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# Development of single nucleotide polymorphisms in key genes of taurine and betaine metabolism in *Crassostrea hongkongensis* and their association with content-related traits

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## Abstract

**Background** Taurine and betaine are important nutrients in the Hong Kong oyster (*Crassostrea hongkongensis*) and have many important biological properties. To investigate the characteristics of taurine and betaine and identify single nucleotide polymorphisms (SNPs) associated with traits in *C. hongkongensis*, we cloned the full-length cDNA of key genes involved in taurine and betaine metabolism (unpublished data), determined taurine and betaine content and gene expression in different tissues and months of the oyster specimen collection, and developed SNPs in the gene coding region.

**Results** We cloned the full-length cDNA of the genes that express cysteine dioxygenase and cysteine sulfite decarboxylase (*ChCSAD* and *ChCDO*, respectively), which are key genes involved in taurine metabolism in *C. hongkongensis*, and found that betaine and taurine contents and the expression of key genes were regulated by seawater salinity. A total of 47 SNP markers were developed in the coding regions of *ChCSAD*, *ChCDO*, choline dehydrogenase (*ChCDH*), betaine aldehyde dehydrogenase (*ChBADH*), and betaine homocysteine methyltransferase (*ChBHMT*) using gene fragment resequencing and FLDAS-PCR. Through association analysis of a population of *C. hongkongensis* in the Maowei Sea, Guangxi, nine SNPs were found to be associated with taurine content, and one SNP was associated with betaine content. Haploid and linkage disequilibrium analyses showed that SNPs in *ChCDO* formed one linkage group with three haplotypes: ACACA, GTACA and GTTTG. The average taurine content of the corresponding individuals was 873.88, 838.99, and 930.72 μg/g, respectively, indicating the GTTTG haplotype has a significant advantage in terms of taurine content.

**Conclusions** SNPs associated with taurine and betaine contents in *C. hongkongensis* were identified for the first time. We found that the GTTTG haplotype in the *ChCDO* coding region has a significant advantage in taurine content. These loci and haplotypes can serve as potential molecular markers for the molecular breeding of *C. hongkongensis*.

**Keywords** *Crassostrea hongkongensis*, Taurine, Betaine, SNP, Association analysis

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## Introduction

With the development of the economy and deepening of health awareness in the general population, an increasing number of people choose seafood with higher nutritional content [1, 2]. Taurine and betaine are important nutrients in Hong Kong oysters (*C. hongkongensis*) and have many important biological properties [3, 4]. Oysters are also one of the most abundant natural sources of taurine. Since the beginning of the twenty-first century, oyster production in China has increased significantly. In 2019, oyster farming production countrywide reached 5.2 million tons, of which *C. hongkongensis* represented approximately 1.8 million tons, accounting for 34.45% of total oyster production in China [5]. Although the production of *C. hongkongensis* is high, it is still difficult to meet the market demand for high-quality oysters; thus, the economic benefits are relatively low. Factors limiting the profitability of oyster production include excessive aquaculture in the sea and changes in the aquaculture environment, which have led to a decline in the quality of *C. hongkongensis*.

Betaine and taurine not only serve as important nutrients in oysters but also give them a unique freshness quality [6, 7]. Research has shown that betaine and taurine buffer other substances in oyster flesh against osmotic pressure fluctuations, thereby improving the tolerance of biological cells to high-salt and high-osmotic environments [8, 9]. Pierce et al. [10] found significant differences in the betaine synthesis rates among populations of *Crassostrea virginica* under different salinity levels. The betaine synthesis rate of oyster populations adapted to high salinities was three to four times greater than that of populations adapted to low salinity. Song et al. [11] found that taurine content in the adductor muscle of *C. hongkongensis* changed significantly with salinity: when *C. hongkongensis* was moved to 30‰ seawater, taurine content increased rapidly; however, 24 h after moving to 6‰ seawater, taurine content decreased by approximately 40%. In the context of the nutritional status of shellfish, glycogen has been studied extensively; however, there have been few genetic analyses of other traits, which can affect the flavor and quality of oysters [12, 13]. The initial goal of shellfish breeding is to select varieties known for their large size and high yield to meet the growing market demand; however, there is not as much attention paid to the quality and flavor of the shellfish [14, 15]. Recently, with the increasing pressure on the environment and food production brought about by industrial transformation, the problem of quality in the oyster industry in China needs to be solved urgently if the goal is to enter the high-end market of consumable shellfish. Taurine and betaine are key components responsible for oyster flavor and taste. Moreover, they are necessary nutrients for human beings and regulate osmotic pressure regulation in oysters; thus, breeding with betaine or taurine as target traits will be of great significance for improving the quality of oysters.

There are two sources of betaine and taurine in oysters: endogenous synthesis and dietary intake. The physiological requirements of the oyster are partly met by endogenous synthesis, and dietary intake provides additional sources of betaine and taurine, especially under high-demand conditions, such as stress and disease [16, 17]. Cysteine dioxygenase (CDO) and cysteine sulfinic acid decarboxylase (CSAD) are key enzymes involved in taurine synthesis [18]. Cysteine, which marine bivalves mainly use as a raw material to maintain taurine content in their bodies [19], is oxidized to cysteine sulfite (CSA) through CDO and decarboxylated to hypotaurine through CSAD. Taurine is obtained via the oxidation of hypotaurine. In marine mollusks, full-length cDNA sequences of CDO and CSAD genes have been cloned from *Crassostrea gigas* and *Bathymodiolus septemdierum*, and the level of expression of the corresponding genes has been found to be related to changes in taurine content [20, 21]. Choline dehydrogenase (CDH) and betaine aldehyde dehydrogenase (BADH) are important enzymes for catalyzing the production of betaine from choline. First, choline is catalyzed by CDH, forming betaine aldehyde, which is then oxidized to betaine by BADH [22, 23]. Betaine homocysteine methyltransferase (BHMT) is a key enzyme in betaine catabolism [24]. Multiple metabolic pathways are involved in the decomposition of betaine in living organisms—the most common being the methionine cycle, which is catalyzed by BHMT [25]. Previous studies have shown that betaine synthesis in *Litopenaeus vannamei* and *Chasmagnathus granulata* depends on the activities of CDH and BADH [26, 27], and the expression of *BHMT* was found to affect the betaine content in *C. gigas* [28]. To date, there have been no reports on the key genes involved in taurine and betaine metabolism in *C. hongkongensis*. Therefore, we took these genes as candidate genes for identification of taurine and betaine related molecular markers.

To explore the relationship between key genes related to taurine and betaine metabolism and content-related traits in *C. hongkongensis*, we first determined the content of taurine and betaine, as well as the level of expression of genes *ChCDO*, *ChCSAD*, *ChBADH*, *ChCDH*, and *ChBHMT* in different tissues and specimen collection months. We then conducted an association analysis to screen single-nucleotide polymorphism (SNP) loci associated with taurine and betaine contents, laying a foundation for the breeding of high-quality oysters.

## Methods

### Oyster collection

Adult oysters were collected from the Sandun culture area of Qinzhou City, Guangxi. Because the nutritional content of adult oysters is relatively stable compared with that of juvenile oysters, we selected adult oysters at the second instar stage (shell height = 84.19 ± 9.66 mm, body weight = 105.42 ± 22.42 g). To avoid differences between groups growing under different environments, the oysters were attached at the same time and grew and developed in the same natural environment. From June 2020 to April 2021, oysters (15 at a time) were collected monthly from the same location, and the salinity was measured. The adductor muscles, gills, and remaining tissues were separated, frozen in liquid nitrogen, and stored at −80 °C. From each oyster, half of the gills were used for RNA extraction, half of the adductor muscle was used for betaine content detection, and the remaining tissues were used for taurine content detection. Samples from April were used for analysis of different tissues, including the adductor muscle, gills, mantle, gonads, digestive glands, and labial palps.

The 105 individual oysters used for SNP development and association analysis were collected from Maowei Sea. The oysters were attached at the same time, and they grew and developed in the same natural environment. Adductor muscle, gill, and remaining tissue were separated, frozen in liquid nitrogen, and stored at -80 °C. Half of the gills were used for RNA extraction, half of the adductor muscle was used for betaine content detection, and the remaining tissues were used for taurine content detection.

### Full-length cDNA cloning of key genes

Total RNA was extracted from the samples using the TRIzol method. The extracted RNA was measured through the use of spectrophotometry with a nucleic acid protein concentration meter (Gene Company Limited, Thermo, China). RNA integrity was detected via 1% agarose gel electrophoresis, and RNA was reverse transcribed into cDNA with a TransGen Biotech reverse transcription kit. Primers were designed for intermediate fragment cloning using the CSAD and CDO sequences of *C. gigas* as references. The 5' and 3' templates used for RACE amplification were synthesized using the SMART 5' RACE & 3' RACE Reagents Kit (TaKaRa, Tokyo, Japan). The extracted total RNA of the oysters (*C. hongkongensis*) was equipped with 5' and 3' end adapter sequences, following the instructions in the reagents kit, and then reverse transcribed into cDNA, which was used as the template for 5' and 3' end amplification. The 5' and 3' RACE amplification was performed using the Tks Gflex™ DNA Polymerase (TaKaRa, Tokyo, Japan), with one end of the primer designed as a 3'- and 5'-specific primer in the cloned intermediate fragment, and the other end as the UPM and UPS primer in the SMART 5' RACE & 3' RACE Reagents Kit (Table 1). The PCR reaction conditions were: 94 °C pre-denaturation for 1 min, 98 °C denaturation for 10 s, annealing and extension: the annealing temperature was set to 60 °C when the average Tm value of the forward and reverse primers was above 55 °C; the annealing temperature was set to 55 °C when the average Tm value of the forward and reverse primers was below 55 °C, and the extension time was set according to the length of the fragment; that is, 1 min/kb. The product was detected by applying 1% agarose gel electrophoresis, connected to the PMD19 clone vector, and transformed into T1 *Escherichia coli* cells. After overnight cultivation and PCR detection, the bacterial suspension was sent to Shanghai Shenggong Biotechnology Co., Ltd. for sequencing.

### Gene sequence and evolutionary analysis

Utilizing ORF Finder online tools (https://www.ncbi.nlm.nih.gov), we predicted the open reading frame (ORF) and amino acid sequences based on the full-length cDNA sequence. We predicted the molecular weight, isoelectric point, hydrophobicity, signal peptide, and structural domain of proteins using online software, namely Expasy (https://web.expasy.org), SignalP (https://services.healthtech.dtu.dk/services/SignalP-5.0/), and NCBI. After using the BLAST tool in the NCBI database for sequence alignment and searching for homologous protein sequences, we performed multiple comparisons of homologous sequences using DNAMAN 7.0 software. The Neighbor-Joining method in MEGA 5.1 software was used to build the evolutionary tree, with the Bootstrap value set to 10,000.

### Analysis of taurine and betaine content

Taurine and betaine contents were analyzed using taurine and betaine enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Meilian Biotechnology Co., Ltd., Shanghai, China). Blank, standard, and sample wells were included in the analysis. We added 50 μL of standard solution into each standard well and then introduced sample diluent (40 μL) and sample solution (10 μL), followed by incubation at 37 ℃ for 30 min and five washes. Next, we added 50 μL of HRP enzyme-linked immunosorbent assay reagent to each well and 100 μL of acidic chromogenic agent for color development for 10 min at 37 ℃ in the dark. Absorbances were measured at 450 nm using an ELISA reader (Molecular Devices, Spectra Max Id5, USA), and the nutrient contents were calculated using a standard curve. Data were expressed as mean ± standard deviation (SD). SPSS 22.0 software was

**Table 1** Primers used for full-length cDNA cloning and quantitative PCR

| Primers | Primer sequences (5′-3′) | Usages |
|---|---|---|
| 15 CSAD-F1 | ATGTGAGGGGTTCAAAGGTCAAAAAG | Fragment cloning |
| 1433 CSAD-R1 | TCATCATTCCCTCCTTGACCTTTGGC | Fragment cloning |
| 430 CSAD-F2 | GGTTTATGAAGGCTCGTGACCGCAA | Fragment cloning |
| 1643 CSAD-R2 | TCATCATTCCCTCCTTGACCTTTGGC | Fragment cloning |
| 193 CDO-F1 | TTTGAGGAAGGAGCTGGGCAATGGT | Fragment cloning |
| 785 CDO-R1 | ACATGAGATTCCAAGCAGGTCCATAA | Fragment cloning |
| 397 CDO-F2 | CATACACAACCACCCGAACTCACAC | Fragment cloning |
| 699 CDO-R2 | ACCACTGAAGTCCTGATCGAATCCCA | Fragment cloning |
| 193 CDO-5-Inner Primer | ACCATTGCCCAGCTCCTTCCTCAAA | 5′ Race |
| 356 CDO-5-Outer | CGTTCCAGCACAGAATCATCAGGTT | 5′ Race |
| 627 CDO-3-Inner | ATCTCTACTCGCCACCATTCACCAC | 3′ Race |
| 577 CDO-3-Outer | CGTGCACAGAGTTGGAAACAGAAGT | 3′ Race |
| 433 CSAD-5-Inner | TCATTGCGGTCACGAGCCTTCATAA | 5′ Race |
| 685 CSAD-5-Outer | AGGGAGAATACAGGGGACACTTCGT | 5′ Race |
| 1455 CSAD-3-Inner | GGCTTCGAGAGAGACATTGACAATC | 3′ Race |
| 1391 CSAD-3-Outer | TCAGTGTGGCCGCAAGAATGACGTT | 3′ Race |
| M13F | TGTAAAACGACGGCCAGT | Universal primer |
| M13R | CAGGAAACAGCTATGACC | Universal primer |
| UPM | CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT | RACE |
| UPS | CTAATACGACTCACTATAGGGC | RACE |
| 400 qPCR-CDO-F1 | GGAGCTCTACAGGAAATCGGA | RT-qPCR |
| 573qPCR-CDO-R1 | TGGTGAATGGTGGCGAGTAGA | RT-qPCR |
| 137qPCR-CSAD-F | AAGCCCACAGAGAAGCAGAGAA | RT-qPCR |
| 345qPCR-CSAD-R | CATTGCGGTCACGAGCCTTCAT | RT-qPCR |
| CDH-qRT-F | CCATCAGGCCACAACACTCTCTT | RT-qPCR |
| CDH-qRT-R | ACGCCGATCTTCTTCATCAC | RT-qPCR |
| BADH-qRT-F | CAGTCCTTGTATCCTCGTCAACT | RT-qPCR |
| BADH-qRT-R | TATTCCTCCTGCCAGTCCTAGTT | RT-qPCR |
| BHMT-qRT-F | TGTATTGGCTTTCACCTATTGC | RT-qPCR |
| BHMT-qRT-R | CGACGGCCCACTCTATCATTT | RT-qPCR |
| LActinF | CTGTGCTACGTTGCCCTGGACTT | RT-qPCR |
| LActinR | TGGGCACCTGAATCGCTCGTT | RT-qPCR |

used to test the normality and variance homogeneity of nutritional data collected in different time periods and different tissues. One-way ANOVA, with Tukey's post-hoc analysis, was used for multiple comparisons of data satisfying normality and homogeneity of variance.

#### Gene expression analysis in different tissues and at different times

Total RNA was extracted from gills collected in different months and various tissue samples from April using the TRIzol method. A 5,000-ng sample of total RNA was reverse transcribed into cDNA using the TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen Biotech Co., Ltd., Beijing, China); the cDNA was diluted to 100 ng/μL for qPCR template. We used PerfectStart® Green qPCR SuperMix (TransGen Biotech Co., Ltd., Beijing, China, SYBR Green as the fluorescent dye) to detect the mRNA expression levels of the key genes. The RT-qPCR reaction conditions were as follows: predenaturation at 95 °C for 30 s; denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, 40 cycles. β-Actin served as a reference gene. The primers used for qRT-PCR are listed in Table 1. RT-qPCR was performed using a fluorescence quantitative PCR instrument (Bio-Rad, CFX96, USA). Each experimental group included three biological replicates and three technical replicates. The relative expression level of the target gene was calculated using the $2^{-\triangle\triangle CT}$ method, with results presented as the mean ± standard deviation (SD). SPSS 22.0 software was used to test the normality and variance homogeneity

of gene expression data in different time periods and different tissues. For the data meeting the normality and homogeneity of variance, one-way ANOVA, with Tukey's post-hoc analysis, was used for multiple comparisons. For data that did not meet the homogeneity of variance index (*ChBHMT and ChCSAD* tissue expression; expression of *ChCDO* and *ChCSAD* in different months), logarithmic conversion was performed so that the data met the homogeneity of variance, and one-way ANOVA was then performed.

### Development of SNPs

Using the full-length cDNA of *ChCSAD*, *ChCDO*, *ChBADH*, *ChCDH*, and *ChBHMT* genes as templates, Oligo 7 Primer Analysis Software was used to design primers for amplifying the ORF region of genes (Table S1). Ten oysters with significant differences in taurine or betaine contents were selected as samples from which to clone the same gene fragment, and the PCR products were sent to Shanghai Shenggong Biotechnology Co., Ltd., for sequencing. We then used sequence comparison in DNAMAN 7.0 software to predict candidate SNPs, followed by fragment length discrepant allele-specific PCR (FLDAS-PCR) and polyacrylamide gel electrophoresis for SNP validation. First, we designed two upstream primers of different lengths, each with a 3' end that paired with two SNP allele bases, using Oligo 7. Simultaneously, we introduced mismatches at the 3rd or 4th base of the 3' end of the two allele-specific primers to increase specificity. We then added base sequences of different lengths at the 5' end; downstream from this end was a universal primer sequence. The lengths of the amplified DNA fragments ranged from 100 to 200 bp. The amplified product was detected via 12% polyacrylamide gel electrophoresis.

### Population genotyping

FLDAS-PCR and polyacrylamide gel electrophoresis were used for genotyping when the SNP density was greater than 1/20 bp. Owing to the different lengths of the upstream primers, the homozygotes were shown as a single band, and the heterozygotes as two bands with a size difference of 8 bp.

Gene fragment resequencing was used for genotyping when SNP density was less than 1/20 bp. First, the target fragments of all samples were cloned, and the PCR products were recovered, purified, and sent to Shanghai Shenggong Biotechnology Co., Ltd., for sequencing. We then compared the sequencing peaks of the same target fragment from different samples and genotyped the candidate SNPs in the population.

### Association analysis

Single factor ANOVA and independent sample t-test in SPSS 20.0 software were used to analyze the association between genotype data and taurine or betaine content traits. All data were presented as the mean ± SD, and $P < 0.05$ was considered to indicate a significant difference.

The false discover rate (FDR) was obtained by correcting for the difference in significance ($p$-values). The Benjamini–Hochberg method was used to perform multiple hypothesis testing correction for hypothesis testing probability ($P$-value) [29]. Selected sites with $P_{FDR} < 0.05$ were considered to be SNPs related to betaine or taurine content.

### Haploid and linkage analysis

We used Haploview 4.2 software to perform haplotype and linkage analyses for the significant SNPs (as determined by the association analysis described above) and ANOVA in SPSS 22.0 to analyze and compare the taurine and betaine content between individuals with different haplotypes.

## Results

### Characteristics of full-length cDNA of *ChCSAD* and *ChCDO*

The total length of *ChCSAD* cDNA was 2140 bp (NCBI login number: OP792983), including a 55-bp 5' untranslated region (UTR), 417-bp 3' UTR, and 668-bp ORF, encoding a total of 555 amino acids. The relative molecular weight of the protein was 63.45 kDa, and its isoelectric point was 7.60. The *ChCSAD* protein was predicted to contain a conserved pyridoxal-deC domain (113–479) (Fig. S1). The total length of the *ChCDO* cDNA was 1,402 bp (NCBI login number: OP792984), including a 196-bp 5' UTR, 705-bp 3' UTR, and 501-bp ORF, together encoding a total of 166 amino acids. The relative molecular weight of the protein was 19.04 kDa, with an isoelectric point of 6.41. The predicted conserved domain in the *ChCDO* protein was a CDOI domain (1–140) (Fig. S2).

### Homology and phylogenetic analysis of ChCSAD and ChCDO

Alignment of the amino acid sequences of ChGSAD and ChCDO in *C. hongkongensis* with those of *C. gigas*, *C. virginica*, and *C. angulata* revealed high amino acid conservation (Fig. 1). The phylogenetic tree showed that ChCSAD and ChCDO in *C. hongkongensis* were closely related to those in other invertebrates such as *C. gigas* and *C. angulata* (Fig. 2).

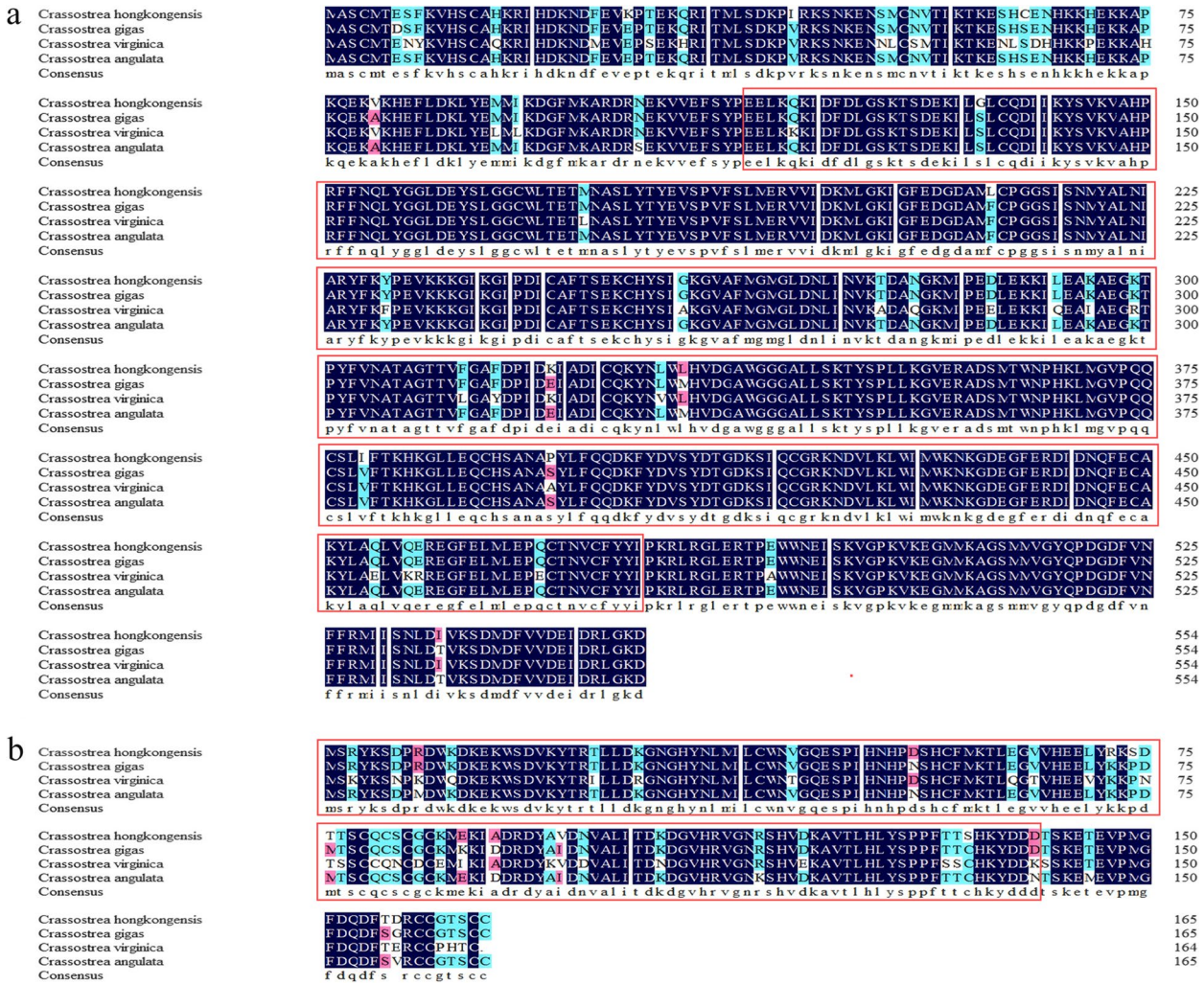


**Fig. 1** Multiple sequence alignments of genes expressing cysteine sulfite decarboxylase (*ChCSAD*) (**A**) and cysteine dioxygenase (*ChCDO*) (**B**). Colors indicate the degree of similarity of the domains: black, high similarity; blue: lower similarity; red, relatively high similarity; red boxes: structural domain. The GenBank accession numbers of ChCSAD amino acid sequence are as follows: *C. hongkongensis* (OP792983.1), *C. gigas* (XP 011418227.3), *C. virginica* (XP 022345949.1), *C. angulata* (XP 52718331.1). The GenBank accession numbers of ChCDO amino acid sequence are as follows: *C. hongkongensis* (OP792984.1), *C. gigas* (XP 011443656.3), *C. virginica* (XP 022309808.1), *C. angulata* (XP 052695037.1)

### Betaine and taurine contents and key gene expression in different tissues

The highest betaine content in *C. hongkongensis* was found in the digestive gland (827.51 μg/g), and this amount was significantly higher than that in other tissues ($P < 0.05$) (Fig. 3a). *ChCDH* and *ChBADH* were expressed at the highest levels in the adductor muscle ($P < 0.05$), whereas *ChBHMT* was highly expressed in the digestive gland ($P < 0.05$) (Fig. 3b–d). Taurine content was the highest in the digestive gland (970.81 μg/g), and this amount was significantly higher than that in other tissues ($P < 0.05$) (Fig. 4a). The expression of *ChCSAD* reached its highest level in the digestive gland ($P < 0.05$), whereas *ChCDO* was the highest in the adductor muscle and digestive gland ($P < 0.05$) (Fig. 4b–c).

### Betaine and taurine contents and key gene expression in different months

The salinity in the Sandun area showed significant seasonal variations (Figs. 5a and 6a). From January to April, the salinity of seawater was above 24 ‰, with the highest salinity of 25.3 ‰ in January. From June to August, the salinity of seawater droped below 18 ‰, with the lowest salinity in August of 12.4 ‰. From September to December, salinity began to rise, with salinity reaching 21.7 ‰ in December. Betaine content in *C. hongkongensis* steadily increased from 754.08 μg/g in February to a

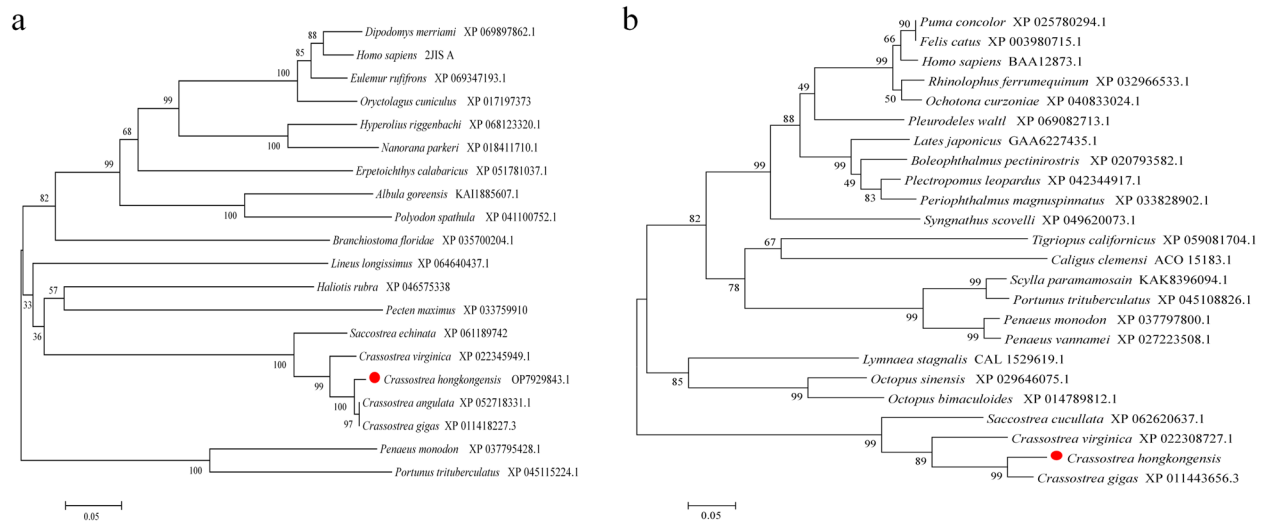


**Fig. 2** Phylogenetic analysis of *ChCSAD* (**A**) and *ChCDO* (**B**). The number next to the internal branch represents the value based on 1,0000 repeated calculations, and 0.05 represents the genetic distance

peak of 999.25 μg/g in April and then rapidly dropped to the lowest level of 743.01 μg/g in December (Fig. 5a). Expression of *ChCDH* was high from January to February, significantly decreased from February to March, and remained low from March to November. Expression of *ChBADH* was high from January to April, decreased significantly from April to June, and remained low from June to November. *ChBHMT* expression was significantly higher in January than in the other months ($P < 0.05$) and remained low after a significant decrease in February (Fig. 5b–d).

Taurine and betaine contents showed a similar trend, with taurine content increasing from February to April and reaching its highest value of 852.32 μg/g in April. From June to December, taurine showed a decreasing trend and dropped to its lowest value of 666.75 μg/g in November (Fig. 6a). *ChCSAD* and *ChCDO* expression levels gradually increased from January, reaching their highest levels in April and March, respectively, and then gradually decreased ($P < 0.05$) (Fig. 6b–c).

### Characteristics of betaine and taurine content in the association analysis population

The taurine content of the experimental individuals ranged from 779.46 to 957.30 μg/g, with an average of 862.95 ± 51.42 μg/g and a coefficient of variation (CV) of 5.96%. The betaine content of the experimental individuals ranged from 809.69 to 1,036.57 μg/g, with an average of 911.02 ± 67.18 μg/g and a CV of 7.37%. The variation in the content of taurine and betaine follows a normal distribution ($P > 0.05$) and is suitable for conducting association analysis.

### SNPs in the coding regions of key genes

Using gene fragment cloning, Sanger sequencing, and multi-sequence alignment, 107 candidate SNPs were predicted for *ChCSAD*, *ChCDO*, *ChBADH*, *ChCDH*, and *ChBHMT*. A total of 107 sets of primers were designed, and 47 SNP loci were validated, all of which were successfully genotyped in the experimental population (Fig. 7). The number of SNPs for each gene is shown in Table S2, and information on the 47 validated SNP loci is shown in Table S3.

### SNPs associated with betaine and taurine contents

Nine SNPs were significantly correlated with taurine content ($P < 0.05$) and one with betaine content ($P < 0.05$) (Table 2). Among these SNPs, three were found to cause coding amino acid changes. SNP *ChCDO*-46 changed lysine (Lys) to glutamate (Glu), *ChCDO*-394 changed threonine (Thr) to serine (Ser), and *ChCDH*-1585 changed asparagine (Asn) to aspartate (Asp) (Table 3).

### Haplotype and linkage analysis

Among the nine SNPs associated with taurine content, five (*ChCDO*-387, *ChCDO*-393, *ChCDO*-394, *ChCDO*-417, *ChCDO*-423) formed a linkage group and three haplotypes (Fig. 8): ACACA, GTTTG, and GTACA, with haplotype frequencies of 0.781, 0.033, and 0.176 respectively. Among them, the GTTTG haplotype had a significant advantage in taurine content (Table 4).

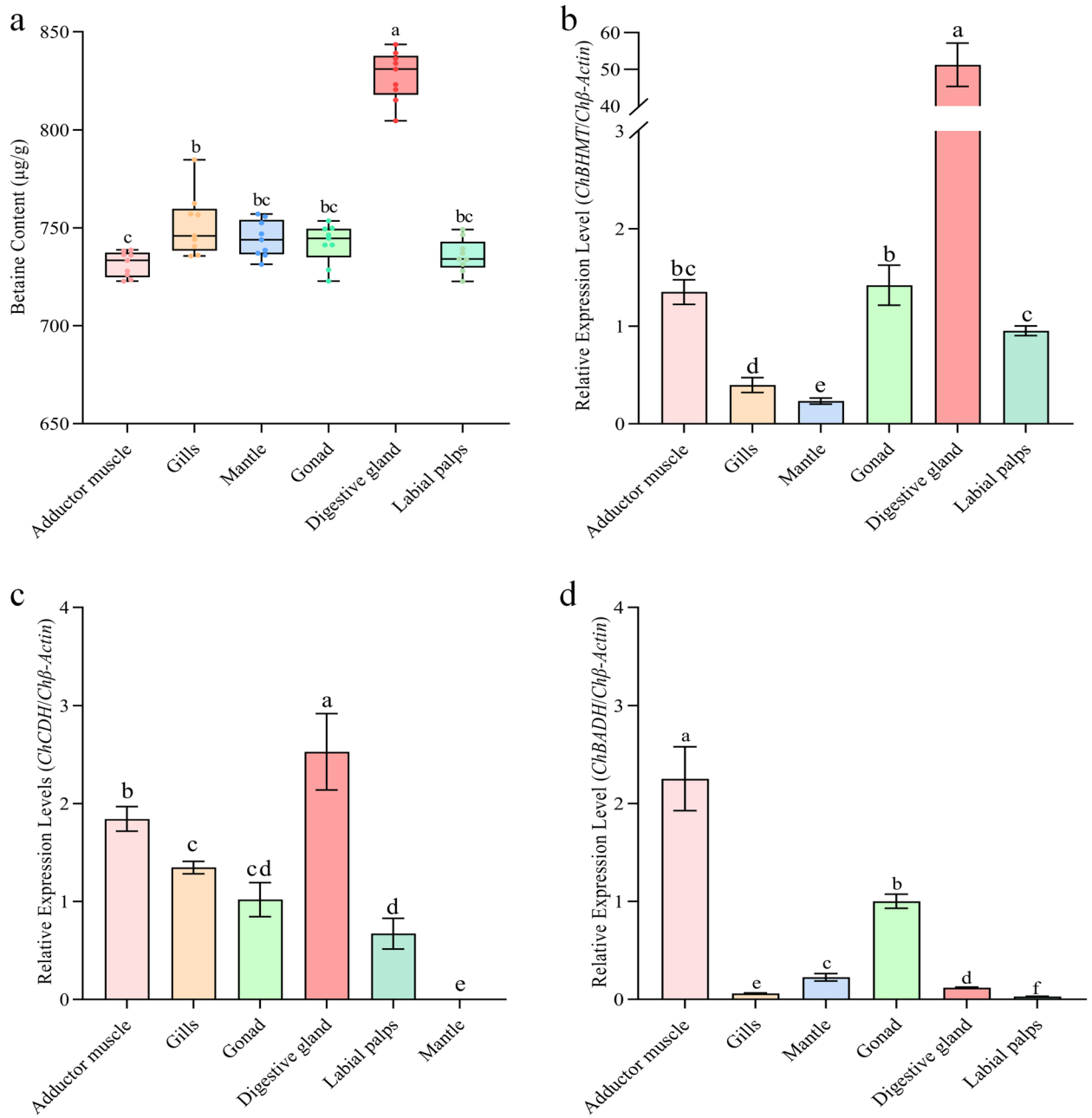


**Fig. 3** Betaine contents and gene expression in different tissues of Hong Kong oyster (*Crassostrea hongkongensis*). **a**, Betaine contents; **b**, *ChBHMT* expression; **c**, *ChCDH* expression; **d**, *ChBADH* expression. Different letters indicate significant differences ($P < 0.05$)

## Discussion

### Characteristics of betaine and taurine contents and key gene expression in *C. hongkongensis*

In our analysis of betaine and taurine content of *C. hongkongensis*, we found that betaine content was higher in the digestive glands than in the other tissues ($P < 0.05$). Given that the digestive gland is the food digestive and energy and fat storage organ, it is likely that the gland is also the main site of betaine synthesis in oysters [30]. *ChCDH* and *ChBHMT* are highly expressed in the digestive gland, whereas *ChBADH* is highly expressed in adductor muscles. The adductor muscle is an important component of oyster muscles, which can control the opening and closing of shells, assist the respiration and feeding of the animal, and play an important role in energy storage, metabolism, reproduction, and development [31,

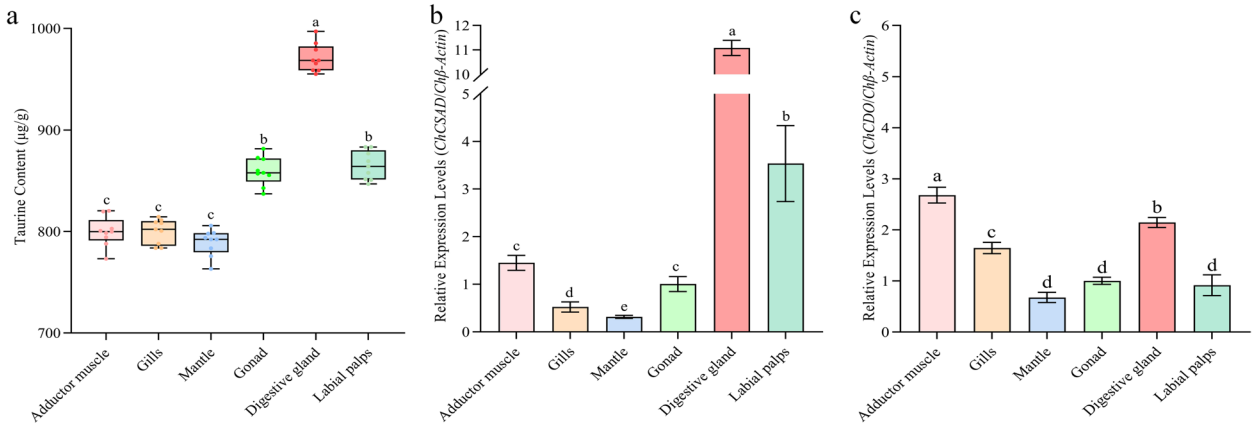


**Fig. 4** Taurine contents and gene expression in different tissues of *C. hongkongensis*. **a**, Taurine contents; **b**, *ChCSAD* expression; **c**, *ChCDO* expression. Different letters indicate significant differences ($P < 0.05$)

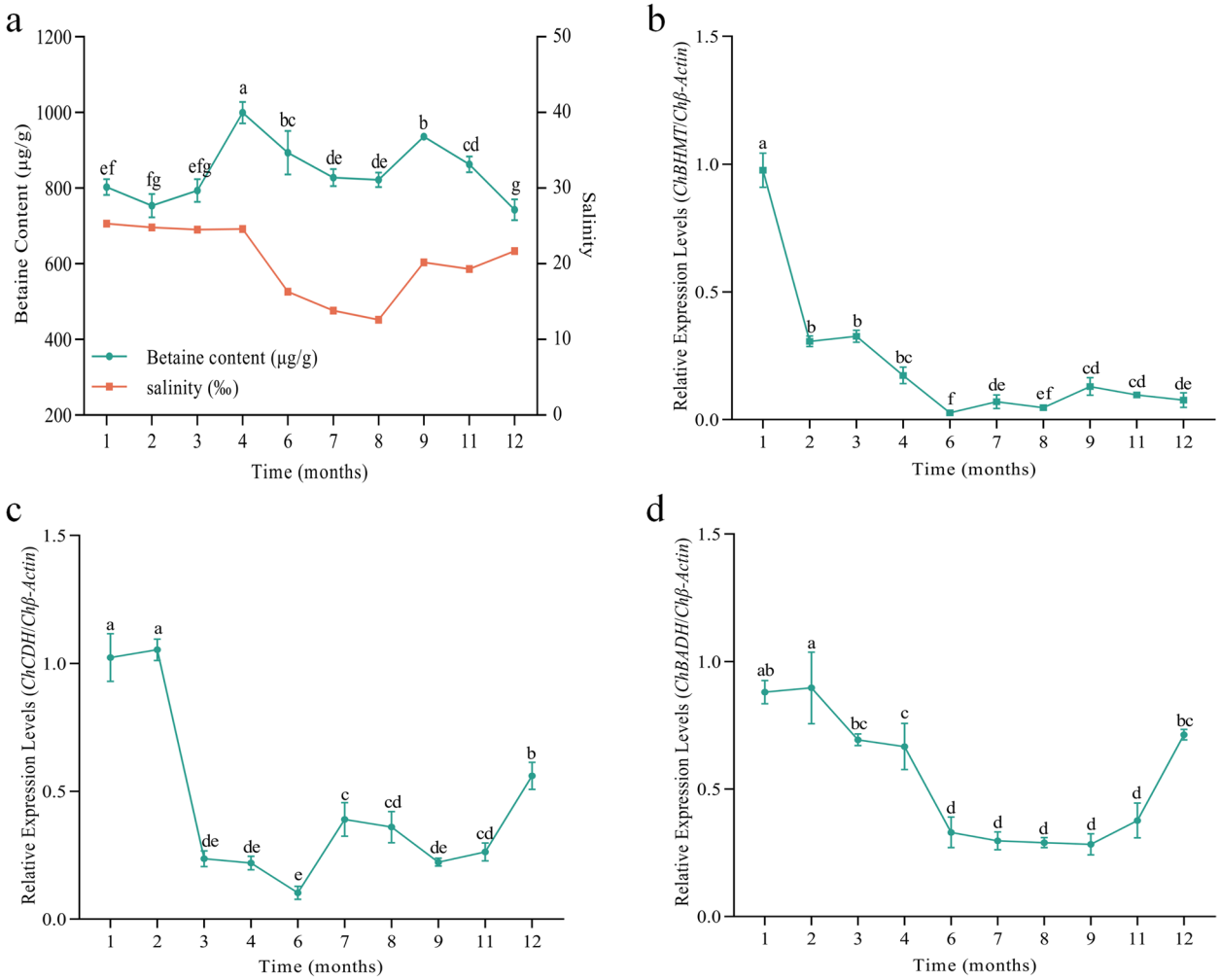


**Fig. 5** Betaine contents and gene expression in different months in *C. hongkongensis*. **a**, Betaine contents; **b**, *ChBHMT* expression; **c**, *ChCDH* expression; **d**, *ChBADH* expression. Different letters indicate significant differences ($P < 0.05$)

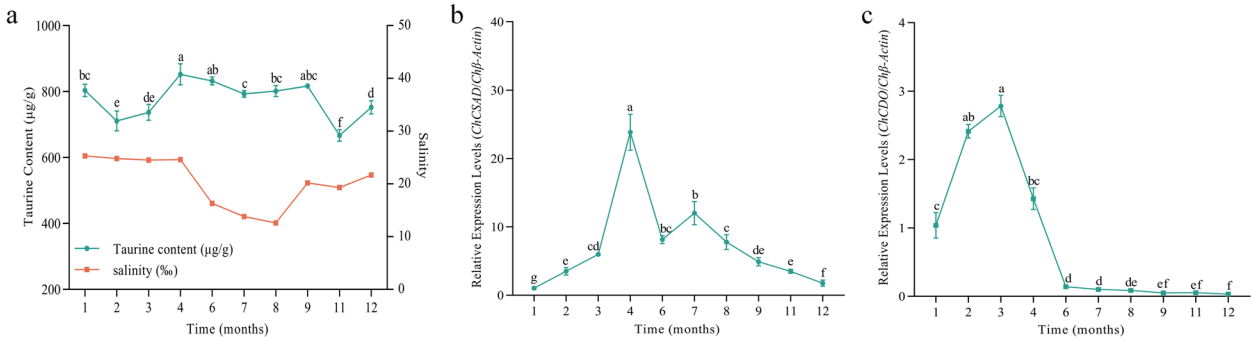


**Fig. 6** Taurine contents and gene expression in different months in *C. hongkongensis*. **a**, Taurine content; **b**, *ChCSAD* expression; **c**, *ChCDO* expression. Different letters indicate significant differences ($P < 0.05$)

32]. Catalysis of choline to betaine occurs in the adductor muscle of the clam [17], and we speculate that this also occurs in the adductor muscle of the oyster. The high expression of *ChBADH* in the adductor muscle indirectly proves that the adductor muscle has the function of synthesizing betaine. Our results indicate that there is comparatively little betaine content in the closed shell muscle, possibly because the adductor muscle is a very active muscle tissue. *ChBADH* is highly expressed in the closed shell muscle, thus protecting the structure and function of muscle cells; however, this does not necessarily imply more betaine is being synthesized. It is possible that *ChBADH* has a stable molecular structure and persists in oyster adductor muscle cells, which leads to its high expression but not necessarily more betaine synthesis [33]. As a filter-feeding animal, *C. hongkongensis* mainly feeds on planktonic algae and organic debris to meet its energy needs. The digestive gland is an important organ that participates in oyster lipid and carbohydrate metabolism [34]. The source of taurine in vertebrates is mainly divided into two aspects: through dietary intake of taurine and through its own synthesis [35]. The main pathway of taurine biosynthesis is cysteine sulfonic acid catalyzed by CDO; the decarboxylase is then catalyzed by CSAD to form taurine, which is further oxidized by taurine monooxygenase [36]. CDO and CSAD are key enzymes in taurine synthesis. In vertebrates, such as rats [37] and mice [38], the liver is the primary site of taurine synthesis. Cells in the liver are rich in enzymes needed to synthesize taurine, such as CDO and CSAD. The digestive gland is a multifunctional organ in invertebrates (such as shellfish and crustaceans) and is equivalent to the combination of liver and pancreas in vertebrates. The digestive gland is the main organ of taurine synthesis in shellfish and is responsible for various metabolic functions, including detoxification and osmotic pressure regulation [39]. Nagasaki et al. [40] found that the hepatopancreas is the main tissue for taurine synthesis in deep-sea mussels, and the precursor of taurine synthesis, cysteine sulfinic acid, is only found in the digestive gland. In the present study, the content of taurine was the highest in the digestive gland. We previously speculated that the digestive gland of oysters has similar functions to the liver of higher animals and is the main organ for the synthesis of taurine [41]. *ChCSAD* is expressed most highly in the digestive gland, suggesting that *ChCSAD* is a key gene involved in the synthesis of taurine in oysters. Chen et al. [42] found that CSAD was widely expressed in all tissues of razor clams, with the highest expression in the liver. *ChCDO* is highly expressed in the adductor muscle, possibly due to its indirect involvement in the regulation of taurine osmotic pressure in oysters, with the adductor muscle serving as the main site for taurine metabolism and accumulation during salinity adaptation. As early as 1966, Lynch [43] found that changes in salinity induced significant changes in the contents of taurine, alanine, glycine, and proline in the shell-closing muscle of American oysters.

### Factors affecting betaine and taurine content and gene expression in *C. hongkongensis*

Betaine acts as a buffer against osmotic pressure fluctuations, thus increasing the tolerance of biological cells to high salt and high osmotic environments [44–46]. Free amino acids (FAAs) play a major role in regulating intracellular osmotic pressure and cell volume [47]. Taurine, one of the main free amino acids in shellfish, plays an important role in maintaining osmotic pressure in oysters. When salinity decreases, oysters release large amounts of taurine as an adaptation to low-osmotic environments. Conversely, taurine accumulates under highly osmotic environments [48]. In our study, we found that the levels of betaine and taurine in *C. hongkongensis* reached their highest levels in April, possibly because of an increase in salinity caused by high temperatures and low rainfall in Guangxi in April. Taurine and betaine

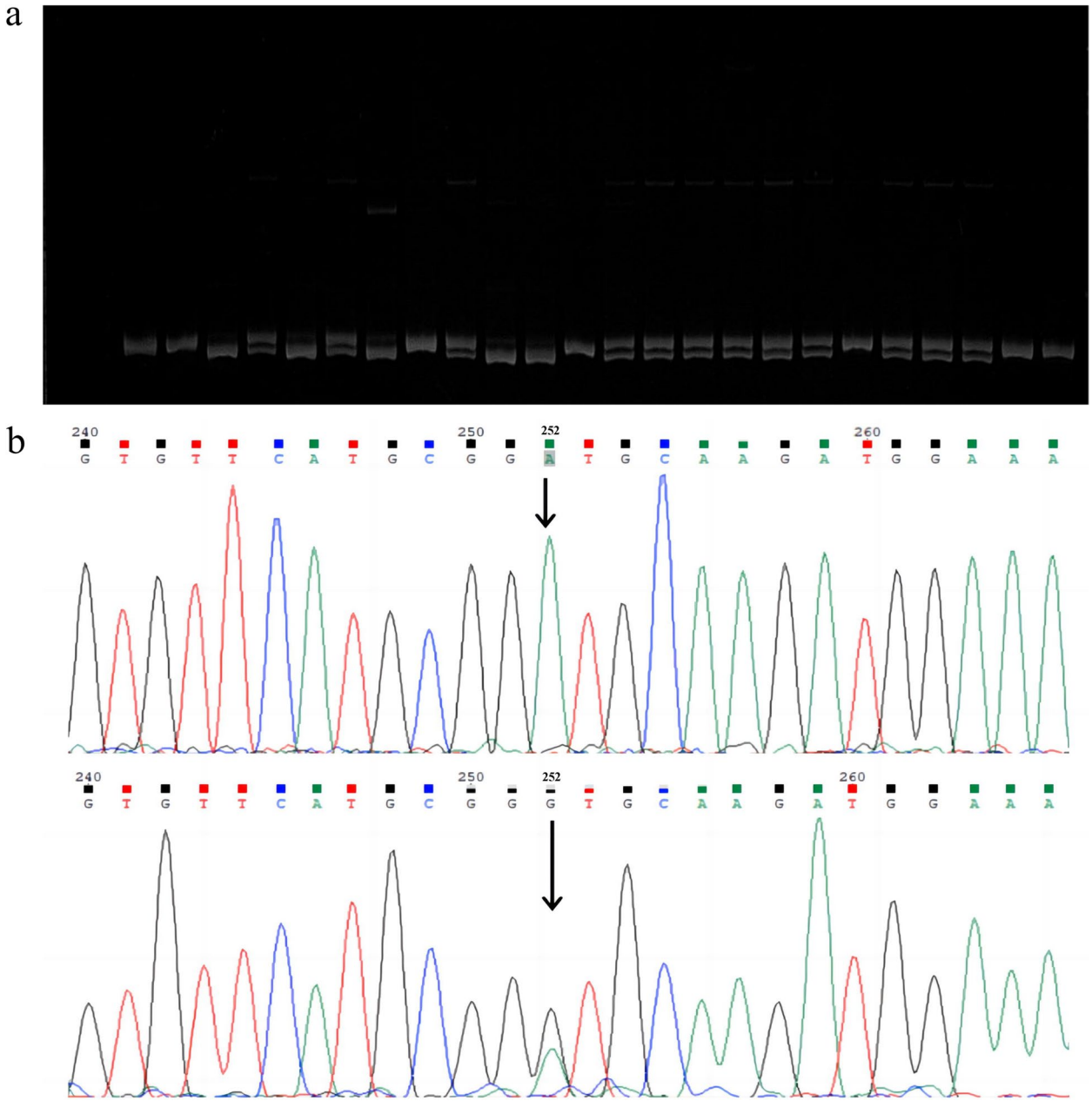


**Fig. 7** SNP genotyping results. **a**, SNP genotyping results based on FLDAS-PCR of the three different genotypes shown; two bands indicate heterozygous and one band indicates homozygous. **b**, SNP genotyping results based on gene fragment resequencing for nucleotide site 252 indicated by the arrow; the single peak in the figure above indicates homozygous, and the double peaks in the figure below indicate heterozygous

contents showed a downward trend from June to December, which may have been due to increased rainfall after May that continued until October, resulting in a decrease in seawater salinity. Lin et al. [49] also found that betaine content in oysters changes when exposed to changes in external salinity. Taurine also participates in osmotic regulation, thus reducing the damage to oysters caused by changes in environmental salinity. Huang et al. [50] found that the taurine content of oysters in high-salinity waters was higher than that in low-salinity waters. In the present study, we also found that the levels of betaine and taurine in oysters changed with changes in seawater salinity, further indicating that betaine and taurine may be involved in osmotic pressure regulation in oysters.

*ChBADH*, *ChCDH*, and *ChBHMT* were found to be the key enzymes involved in the synthesis and decomposition

**Table 2** SNPs associated with taurine and betaine contents

| Number | SNPs | Genotype | Content (μg/g) | *P*-value | $P_{FDR}$ | Associated trait |
|---|---|---|---|---|---|---|
| 1 | *ChCDO*-46 | AA | 873.16 | 0.004 | 0.033 | taurine |
| | | AG | 843.39 | | | |
| 2 | *ChCDO*-252 | AA | 872.15 | 0.010 | 0.033 | taurine |
| | | AG | 845.32 | | | |
| 3 | *ChCDO*-375 | CC | 873.45 | 0.027 | 0.036 | taurine |
| | | CT | 845.78 | | | |
| | | TT | 846.66 | | | |
| 4 | *ChCDO*-384 | GG | 872.37 | 0.025 | 0.036 | taurine |
| | | GT | 844.78 | | | |
| | | TT | 882.99 | | | |
| 5 | *ChCDO*-387 | AA | 872.76 | | | |
| | | AG | 846.31 | 0.038 | 0.038 | taurine |
| | | GG | 846.66 | | | |
| 6 | *ChCDO*-393 | CC | 872.76 | 0.038 | 0.038 | taurine |
| | | CT | 846.31 | | | |
| | | TT | 846.66 | | | |
| 7 | *ChCDO*-394 | AA | 872.34 | 0.029 | 0.036 | taurine |
| | | AT | 844.17 | | | |
| | | TT | 872.43 | | | |
| 8 | *ChCDO*-417 | CC | 872.34 | 0.009 | 0.033 | taurine |
| | | CT | 843.34 | | | |
| 9 | *ChCDO*-423 | AA | 872.34 | 0.017 | 0.034 | taurine |
| | | AG | 843.34 | | | |
| | | GG | 930.72 | | | |
| 10 | *ChCDH*-1585 | G/G | 905.48 | 0.017 | 0.034 | betaine |
| | | A/G | 838.26 | | | |

$P_{FDR}$ is the *P*-value corrected by FDR

of betaine in oysters. *ChBADH* and *ChCDH* levels were high from January to April and from January to February, respectively. Other researchers have proposed that when oysters are subjected to external salinity stress, the expression levels of *ChBADH* and *ChCDH* increase, and betaine content increases accordingly [51]. *ChCDH* only remained at a high level from January to February, possibly because of its role as a pre-catalytic enzyme in the betaine synthesis pathway, and because its expression time was earlier than that of *ChBADH* [26]. The expression level of *ChBHMT* was high in January, possibly because of the continuous increase in salinity in the Maowei Sea of Guangxi in that month. Under high salt stress, *ChBHMT* expression decreases in oysters to maintain the corresponding osmotic pressure. In recent years, BHMT has been reported to maintain the osmotic pressure balance in various aquatic animals, such as sea cucumber (*Apostichopus japonicus*) [52] and Atlantic salmon [53]. In the present study, we found that taurine content was the highest in April, and the expression levels of *ChCDO* and *ChCSAD* reached their highest levels in March and April, respectively. At this time, salinity is also at its highest level. This may be due to increased taurine synthesis or uptake by aquatic organisms when salinity is elevated. Taurine, as an important organic osmotic regulator, plays a key role in maintaining the balance of osmotic pressure inside and outside cells and protecting cells from the damage of high salt environment [54, 55]. Consequently, under high salt conditions, oysters need more taurine to maintain osmotic pressure balance inside and outside the cell. *ChCDO* and *ChCSAD* are the key enzymes of taurine synthesis [18]. High salt environment can significantly induce the expression of

**Table 3** Information on non-synonymous mutation SNPs

| SNPs | Nucleotide variant | Amino acid variant |
|---|---|---|
| *ChCDO-46* | A → G | Lys → Glu |
| *ChCDO-394* | A → T | Thr → Ser |
| *ChCDH-1585* | A → G | Asn → Asp |

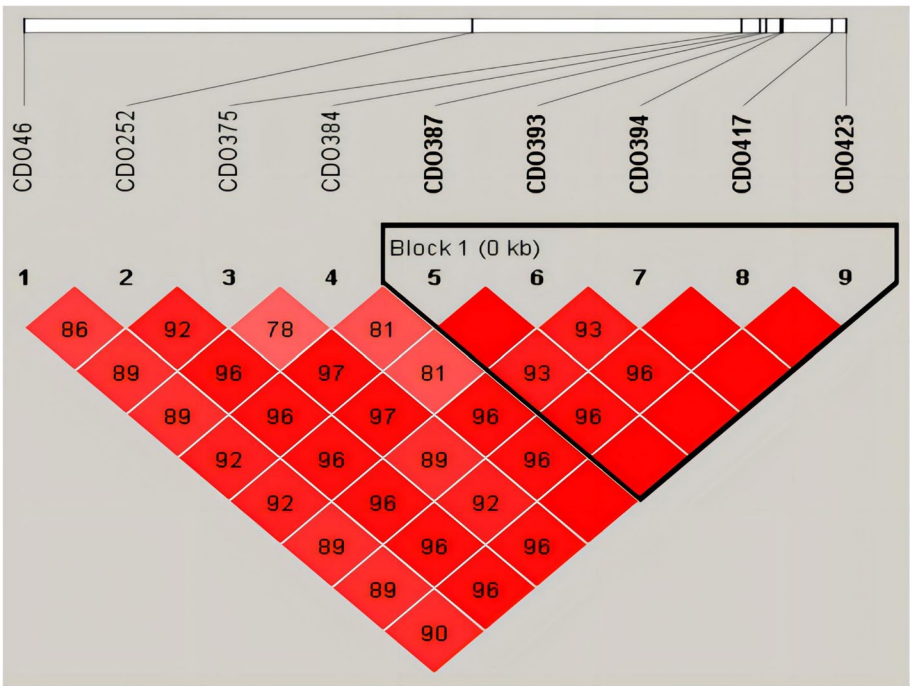


**Fig. 8** Linkage disequilibrium (LD) for the nine SNPs associated with taurine. SNP IDs are shown in the upper panels. D'>80 was the threshold of linkage disequilibrium. Darker colors indicate stronger linkage disequilibrium of SNPs

**Table 4** Results of the haplotype analysis

| Haplotype | CDO 387 | CDO 393 | CDO 394 | CDO 417 | CDO 423 | Frequency | Taurine content (μg/g) |
|---|---|---|---|---|---|---|---|
| H1 | A | C | A | C | A | 0.781 | 873.88 |
| H2 | G | T | T | T | G | 0.033 | 930.72 |
| H3 | G | T | A | C | A | 0.176 | 838.99 |

*ChCDO*, the key enzyme in the first step of taurine synthesis. When cysteine is oxidized to cysteine sulfinic acid through *ChCDO* under high salt stress, the increased level of ChCDO promotes taurine synthesis in the oyster. *ChCSAD* expression is also induced in a high-salt environment, given that ChCSAD is a second-step rate-limiting enzyme in taurine synthesis, helping convert cysteine sulfinic acid to taurine [56]. This indirectly suggests that *ChCDO* and *ChCSAD* are involved in the regulation of osmotic pressure in *C. hongkongensis.* The expression of *ChCDO* peaked before that of *ChCSAD*, possibly because the former is an upstream regulatory factor in the taurine synthesis pathway.

### Coding region SNPs and development efficiency

Approximately 90% of the mutations in DNA sequences are SNPs, which contribute to the rich genetic diversity of organisms [57]. Most SNPs are located in the non-coding regions of the genome and are of interest in terms of population genetics and evolutionary studies [58, 59]; in contrast, SNP loci in the coding region have high genetic stability [60]. In this study, 107 SNP loci were detected in five key gene-coding regions of *C. hongkongensis*, and 47 SNP markers were successfully developed using FLDAS-PCR and polyacrylamide gel technology. The success rate of SNP development was 43.9% (47/107). In comparison, the success rate of SNP development using high-resolution melting (HRM) technology for *C. gigas* glycogen phosphorylase was 33.3% (14/42) [61], reflecting the relatively high success rate of FLDAS-PCR technology in SNP development. However, FLDAS-PCR technology has low throughput and is not suitable for large-scale SNP detection and development.

### Correlations between SNPs and trait content

SNP sites with synonymous mutations do not alter the encoded amino acid sequence; however, synonymous mutations can affect phenotypes by altering mRNA secondary structure stability, translation efficiency, and protein folding [62]. Non-synonymous mutations are single-nucleotide mutations that cause changes in amino acid sequences that are likely to affect DNA transcription and subsequent translation processes, thereby affecting the structure and function of proteins; these are known as functional SNPs [63]. In this study, we identified nine SNP sites associated with taurine content in the *ChCDO* coding region of *C. hongkongensis*. *ChCDO*-46 and *ChCDO*-394 are non-synonymous mutations that cause amino acid changes from lysine to glutamate and from threonine to serine, respectively. Only one SNP, *ChCDH*-1585, was found to be associated with betaine content in the *ChCDH* coding region of *C. hongkongensis*. *ChCDH*-1585 is a non-synonymous mutation that causes an asparagine-to-aspartate change. Haploid-level analysis is considered more robust than single-marker allele-level analysis [64], and haplotype linkage disequilibrium and taurine association analyses can better illustrate the correlation between SNPs and taurine content. The results of our study showed that the five SNP loci that were significantly associated with taurine content formed a linkage group and resulted in three haplotypes, among which the GTTTG haplotype had a significant advantage in taurine content.

## Conclusions

We cloned the full-length cDNA of *ChCDO* and *ChCSAD*, key genes involved in taurine metabolism in *C. hongkongensis*, and found that betaine and taurine content and the expression of key genes were regulated by seawater salinity. Using Sanger sequencing and multiple sequence alignment, 107 candidate SNPs were predicted in the coding regions of the key genes involved in betaine and taurine metabolism in *C. hongkongensis*, including *ChCDH*, *ChBADH*, *ChBHMT*, *ChCDO*, and *ChCSAD*. Forty-seven SNPs were successfully validated and genotyped using FLDAS-PCR and gene fragment resequencing. Nine SNPs were associated with taurine content in the *ChCDO* coding region ($P < 0.05$), and one SNP was associated with betaine content in the *ChCDH* coding region ($P < 0.05$). The results of linkage disequilibrium analysis showed that the five SNPs in the coding region of *ChCDO* form a linkage group and result in three haplotypes, among which the haplotype GTTTG has a significant advantage. These loci and haplotypes can serve as potential molecular markers for the molecular breeding of *C. hongkongensis*.

#### Abbreviations

| | |
|---|---|
| ANOVA | Analysis of variance |
| BADH | Betaine aldehyde dehydrogenase |
| BHMT | Betaine homocysteine methyltransferase |
| CDH | Choline dehydrogenase |
| CDO | Cysteine dioxygenase |
| CSA | Cysteine sulfite |
| CSAD | Cysteine sulfinic acid decarboxylase |
| ELISA | Enzyme-linked immunosorbent assay |
| FLDAS-PCR | Fragment length discrepant allele specific PCR |
| HRM | High resolution melting |
| ORF | Open reading frame |
| UTR | Untranslated region |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11373-8.

Supplementary Material 1.

#### Authors' contributions

All authors contributed to the study conception and design. Z.S., Y.X., and P.Z. were responsible for experiment design, methodology, and resources. L.K. performed the investigation and gene expression analysis. Material preparation was performed by Z.C., Z.J., and Q.D. The draft of the manuscript was written by L.K., and all authors reviewed and approved the final manuscript.

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#### Data availability

All data generated during this study or analysis are included in the published articles, sequence data is available in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) under accession number OP792983.1 and OP792984.1.

### Declarations

#### Ethics approval and consent to participate

The oysters used in this study were marine-cultured animals, and all experiments were conducted according to the regulations established by the local and central governments. No specific permissions were required to collect oysters or conduct the experiments described.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare no competing interests.

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