaria japonica*, and compound feed, and ensured that the amount of feeding was excessive every day. The experiment lasted for one month. After that, the abalone was divided into male and female, and data such as shell length were recorded according to the corresponding group, and then the foot muscle was weighed. Then, we remove the shell of every experimental abalone, dissect the viscera, weigh it, freeze it in liquid nitrogen, and store it at −80°C until processed. In each experimental group, 5 male and 5 female abalones were randomly selected and ground to extract crude enzymes. In this study, the Coomassie Brilliant Blue method was used to determine the protein concentration and was improved by the method of Li et al. [21]. Referring to the methods of Bansemer et al. [22] and Yang et al. [23], the DNS (3,5-dinitrosalicylic acid) method was appropriately modified to determine the activity of alginate lyase, taking 1 mL of enzyme solution and adding 1 mL of 1% sodium alginate solution (prepared with Na_2HPO_4-NaH_2PO_4 buffer solution of 0.05 mol/L, pH 7.0), react at 40°C for 30 min, add 1 mL of DNS solution, react in boiling water bath for 5 min, and then proceed to UV spectroscopy. Absorbance was measured at 520 nm with a photometer. The method to determine the activity of cellulase is to add 0.5 ml of the corresponding enzyme solution to the control group and then add 1.5 ml of DNS to inactivate the enzyme. We add 0.5 ml of the corresponding enzyme solution and 1.5 ml of 0.51% CMC citrate buffer solution to the experimental group, shake well, react with the control group at 50°C for 30 minutes, and then add 1.5 ml of 0.51% CMC citrate buffer solution to the control group. The experimental group was added 1.5 ml DNS, shaken well, reacted at 50°C for 30 min, and boiled in boiling water for 5 min to terminate the reaction completely and measure the change of absorbance at 540 nm wavelength. The amylase activity determination method is to take 0.5 ml of the corresponding enzyme solution and 1.5 ml of 1% maltose, shake well, first measure the α-amylase activity in a water bath at 70°C for 15 minutes, and then place it in a constant temperature water bath at 40°C together with the total amylase. We incubate for 30 min. Finally, we boil in boiling water for 5 min, completely stop the reaction, and measure the change of absorbance at 540 nm wavelength.

2.4. RNA and DNA Extraction and the First-Strand Synthesis. Total RNA of *Haliotis diversicolor* was isolated using a TRIZOL kit (Invitrogen, USA). In reverse transcription

experiments, Takara PrimeScript RT reagent kits were used (Takara, Japan). A total RNA sample was reverse transcribed into cDNA, and the integrity and quantity of RNA were measured [24]. To carry out further PCR analysis, first-strand cDNA was synthesized. In accordance with the instructions provided by the manufacturer, DNA was extracted from the foot muscle using a marine animal tissue genomic DNA extraction kit (TIANGEN, Beijing).

2.5. Molecular Cloning of Alginate Lyase, Cellulase, and Amylase Genes and Sequence Analysis. Previously published conserved nucleotide sequences of *Haliotis diversicolor* were retrieved from the NCBI nucleotide and protein sequence database (https://www.ncbi.nlm.nih.gov/). A segment was generated using degenerate primers, upstream and downstream, based on the conserved sequence. PCR was carried out at 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 30 seconds. Using a cloning vector pMD-18T, the amplified segment was inserted into E. coli DH 5α. Sequencing was then performed on a positive clone. By using 5′ RACE and 3′ RACE primers, we were able to get the complete sequence of the cDNA (5′RACE Inner Primer; 5′-CGCGGATCCACA GCCTACTGATGATCAGTCGATG-3′ and 3′RACE Inner Primer; 5′-CGCGGATCCTCCACTAGTGATTTCACT ATAGG-3′ having GenBank accession numbers of NCBI alginate lyase; OQ503646, cellulase; OQ503647, and amylase; OQ503648).

BLAST was used to analyze nucleotide sequences (https://www.ncbi.nlm.nih.gov/BLAST). The encoding protein was identified using DNAMAN (DNAMAN Lynnon Biosoft, Santa Clara, USA). To predict the protein's isoelectric point and molecular weight, Expasy Protparam was used, as well as SMART (https://smart.embl-heidelberg.de/). In order to construct the phylogenetic tree, Mega 5.1 was used in conjunction with the neighbor-joining clustering method based on a PAM Matrix. A bootstrap value of 1,000 replications was calculated.

2.6. Alginate Lyase, Cellulase, and Amylase Expression Analysis. The expression of genes in different tissues and stages of larvae development was quantified using real-time qPCR, from unfertilized eggs to creeping larvae. cDNA obtained from the extracted mRNA templates was diluted at 1 : 10, and 2 μL was used for qPCR using the ABI 7500 fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the SYBR Green qPCR kit (ThermoFisher Scientific, Waltham, MA, USA).

For each reference cDNA sample, standard curves were constructed using Microsoft Office Excel 2007. Data of competitive real-time PCR analysis subjected to one-way analysis of variance (ANOVA) followed by the LSD t test was used to determine the difference in mean values with SPSS 17.0 software. The P value for significance was set at $P < 0.05$.

2.7. SNP Screening of Alginate Lyase. This study extracted genomic DNA of 100 abalone foot muscle tissue. The database construction referred to 16S Metagenomic Sequencing Library Preparation operating instructions. After mixing the first and second rounds of PCR products, sequencing was performed on a Miseq high-throughput sequencing platform (Illumina, USA). The data obtained by sequencing were filtered, and the candidate SNP loci were searched and genotyped. Finally, the association between the growth traits and the SNP loci of 99 isolates was analyzed using the R language and scripting, and then the SNPs with a significant difference were found.

3. Results

3.1. The Effect of Alginate Lyase Enzyme Activity on Four Different Diets. Even though the experimental groups were fed four different diets, there were no significant differences in alginate lyase activity between the two groups (Figure 1). In the group that the abalone was fed with the same diet, the enzymatic activity of alginate lyase in female abalone was about 1.7 times that in male abalone, but there was no significant difference. In the abalone group fed with Gracilaria, the enzymatic activity of alginate lyase in the male abalone was about 1.98 times that in the female abalone, but there was no significant difference when they were fed with other diets. The difference in the enzymatic activity of alginate lyase between the female abalone group fed with Gracilaria and the female abalone group fed with Laminaria japonica was the highest; the enzymatic activity of alginate lyase in the female abalone group fed with Laminaria japonica was about 2.73 times of that in the female abalone group fed with Gracilaria.

3.2. The Effect of Cellulase Enzyme Activity on Four Different Diets. There was no significant difference in cellulase activity among the four diets; in abalone treated with each diet, the alginate lyase activity of males and females differed (Figure 2). There were significant differences between males and females among the 3 groups. The cellulase activity of males in laver was about 1.22 times that of females and that of females in kelp was about 1.29 times that of males.

3.3. The Effect of Amylase Enzyme Activity on Four Different Diets. There are two isoforms of amylase in abalone, alpha-amylase and beta-amylase; for alpha-amylase, there was no significant difference between the four diets, while for total amylase and beta amylase, the two have the same trend, and there are significant differences between the four diets (Figure 3). Among them, the amylase activity of the Compound feed group is the highest, and the amylase activity of the asparagus is the lowest. The specific activity of β-amylase was 4.39 times higher; α-amylase was not significantly different between males and females of the four diets; the total amylase of the abalone treated with each diet was different between males and females, except. In addition to the Compound feed, there were significant differences between males and females among the three groups of seaweed, kelp, and asparagus; the amylase activities of males and females were different in abalones treated with each diet of β-amylase, except for the Compound feed and seaweed. In

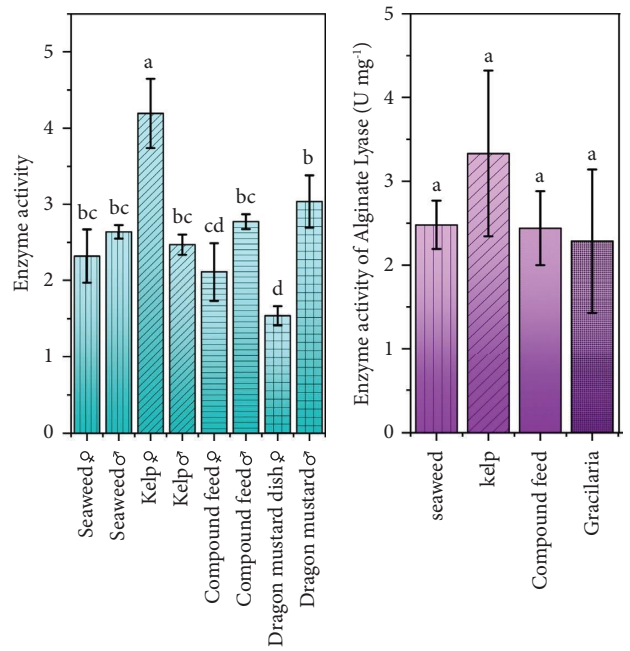

FIGURE 1: The enzyme activity of alginate lyase. Means values ± SD ($n = 3$); different lowercased letters in each subfigure represent statistically significant differences (least significant difference (LSD) test, $P \leq 0.05$).

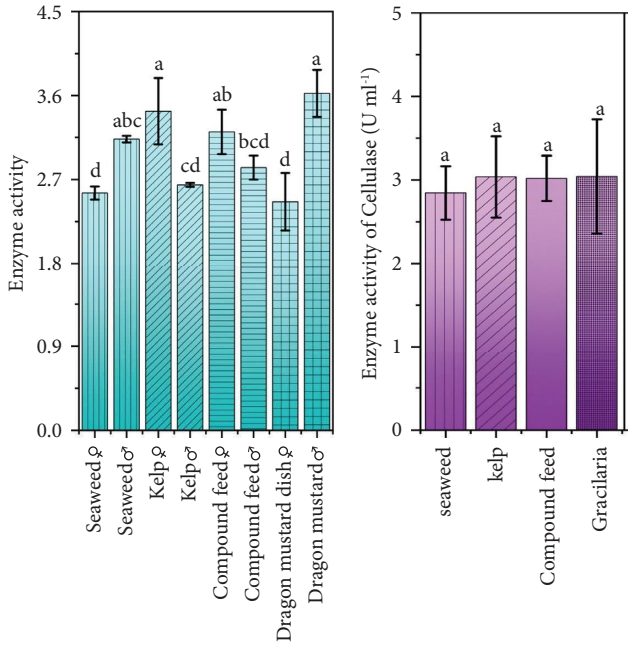

FIGURE 2: The enzyme activity of cellulase. Means values ± SD ($n = 3$); different lowercased letters in each subfigure represent statistically significant differences (least significant difference (LSD) test, $P \leq 0.05$).

addition, there were significant differences between males and females between the two groups of kelp and Astragalus; in total amylase, the female abalone with compound feed had the greatest difference in activity with the male abalone of Astragalus and the former was about 3.14 times that of the latter; among the β-amylases, the female abalone with the Compound feed had the greatest difference in the activity of the male abalone with Astragalus and the former was about 5.84 times that of the latter.

3.4. Sequence Analysis of Alginate Lyase. In the present study, the alginate lyase gene was cloned for the first time from *Haliotis diversicolor*. The complete alginate lyase cDNA sequence was 927 bp (Figure S1). The sequence was imported to NCBI, a predicted 720 bp ORF was found, and a 239-amino acid protein was encoded (Figure S2). The 3′-untranslated region of the cDNA was 70 bp, and there was a typical polyadenylation signal (AATAAA) and polyadenylation sequence (polyA). The 5′-untranslated region of the alginate lyase cDNA was 137 bp. The length of the upstream sequence of alginate lyase in *H.diversicolor* was 5052 bp.

Phylogenetic analysis of the amino acid sequences between these and other alginate lyase indicated that the alginate lyase gene in *H. diversicolor* is similar to sequences in several other abalones. Alginate lyase gene in *H. diversicolor* had the maximal identity of 82.72% with *Haliotis tuberculate*, the identity of 77.29% with Haliotis discus, and the identity of 54.95% with Haliotis discus hannai. But the similarity with other gastropoda species is relatively low. For example, the alginate lyase gene in *H. diversicolor* had a relatively low identity of 34.56% with *Aplysia kurodai* and the similarity with vertebrates is even lower only 5.69%–8.30% (Figure 4). A molecular phylogenetic tree was constructed to analyze the evolutionary relationship among the alginate lyase amino acid sequence; the results of cluster analysis show that in the mollusc branch, *H. diversicolor* and several other abalones and other gastropods were clustered to one branch, vertebrates are first clustered, fungi and other microorganisms clustering alone.

3.5. Sequence Analysis of Cellulase. The complete cellulase cDNA sequence was 1962 bp (Figure S3). The sequence was imported to NCBI, a predicted 1782 bp ORF was found, and a 593-amino acid protein was encoded. The 3′-untranslated region of the cDNA was 134 bp, and there was a typical polyadenylation signal (AATAAA) and polyadenylation sequence (polyA). The 5′-untranslated region of the alginate lyase cDNA was 46 bp. The length of the upstream sequence of cellulase in *H. diversicolor* was 719 bp, and the full length of cellulase gene DNA was 5649 bp, containing 9 exons and 8 introns.

Using SMART software analysis, it was found that the N-terminus of the amino acid sequence of the cellulase gene contains a cellulose-binding domain CBD_II, from the 14th amino acid to the 114th amino acid (Figure S4). After analysis by Expasy prosite software, a predicted carbohydrate-binding site CBM2 (carbohydrate-binding type-2) domain and a catalytic activation site Active site were obtained.

The multiple sequence alignment of amino acids in DNAMAN software (as shown in Figure 5) found that cellulase amino acid similarity of *H. diversicolor* and several

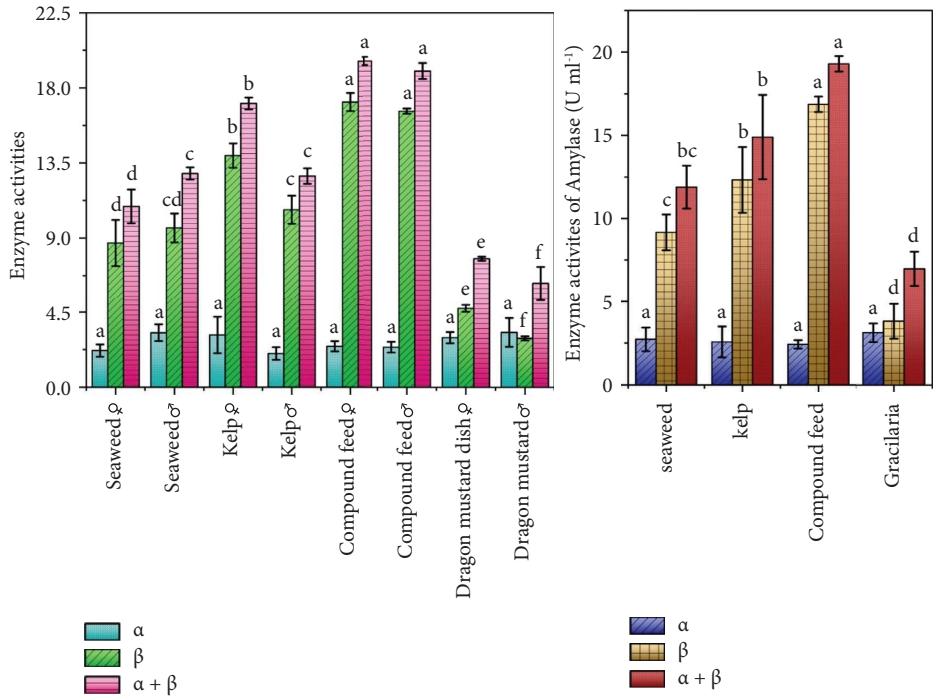

FIGURE 3: The enzyme activity of amylase. Means values ± SD ($n = 3$); different lowercased letters in each subfigure represent statistically significant differences (least significant difference (LSD) test, $P \leq 0.05$).

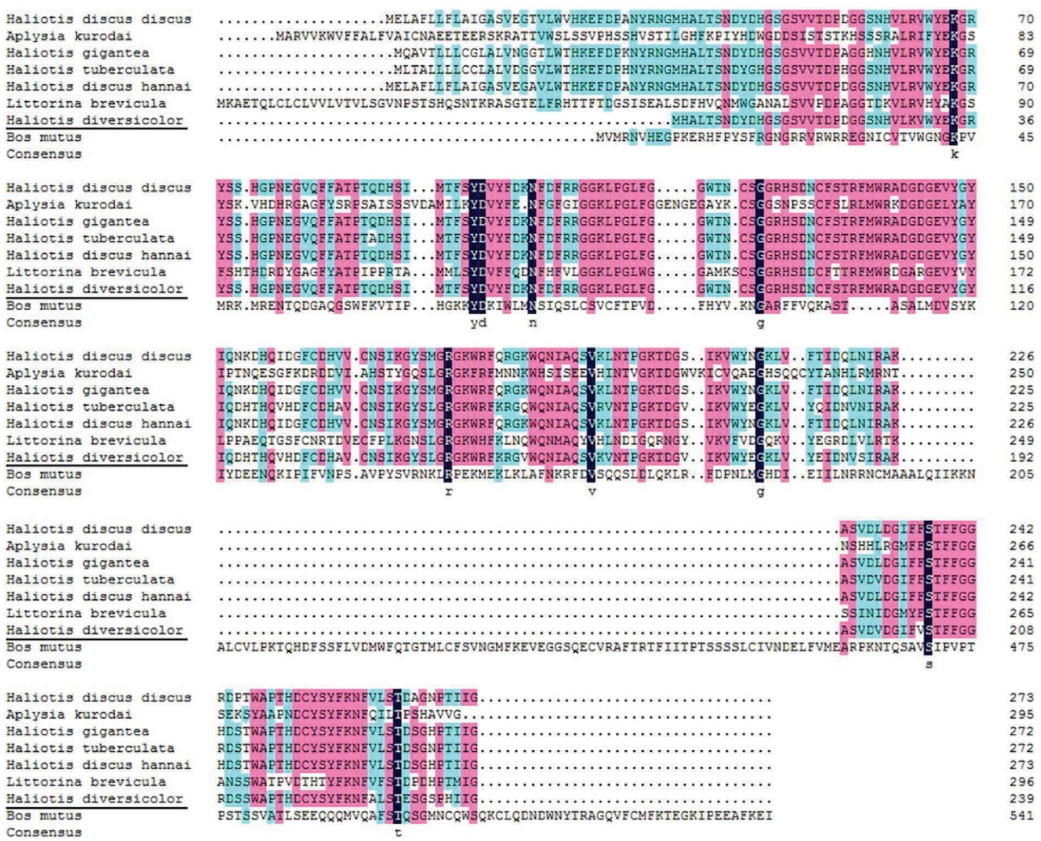

FIGURE 4: Multiple alignments of the amino acid sequence of alginate lyase between *H. diversicolor* and other published animal species. (The amino acid boxed in black indicates conservation of identical residues in all sequences. The amino acid boxed in pink indicates the conservation of residues with above 75% consistency. Amino acid residues are numbered to the right of each sequence, and dots represent indels. Alginate lyase amino acid sequences are obtained from GenBank as follows: *Haliotis discus discus*, BAE45131.1; *Haliotis discus hannai*, BAE81787.1; *Haliotis gigantean*, AFQ98373.1; *Haliotis tuberculate*, AFQ98374.1; *Aplysia Kuroda*, BAJ72675.1; *Littorina brevicula*, BAM17303.1; *Bos mutus*, XP_01zz4335099.1.).

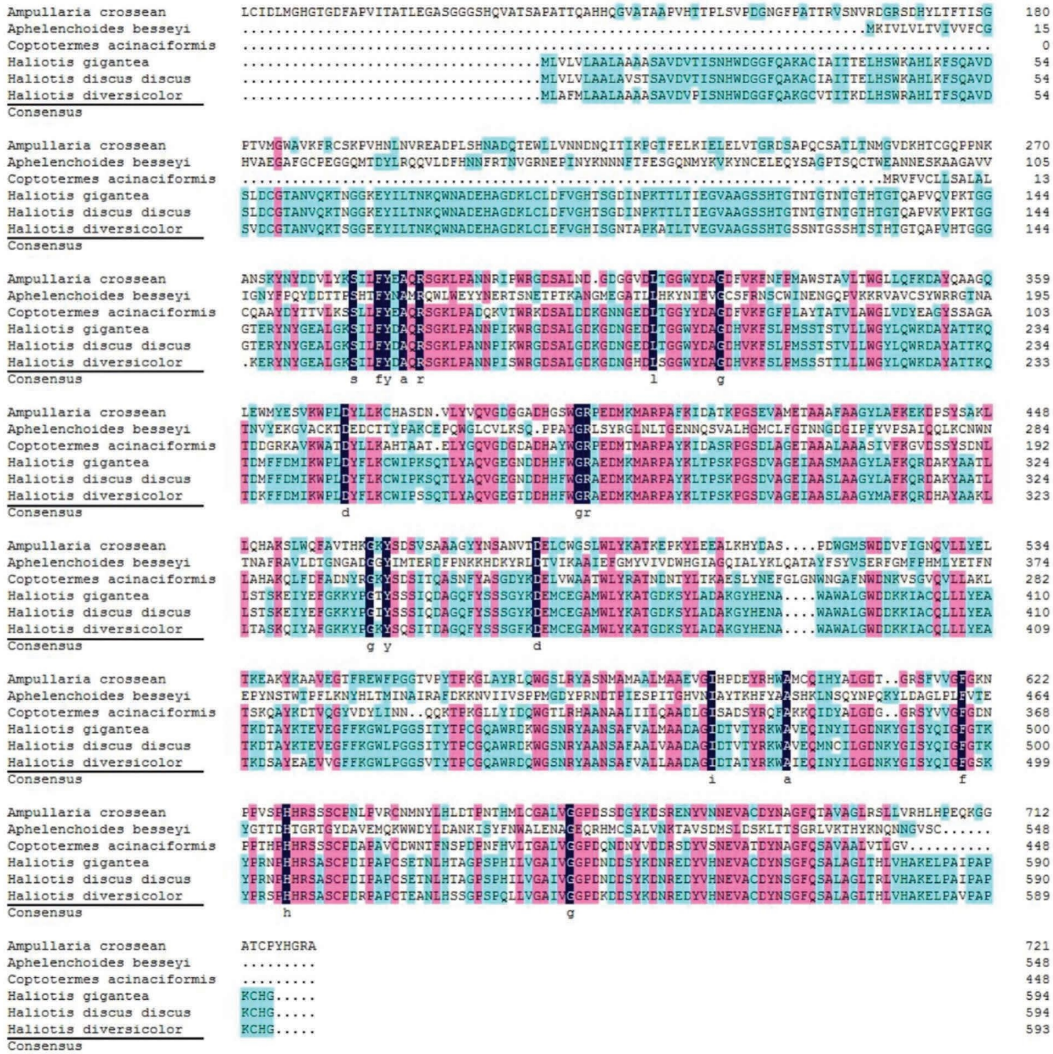

Figure 5: Multiple alignments of the amino acid sequence of cellulase between *H. diversicolor* and other published animal species (the accession numbers of the cellulase amino acid sequences of each species in the figure are as follows: *Haliotis gigantea*, AFQ98380.1; *Haliotis discus discus*, BAD44734.1; *Coptotermes acinaciformis*, AAK12339.1; *Ampullaria crossean*, ABD24280.1; *Aphelenchoides besseyi*, AIK28470.2.).

abalones belonging to the genus Abalone is relatively high. The similarity between *H. diversicolor* and *Haliotis gigantea* is 87.58%, and it is 86.41% with *Haliotis discus*. The similarity with *Ampullaria* crossing, which is also a mollusk gastropod, is also lower than the previous ones, at 41.6%; the similarity with the invertebrate *Coptotermes acinaciformis* is 35.51%, and the similarity with Bacillus subtilis is 35.51%. *Bacillus subtilis* was 11.27%, the similarity with *Mesosa myops* was 9.03%, the similarity with *Tachypleus tridentatus* was 12.22%, and the similarity with *Aphelenchoides besseyi* was 10.84%. A molecular phylogenetic tree was constructed to analyze the evolutionary relationship among the cellulase amino acid sequence. From the phylogenetic tree, we can see that in the mollusk branch, *H. diversicolor* is grouped with several other abalones and invertebrates such as *Ampullaria crossean* and *Coptotermes acinaciformis*.

3.6. Sequence Analysis of Amylase. The complete amylase cDNA sequence was 2195 bp (Figure S5). The sequence was imported to NCBI, a predicted 2097 bp ORF was found, and a 698-amino acid protein was encoded. The 3′-untranslated region of the cDNA was 84 bp, and there was a typical polyadenylation signal (AATAAA) and polyadenylation sequence (polyA). The 5′-untranslated region of the alginate lyase cDNA was 14 bp. The length of the upstream sequence of amylase in *H. diversicolor* was 2340 bp, and the full length of cellulase gene DNA was 4302 bp, containing 4 exons and 3 introns.

Using SMART software analysis, it was found that the N-terminus of the amino acid sequence of the MNK gene of abalone contained an Aamy domain, and a predicted typical Aamy_C domain appeared in the sequence (Figure S6).

The multiple amino acid sequence alignment of DNAMAN software (Figure 6) found that the amino acid similarity between *H. diversicolor* and several abalones belonging to the same genus was higher than that of other species, but the differences were obvious. The similarity was 36.10% with *Haliotis gigantea*, 36.96% with *Haliotis tuberculata*, and 87.70% with *Haliotis discus discus*. The *H. diversicolor* is 34.85% with Spondylus violaceus, the same mollusk, 35.34% with Crassostrea gigas, 34.74% with *Pteria penguin*, and 53.18% with *Litopenaeus vannamei*, and the similarities with other invertebrates are Italian honey bee 31.33%, cotton bollworm 30.73%, and fruit fly 32.36%. Comparison of similarities between *H. diversicolor* and vertebrates: *Siniperca chuatsi* 29.48%, *Tetraodon nigroviridis* 31.29%, *Cynoglossus semibreves* 31.56%, *Anguilla japonica* 32.65%, *Xenopus laevis* 33.38%, *Gallus gallus* 32.23%, *Homo sapiens* 33.24%, *Pan troglodytes* 32.97%, *Sus scrofa* 32.97%, and *Mus musculus* 32.41%. A molecular phylogenetic tree was constructed to analyze the evolutionary relationship among the cellulase amino acid sequence. It can be seen from the phylogenetic tree that the variegated abalone is grouped with other invertebrates, and other vertebrates are grouped into another group.

3.7. Alginate Lyase Expression in Different Developmental Larval Stages and Different Tissues. Alginate lyase gene was started expressing in early creep, and it was not expressed in the stage from eggs to the late veliger larva, and the expression of the alginate lyase gene began to decrease in mid creep. The expression in the early creep was about 1.2 times that in mid creep (Figure 7). The expression of the alginate lyase gene in adults has tissue-specificity. The gene expression in the hepatopancreas was much higher than in the testis and ovary. The expression level in the hepatopancreas was about 45.57 times that in the ovary and 24.82 times that in the testis, while the expression difference between ovarian and testis is small. The expression in the spermary was about 1.84 times that in the ovary (Figure 7).

3.8. Cellulase Expression in Different Developmental Larval Stages and Different Tissues. The cellulase gene started expressing in early creep, and it was not expressed in the stage from eggs to the late veliger larva, and the expression of the cellulase gene began to decrease in mid creep. The expression in the early creep was about 3.08 times that in mid creep (Figure 8(a)). The expression of the cellulase gene in adults has tissue-specificity. The expression of the gene in the hepatopancreas was much higher than that in the testis and ovary, and the expression level in the hepatopancreas was about 14.26 times that in the testis and 10.52 times of that in the ovary, while the expression difference between in ovarian and ovary is small, and the expression in spermary was about 1.36 times of that in testis (Figure 8(b)).

3.9. Amylase Expression in Different Developmental Larval Stages and Different Tissues. The amylase gene started expressing in early creep, and it was not expressed in the stage from eggs to the late veliger larva, and the expression of the amylase gene began to decrease in mid creep. The expression in the early creep was about 3.08 times that in mid creep (Figure 9(a)). The expression of the amylase gene in adults has tissue-specificity. The gene expression in the hepatopancreas was much higher than that in the testis, and the expression level in the hepatopancreas was about 48.44 times that in the testis and not expressed in the ovary (Figure 9(b)).

3.10. Screening the SNPs of Alginate Lyase Associated with Growth Traits. After high-throughput sequecing, SNPs were initially screened, and 84 SNPs with high likelihood were screened. Five SNPs were located in the coding region, ALG5313, ALG5525, ALG5609, ALG5633, and ALG5659, and the other 79 SNPs were all located in the upstream regulatory region. Genotyping and the association analysis between SNP loci and growth traits showed that two SNP loci, ALG1016 and ALG1544, were significantly correlated with the growth traits ($P < 0.05$). ALG1016 and ALG1544 were significantly correlated with shell length. ALG1016 was significantly correlated with shell width, and ALG1544 correlated significantly with shell width. ALG1016 was very significantly correlated with shell height. ALG1016 and ALG1544 were significantly correlated with total weight. The analysis results by PopGen32 software show that the two SNP loci conform to Hardy–Weinberg equilibrium. In addition, the SNP loci ALG1541, ALG3132, and ALG3958 were more significantly ($P < 0.1$) correlated with growth traits, the correlation with the shell width was significant, and the association between ALG3132 and whole weight was significant.

We can arrive at the conclusions about the alginate lyase gene in the Dadeng pedigree (Table 1) that the individuals with GG genotype in the SNP locus ALG1016 had higher growth traits than those of the individuals with GT genotype. The shell width and the total weight of the individuals with GG genotype were significantly higher than those with GT genotype ($P < 0.05$). The individuals with GG genotype were significantly higher than those with GT genotype ($P < 0.01$), but there was no significant difference between individuals with GG genotype and those with GT genotype. The growth traits of the individuals with AA genotype in the SNP locus ALG1544 were higher than those of the individuals with AC genotype, and the length and the total weight of the individuals with AA genotype were significantly higher than those of the individuals with AC genotype ($P < 0.05$). The individuals with the AA genotype were significantly higher than those with the AC genotype ($P < 0.001$), but there was no significant difference between the individuals with the AA genotype and those with the AC genotype.

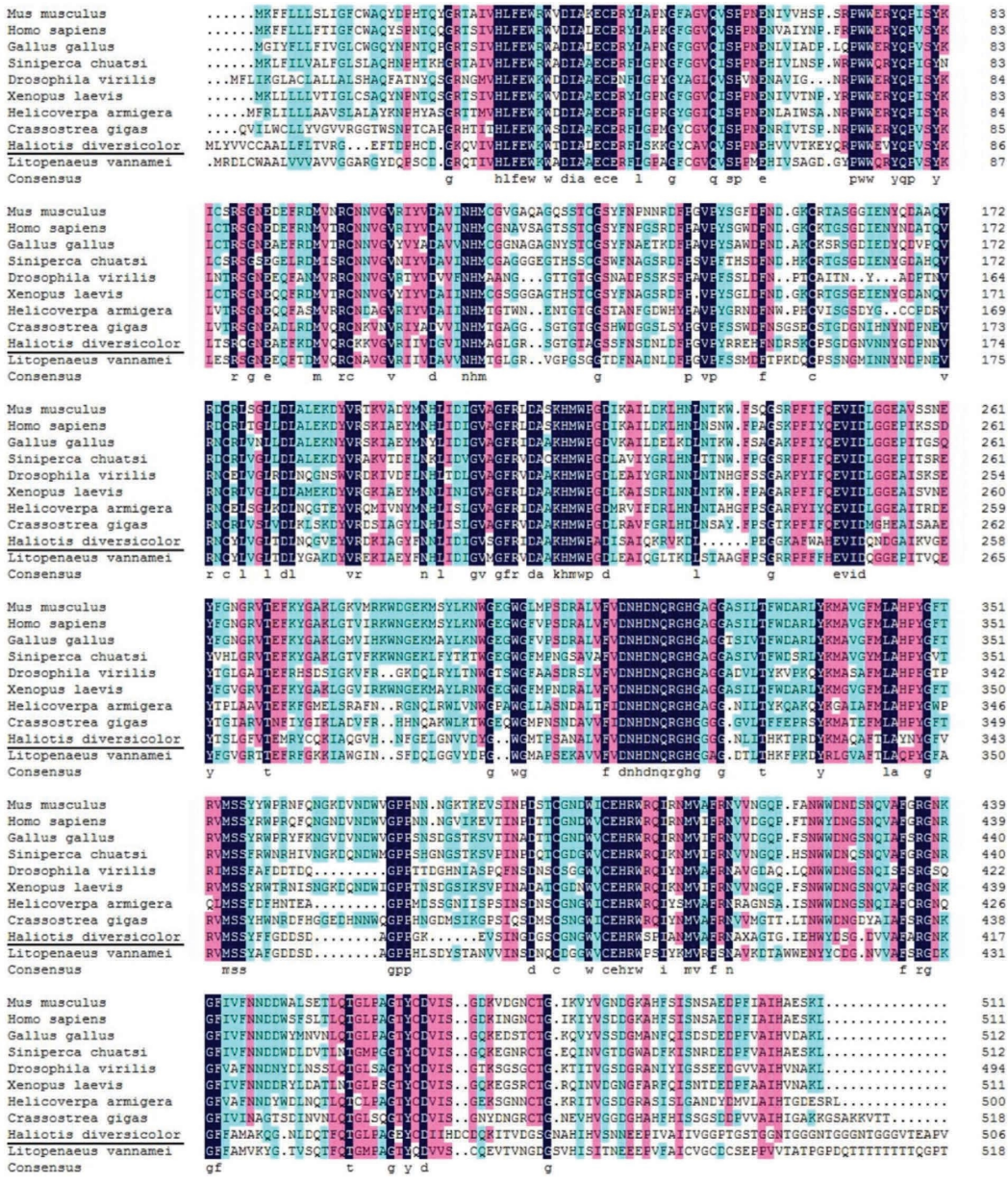

FIGURE 6: Multiple alignments of the amino acid sequence of amylase between *H. diversicolor* and other published animal species (the accession numbers of the amylase amino acid sequences of each species in the figure are as follows: *Mus musculus*, NP_031472.2; *Homo sapiens*, AAA51724.1; *Xenopus laevis*, NP_001079910.1; *Gallus gallus*, NP_001001528.2; *Siniperca chuatsi*, ACJ06746.1; *Helicoverpa armigera*, ACB54942.1; *Drosophila virilis*, XP_002059348.1; *Crassostrea gigas*, CAA69658.1; *Litopenaeus vannamei*, AIJ02080.1.).

4. Discussion

There was no significant difference in alginate lyase activity among the four diets, but if males and females were treated for each diet, it was found that the activity of males and females was different, and the difference was significant within and between groups. Similar to the results of this experiment, Zhao et al. [20] found that during the development of abalone gonads, the enzyme activities of several digestive enzymes in the hepatopancreas and digestive tract of abalone were higher in females than in males, and some of them were significantly different.

Cellulase is a multicomponent enzyme, including C1, Cx, and β-glucosidase, commonly found in the abalone [25]. In this experiment, the activity of the Cx enzyme was detected, and it was found that there was no significant difference in the Cx enzyme among the four diets, but the cellulase activity of males and females was different. Except

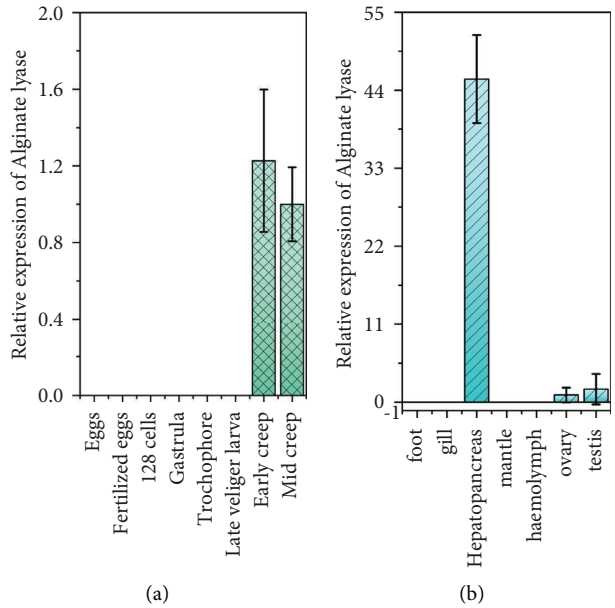

Figure 7: The qRT-PCR for alginate lyase gene in (a) 8 different periods in larvae; (b) 7 different tissues in adults.

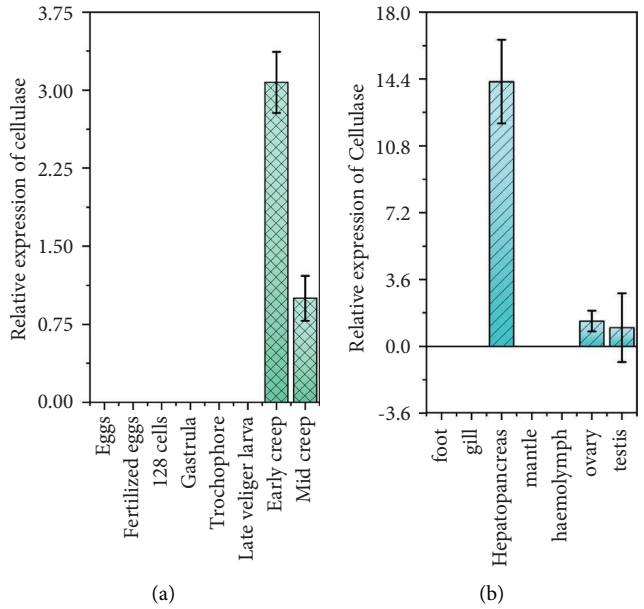

Figure 8: The qRT-PCR for cellulase gene in (a) 8 different periods in larvae; (b) 7 different tissues in adults.

for the Compound feed, there were significant differences between males and females among the three groups of *Porphyra, Laminaria japonica,* and *Gracilaria*. Similar to the results of this study, Zhao et al. [20] found that during the development of abalone gonads, the enzymatic activities of several digestive enzymes in the hepatopancreas and digestive tract of abalone were higher in females than in males.

The prominent polysaccharide-degrading enzymes in abalone digestive juice are alginate lyase, cellulase, and α-amylase [10–12, 26], among which α-amylase was considered to digest the α-amylase contained in red and green seaweeds. α-Amylase was thought to play an important role in digesting α-glucans contained in red and green seaweeds. In this experiment, both α-amylase and β-amylase were detected by the amylase detection method in the crude enzyme liquid extracted from the body, indicating that there are two subspecies of amylase in the variegated abalone, and β-amylase activity was higher than that of α-amylase. There is no report that abalone contains β-amylase, but it is still analyzed from the perspective of α-amylase, and the detection method of enzyme activity is still limited to the related methods of α-amylase. Nikapitiya et al. [26] stated that the current research on amylase in abalone is mainly based on recombinant enzymes rather than enzymes directly

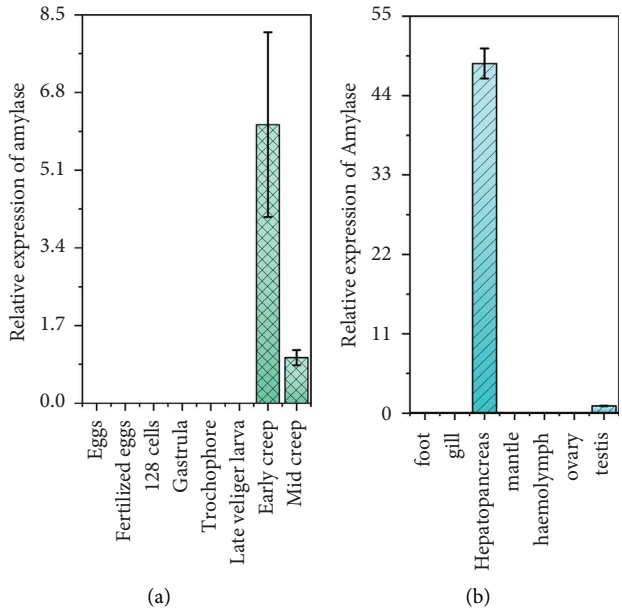

Figure 9: The qRT-PCR for amylase gene in (a) 8 different periods in larvae; (b) 7 different tissues in adults.

Table 1: Growth traits of *H. diversicolor* with different genotypes in Dadeng pedigree.

Locus	Genotype	*N*	Frequency	Shell length	Shell width (mm)	Shell height	Shell height (g)
ALG 1016	GG	23	23.2	47.15 ± 6.32	29.77 ± 3.79	9.28 ± 2.47	13.23 ± 4.57
	GT	76	76.8	43.61 ± 7.81	27.13 ± 4.73	7.95 ± 1.32	10.25 ± 4.90
ALG 1544	AA	85	85.9	44.97 ± 7.59	28.32 ± 4.14	8.36 ± 1.80	11.35 ± 4.99
	AC	14	14.1	40.86 ± 7.02	23.92 ± 6.04	7.57 ± 1.07	8.27 ± 4.05

Note. *N* represents the number of individuals. Growth traits were expressed as the mean ± standard deviation.

extracted from the body. Most of the α-amylase gene has been cloned, but no β-amylase gene has been cloned; the possible reason is based on this.

In the natural state, *H. diversicolor* mainly feeds on brown algae, and alginate lyase is an important hydrolase for the digestion of brown algae. The number of studies about alginate lyase in abalone has been increasing, and the related studies in *Haliotis discus discus* and *Haliotis rufescens* have been published, but the alginate lyase gene in *H. diversicolor* has not been cloned yet. In the present study, the full-length cDNA sequence of the alginate lyase gene was cloned from *H. diversicolor* for the first time. We also obtained the genomic DNA sequences and upstream sequences of the alginate lyase gene from *H. diversicolor*. The multiple alignments of amino acid sequences showed that the genes encoding alginate lyase were relatively conserved among shellfish species. Phylogenetic tree results showed that the alginate lyase amino acid sequence in *H. diversicolor* was highly homologous to the mollusks such as the *Aplysia kurodai Baba and Littorina brevicula*, and the genetic distance between *H .diversicolor* and vertebrates as well as fungi and other micro-organisms is far away, which is consistent with the law of genetic evolution. In addition, the alginate lyase gene is very rare in vertebrates, and the reference gene sequences are very few. Therefore, it is speculated that the digestion of brown algae in vertebrates may not be through their enzymes but symbiotic intestinal microbes. It can be seen from the above that the alginate lyase gene was different among different species, and the alginate lyase amino acid sequence in *H. diversicolor* is similar to other abalone species as well as other marine mollusks such as *Apocynia jurodai Baba and Littorina brevicula*, but there also exist some differences, which may be related to feeding habit. Dunaevsky et al. [27] found that the digestive enzyme activity varied with the tissue structure of the digestive organ and digestive function. Under normal circumstances, the digestive tract of herbivorous fish was longer than carnivorous fish, and the sugar-metabolic enzymes were strong, while carnivorous fish have stronger protease activity. Many studies have shown that cellulase activity in abalone is very low and cannot digest the cellulose effectively. With the increase of cellulose content in the feed, the weight gain rate, feed conversion efficiency, and protein efficiency ratios of abalone would decrease significantly. So, under natural conditions, the differences in feeding habits may lead to differences in genes between abalones and close relatives.

Cellulase is a very important digestive enzyme for decomposing seaweed components in *H. diversicolor*. The research of cellulase in abalone has gradually attracted attention, and related research is also in progress; and the cellulase gene has been obtained in many abalone and mollusks. For example, Wang et al. isolated and purified

multifunctional cellulose EGX from *Ampullaria crossean*, and the fragment cloned from the ovary to this gene consists of 9 exons and 8 introns [28]. Like its research results, the cellulase gene cloned in this study also contains 9 exons and 8 introns and has a high similarity coefficient on the phylogenetic tree, clustering into the same class. Through clustering with other species and multiple alignments of amino acid sequences, it was found that the amino acid sequence of the cellulase gene of *H. diversicolor* was highly similar to the cellulase amino acid sequences of other known abalones and other marine shellfish; it shows that the gene sequence encoding cellulase has high homology among shellfish species and is relatively conserved, while other vertebrates and microorganisms such as fungi are clustered into a single group, indicating that the genetic distance between them is relatively long, which is in line with the evolutionary relationship. In addition, it was found that the cellulase gene is very rare in vertebrates, and it is speculated that vertebrates may not need to digest brown algae through their enzymes but digest brown algae through symbiotic gut microbes. It differs from similar shellfish, such as Haliotis gigantea and Ampullaria *crossean*, which may be related to food habits. Many studies have shown that cellulase activity in abalone is very low and cannot effectively digest and degrade cellulose. With the increase of cellulose content in the feed, the weight gain rate, feed conversion efficiency, and protein efficiency of abalone will decrease significantly. Therefore, differences in feeding habits under natural conditions may lead to genetic differences between abalone and similar species. This study is also the first time to obtain the upstream sequence in *H. diversicolor*, and through the sequence splicing of the upstream sequence and the sequence obtained by the RACE method, it is found that the 5′ end of the cDNA sequence obtained by the RACE method has a sequence similar in sequence. However, it did not match the upstream sequence and was found to be the cap structure Caps in the mRNA. Through bioinformatics analysis, it can be seen that the cellulase of *H. diversicolor* contains a predicted carbohydrate-binding site CBM2 (carbohydrate-binding type-2) domain and a catalytic activation site. The active site has complete substrate binding and catalytic sites; it is a multifunctional cellulase.

Amylase is a very important digestive enzyme for decomposing seaweed components in *H. diversicolor* and the most important enzyme for decomposing cellulose components. The amylase gene has been cloned in the abalone [10, 11]. In this study, the full-length cDNA sequence, the genomic DNA sequence, and the upstream sequence of the amylase gene were cloned from *H. diversicolor*, and the amino acid sequence and the upstream promoter region of the gene were deduced. This study is also the first time to obtain the upstream sequence in *H. diversicolor*, and we found that the 5′-end of the cDNA sequence obtained by the RACE method has a sequence similar in sequence, but it does not match the upstream sequence, which is the cap structure Caps in the mRNA.

After multiple alignments of amino acid sequences, it was found that the amino acid sequence of the amylase gene of *H. diversicolor* was more similar to the amino acid sequence of other known amylases of the abalone family and other marine shellfish such as Crassostrea gigas and other invertebrates such as *Litopenaeus vannamei*. It shows that the gene sequence encoding amylase is relatively conserved between shellfish and other similar species, but there are also differences, which may be related to feeding habits. Many studies have shown that cellulase activity in abalone is very low and cannot effectively digest and degrade cellulose. With the increase of cellulose content in the feed, the weight gain rate, feed conversion efficiency, and protein efficiency of abalone will decrease significantly. Therefore, under natural conditions, the difference between abalone and similar species may be caused by different food habits.

By constructing a phylogenetic tree, it was found that in the mollusk branch, the *H. diversicolor* was clustered with invertebrates such as *Crassostrea gigas* and *Litopenaeus vannamei*, while the other branch was clustered with vertebrates. It shows that the amino acid sequence of amylase in *H. diversicolor* has a high degree of homology with invertebrate species such as shellfish but has a long genetic distance from vertebrates which is in line with the evolutionary relationship.

The three genes of alginate lyase, cellulase, and amylase were all expressed from the early stage of the prostrate, and there was no expression level in the previous period, indicating that they are not constitutive enzymes but inducible enzymes. *H. diversicolor* attaches and feeds from the outside from the creeping stage, so, realistically, the gene expression of the enzyme starts at this time. The expression levels of the three genes surged in the early stage of creeping but decreased significantly in the middle stage, especially the cellulase and amylase genes. When the expression starts at a specific time under the existing conditions, it may be overexpressed for the continuous progress of the later process. Then, the expression level is correspondingly reduced according to the demand for the enzyme amount of self-feeding and the number of algae in the environment. The later change of the expression level will gradually go stable. From the changes in the expression levels in seven different tissues of adults, the three enzyme genes are tissue-specific and the three genes are highly expressed in the hepatopancreas. It indicated that the expression and secretion of alginate lyase, cellulase, and amylase were mainly carried out in the hepatopancreas. The three enzyme genes were also expressed in very small amounts in the gonads. But we also do not rule out that the gonad is contaminated by visceral mass because the three enzymes are all enzymes that digest polysaccharides, so the expression levels are also very similar. Amylase is not expressed in the ovary, while other enzyme genes are in the testis. There is little difference between the expression level and the ovary, and the expression level is extremely low. We speculate that the sampling site of the gonad is relatively close to the visceral mass during sampling, and there may be a little contamination of the visceral mass, resulting in a very small amount of expression.

The average density of SNP loci in the full-length and upstream regions of the genes in *H. diversicolor* was 1/71 (bp), and in the coding region, it was 1/185 (bp), while in the

upstream noncoding regulatory region, it was 1/64 (bp). The SNP loci of the Alginate gene in *H. diversicolor* had a higher average density in the upstream noncoding regulatory region relative to the coding region. Although SNP loci exist widely in the genome, the distribution frequency in different genomes is inconsistent. Even in the same genome, the distribution frequency of the coding and noncoding regions is also different [29]. There are also large differences in the distribution frequency of SNP loci in aquatic animals. SNP loci in the EST sequence of Pacific oyster appears in the frequency of 1/40 (bp) [30, 31], while the SNP loci had an average density of 1/100 (bp) in the EST sequence of *Haliotis discus* [32], and the average density of SNP loci in *Crassostrea virginica* is 1/20 (bp) [33]. The proportion of variation in aquatic animals is very high. In this study, the R language program was used to correlate the SNP loci with the growth traits of the *H. diversicolor*. It was found that two SNPs were significantly associated with the growth traits. SNPs associated with growth traits are important for the cultivation of *H. diversicolor*.

5. Conclusions

In conclusion, our results provided biochemical and molecular information on alginate lyase, cellulase, and amylase from *H .diversicolor* that is essential for understanding its molecular mechanisms regulating growth traits to cultivate new varieties with excellent traits. In this study, the cDNA and full-length genes of alginate lyase, cellulase, and amylase genes were cloned and obtained from *H .diversicolor* for the first time (the amylase gene obtained some introns), and the upstream regulatory sequences of the three genes were obtained by amplification for the first time. The function and promoter sequence of the gene was predicted, and the SNP loci that were significantly associated with the growth traits of variegated abalone were obtained by screening. It can be used for later molecular marker-assisted breeding, which has certain guiding significance for practical application.

Data Availability

The data are included within the article in Supplementary file.

Ethical Approval

This article does not contain any studies involving animals performed by any of the authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

W.L., S.M., and H.C. conceptualized the study; W.L. and W.X. developed the methodology; S.M. handled software; W.L., S.M., and M.M. validated the study; W.L. and W.Y. carried out the formal analysis; W.A., W.L., and S.M. investigated the study; S.M. wrote and prepared the original draft of the study; W.A. wrote, reviewed, and edited the study; S.M. supervised the study; W.L. carried out the funding acquisition. All authors have read and agreed to the published version of the manuscript. Weidong Li and Huaifeng Cao contributed equally to this work.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (31860728).

Supplementary Materials

Figure S1: the cDNA sequence and deduced amino acid sequence of alginate lyase in *H. diversicolor*. (The open reading frame is marked in gray. The start and stop codons are marked in red. The polyadenylation signal is underlined in blue). Figure S2: phylogenetic tree of the alginate lyase amino acid sequence between *H. diversicolor* and other species. (Neighbor-joining (NJ) phylogenetic tree for initiator caspase proteins using MEGA 5.0. Alginate lyase from *H. diversicolor* is indicated by a red underline. Numbers next to the branches indicate the bootstrap value of each internal branch in the phylogenetic tree nodes from 1,000 replicates. Alginate lyase amino acid sequences are obtained from GenBank as follows: *Haliotis discus discus*, BAE45131.1; *Haliotis discus hannai*, BAE81787.1; *Haliotis gigantean*, FQ98373.1; *Haliotis tuberculate*, AFQ98374.1; *Aplysia kurodai*, BAJ72675.1; *Littorina brevicula*, BAM17303.1; *Lottia gigantean*, XP_009052194.1; *Cryptococcus neoformans*, P_012051769.1; *Drosophila melanogaster*, NP_001097832.1; *Moniliophthora roreri*, XP_007848974.1; *Bos mutus*, XP_014335099.1; *Panthera tigris altaica*, XP_007089060.1.). Figure S3: the cDNA sequence and deduced amino acid sequence of cellulase from *H. diversicolor* (the predicted cellulose-binding domain CBD_II is indicated by a red box; the predicted carbohydrate-binding site CBM2 (carbohydrate-binding type-2) domain is indicated by gray shading; the activation site active site is marked with a blue border; start and stop codons are highlighted in bold red; the AATAAA tailed signal sequence is underlined in blue.). Figure S4: phylogenetic tree of the cellulase amino acid sequence between *H. diversicolor* and other species (the accession numbers of the cellulase amino acid sequences of each species in the figure are as follows: *Haliotis gigantea*, AFQ98380.1; *Haliotis discus discus*, BAD44734.1; *Coptotermes acinaciformis*, AAK12339.1; *Ampullaria crossean*, ABD24280.1; *Bacillus subtilis*, BAL46915.1 *Mesosa myops*, AMA76416.1; *Tachypleus tridentatus*, BAA04044.1; *Aphelenchoides besseyi*, AIK28470.2; *Bursaphelenchus xylophilus*, ACM44323.1.). Figure S5: the cDNA sequence and deduced amino acid sequence of Amylase from *H.diversicolor* (the predicted Aamy domain is marked with a red box; the Aamy_C domain is marked with a blue box; the start and stop codons are marked with red bold font and a black box; the AATAAA tailed signal sequence is underlined in blue.). Figure S6: phylogenetic tree of the Amylase amino acid sequence between *H. diversicolor* and other species (the accession numbers of the amylase amino acid sequences of

each species in the figure on NCBI are as follows: *Mus musculus*, NP_031472.2; *Sus scrofa*, NP_999360.1; *Pan troglodytes*, NP_001103627.1; *Homo sapiens*, AAA51724.1; *Gallus gallus*, NP_001001528.2; *Xenopus laevis*, NP_001079910.1; *Anguilla japonica*, BAB85635.1; *Cynoglossus semilaevis*, AGT37610.1; *Tetraodon nigroviridis*, CAD20263.; *Siniperca chuatsi*, ACJ06746.1; *Helicoverpa armigera*, ACB54942.1; *Apis mellifera*, BAA86909.1; *Drosophila virilis*, XP_002059348.1; *Haliotis gigantea*, AFQ98378.1; *Haliotis tuberculate*, AFQ98377.1; *Spondylus violaceus*, AFE48186.1; *Crassostrea gigas*, CAA69658.1; *Pteria penguin*, AEI58894.1; *Litopenaeus vannamei*, AIJ02080.1; *Haliotis discus discus*, ABO26611.1.). (*Supplementary Materials*)

References

[1] W. Li, W. You, W. Chen et al., "Characterization of ornithine decarboxylase, a potential selective breeding marker, from small abalone, Haliotis diversicolor," *Journal of the World Aquaculture Society*, vol. 41, no. 5, pp. 721–732, 2010.

[2] G. F. Zhang, H. Y. Que, X. Liu, and H. S. Xu, "Abalone mariculture in China," *Journal of Shellfish Research*, vol. 23, no. 4, pp. 947–950, 2004.

[3] P. C. Liu, Y. C. Chen, and K. K. Lee, "Pathogenicity of Vibrio alginolyticus isolated from diseased small abalone Haliotis diversicolor supertexta," *Microbios*, vol. 104, no. 408, pp. 71–77, 2001.

[4] J. L. Nicolas, O. Basuyaux, J. Mazurié, and A. Thébault, "Vibrio carchariae, a pathogen of the abalone Haliotis tuberculata," *Diseases of Aquatic Organisms*, vol. 50, pp. 35–43, 2002.

[5] M. A. Travers, M. Koken, S. Huchette, and C. Paillard, "Vibrio carchariae detection and interaction with Haliotis tuberculata haemocytes," *Vibrio*, 2005.

[6] H. S. Doh, J. A. Ko, and H. J. Park, "Preparation of novel iodized salt with natural iodine-rich sources by spray drying," *Journal of Food Science*, vol. 83, no. 6, pp. 1676–1684, 2018.

[7] L. Ford, K. Theodoridou, G. N. Sheldrake, and P. J. Walsh, "A critical review of analytical methods used for the chemical characterisation and quantification of phlorotannin compounds in brown seaweeds," *Phytochemical Analysis*, vol. 30, no. 6, pp. 587–599, 2019.

[8] A. M. Mannino, F. Borfecchia, and C. Micheli, "Tracking marine alien macroalgae in the mediterranean sea: the contribution of citizen science and remote sensing," *Journal of Marine Science and Engineering*, vol. 9, no. 3, p. 288, 2021.

[9] B. Schaffelke, J. E. Smith, and C. L. Hewitt, "Introduced macroalgae - a growing concern," *Journal of Applied Phycology*, vol. 18, no. 3-5, pp. 529–541, 2006.

[10] E. Shimizu, T. Ojima, and K. Nishita, "cDNA cloning of an alginate lyase from abalone, Haliotis discus hannai," *Carbohydrate Research*, vol. 338, no. 24, pp. 2841–2852, 2003.

[11] H. Suzuki, K. Suzuki, A. Inoue, and T. Ojima, "A novel oligoalginate lyase from abalone, Haliotis discus hannai, that releases disaccharide from alginate polymer in an exolytic manner," *Carbohydrate Research*, vol. 341, no. 11, pp. 1809–1819, 2006.

[12] K. I. Suzuki, T. Ojima, and K. Nishita, "Purification and cDNA cloning of a cellulase from abalone Haliotis discus hannai," *European Journal of Biochemistry*, vol. 270, no. 4, pp. 771–778, 2003.

[13] T. Sawabe, H. Takahasi, H. Saeki, K. Niwa, and H. Aono, "Enhanced expression of active recombinant alginate lyase AlyPEEC cloned from a marine bacterium Pseudoalteromonas elyakovii in Escherichia coli by calcium compounds," *Enzyme and Microbial Technology*, vol. 40, no. 2, pp. 285–291, 2007.

[14] I. W. Davidson, I. W. Sutherland, and C. J. Lawson, "Purification and properties of an alginate lyase from a marine bacterium," *Biochemical Journal*, vol. 159, no. 3, pp. 707–713, 1976.

[15] L. Li, X. Jiang, H. Guan, P. Wang, and H. Guo, "Three alginate lyases from marine bacterium Pseudomonas fluorescens HZJ216: purification and characterization," *Applied Biochemistry and Biotechnology*, vol. 164, no. 3, pp. 305–317, 2011.

[16] Y. Matsubara, R. Kawada, K. I. Iwasaki, T. Oda, and T. Muramatsu, "Extracellular poly(α-L-guluronate)lyase from Corynebacterium sp.: purification, characteristics, and conformational properties," *Journal of Protein Chemistry*, vol. 17, no. 1, pp. 29–36, 1998.

[17] J. C. Tang, H. Taniguchi, H. Chu, Q. Zhou, and S. Nagata, "Isolation and characterization of alginate-degrading bacteria for disposal of seaweed wastes," *Letters in Applied Microbiology*, vol. 48, no. 1, pp. 38–43, 2009.

[18] P. Liu, A. Lin, G. Zhang et al., "Enhancement of cellulase production in Trichoderma reesei RUT-C30 by comparative genomic screening," *Microbial Cell Factories*, vol. 18, no. 1, p. 81, 2019.

[19] S. Singh, J. K. Brar, D. K. Sandhu, and A. Kaur, "Isozyme polymorphism of cellulases in Aspergillus terreus," *Journal of Basic Microbiology*, vol. 36, no. 4, pp. 289–296, 1996.

[20] J. Zhao, B. Shi, Q. Jiang, and C. Ke, "Changes in gut-associated flora and bacterial digestive enzymes during the development stages of abalone (Haliotis diversicolor)," *Aquaculture*, vol. 338–341, pp. 147–153, 2012.

[21] J. Li, H. L. Zhang, Z. Wang et al., "Determination content of the antidepressant extraction and analysis the trace elements from Morinda officinalis," *Zhong Yao Cai*, vol. 31, no. 9, pp. 1337–1340, 2008.

[22] M. S. Bansemer, J. G. Qin, J. O. Harris et al., "Age-dependent response of digestive enzyme activities to dietary protein level and water temperature in greenlip abalone (Haliotis laevigata)," *Aquaculture*, vol. 451, pp. 451–456, 2016.

[23] P. Yang, G. Yan, and J. Tang, "Screening of alginate lyase synthase producing strain and optimization of its fermentation conditions," *Advances in Microbiology*, vol. 07, no. 03, pp. 87–97, 2018.

[24] B. Yang, J. Qin, B. Shi et al., "Molecular characterization and functional analysis of adrenergic like receptor during larval metamorphosis in Crassostrea angulata," *Aquaculture*, vol. 366-367, pp. 54–61, 2012.

[25] N. T. Hamdan and H. M. Jasim, "Purification and Characterization of Cellulase enzyme from Trichoderma longibrachiatum isolated in Iraqi soil," *Journal of Biotechnology Biochemistry*, vol. 4, no. 1, pp. 32–41, 2018.

[26] C. Nikapitiya, C. Oh, I. Whang et al., "Molecular characterization, gene expression analysis and biochemical properties of α-amylase from the disk abalone, Haliotis discus discus," *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, vol. 152, no. 3, pp. 271–281, 2009.

[27] Y. E. Dunaevsky, V. F. Tereshchenkova, B. Oppert, M. A. Belozersky, I. Y. Filippova, and E. N. Elpidina, "Human proline specific peptidases: a comprehensive analysis,"

Biochimica et Biophysica Acta (BBA) - General Subjects, vol. 1864, no. 9, Article ID 129636, 2020.

[28] J. Wang, M. Ding, Y. H. Li, Q. X. Chen, G. J. Xu, and F. K. Zhao, "A monovalent anion affected multi-functional cellulase EGX from the mollusca, Ampullaria crossean," *Protein Expression and Purification*, vol. 31, no. 1, pp. 108–114, 2003.

[29] L. Dupont, "Perspectives on the application of molecular genetics to earthworm ecology," *Pedobiologia*, vol. 52, no. 3, pp. 191–205, 2009.

[30] W. Jiao, X. Fu, J. Li et al., "Large-scale development of gene-associated single-nucleotide polymorphism markers for molluscan population genomic, comparative genomic, and genome-wide association studies," *DNA Research*, vol. 21, no. 2, pp. 183–193, 2014.

[31] C. Sauvage, N. Bierne, S. Lapègue, and P. Boudry, "Single Nucleotide polymorphisms and their relationship to codon usage bias in the Pacific oyster Crassostrea gigas," *Gene*, vol. 406, no. 1-2, pp. 13–22, 2007.

[32] H. Qi, X. Liu, G. Zhang, and F. Wu, "Mining expressed sequences for single nucleotide polymorphisms in Pacific abalone Haliotis discus hannai," *Aquaculture Research*, vol. 40, no. 14, pp. 1661–1667, 2009.

[33] L. Zhang and X. Guo, "Development and validation of single nucleotide polymorphism markers in the eastern oyster Crassostrea virginica Gmelin by mining ESTs and resequencing," *Aquaculture*, vol. 302, no. 1-2, pp. 124–129, 2010.