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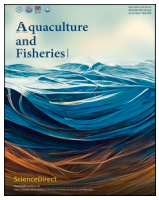

Original research article

Polymorphisms in *cathepsin b* is associated with growth in Asian seabass

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ABSTRACT

This study investigates the relationship between polymorphisms in the cathepsin B (*ctsb*) gene and body weight in Asian seabass (*Lates calcarifer*). Despite the economic significance of this species, the genetic factors that influence growth performance in fish remain poorly understood. CTSB, a lysosomal cysteine protease involved in protein degradation and tissue remodelling, is a crucial regulator of growth. We analysed the full-length cDNA of *ctsb* and mapped it to the major quantitative trait locus (QTL) for growth on linkage group 2 in *L. calcarifer*. Single nucleotide polymorphisms (SNPs) within ctsb were identified and their associations with body weight were analysed in 298 individuals. Results revealed that SNP1 (C > T) in intron 2 of *ctsb* was significantly correlated with body weight. Quantitative real-time PCR (qRT-PCR) showed that *ctsb* was expressed in all tissues, with the highest expression in the gills and kidney of developing seabass. Knockdown/overexpression of *ctsb* in cell lines respectively promoted/inhibited cell proliferation without altering cell size. These findings suggest that *ctsb* plays a significant role in growth regulation in Asian seabass. This study provides a potential molecular marker for selecting fast-growing Asian seabass. Future research should focus on identifying causal variants in ctsb associated with accelerated growth, conducting in vivo studies, and exploring related signalling pathways.

1. Introduction

The Asian seabass (*Lates calcarifer*), also known as barramundi, is a key species in aquaculture due to its economic significance and high nutritional value (Grey, 1987; Yue et al., 2024). Its broad distribution across the Indo-West Pacific region, rapid growth rate, desirable flesh quality, and adaptability to diverse environmental conditions, makes this species an ideal candidate for aquaculture development (Grey, 1987; Yue et al., 2009). Despite its commercial importance, challenges remain in maximizing its growth potential, particularly in relation to genetic factors influencing growth performance (Yue et al., 2024) (Table 1).

Genetic variation is key to improving economically important traits in aquaculture species (Ferez-Puche et al., 2024; Houston et al., 2020; Ulloa et al., 2024; Wong et al., 2024; Xia et al., 2014; Yue et al., 2024). DNA polymorphisms influence traits such as growth in aquatic organisms, including the Asian seabass (Wong et al., 2024; Xia et al., 2013). Understanding the genetic mechanisms governing growth traits is essential for effective selective breeding programs aimed at improving productivity and profitability in aquaculture (Yue et al., 2024).

Cathepsin B (*ctsb*) has emerged as a promising candidate gene due to its role in protein degradation, tissue remodelling, and growth regulation (Delbarre-Ladrat et al., 2004; Piorkowska et al., 2012) besides other know genes for growth, such as IGF1 and myostatin (Yang et al., 2025). While the *ctsb* gene has not been extensively studied in aquaculture species, its involvement in growth-related processes suggests that genetic variations within this gene may impact growth performance in Asian seabass. Polymorphisms in the *ctsb* gene have been reported in various species (Aggarwal and Sloane, 2014; Piorkowska et al., 2012; Salmerón et al., 2015; Yun et al., 2020) and are associated with diverse phenotypic traits, including growth (Ma et al., 2021; Mahurkar et al., 2006; Piorkowska et al., 2012; Russo et al., 2008). These variations can influence gene expression, protein structure, and enzymatic activity (Aggarwal and Sloane, 2014; Salmerón et al., 2015; Wang et al., 2023; Yun et al., 2020), thereby affecting the physiological processes underlying growth and development.

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Table 1
Body weight of Asian seabass at 90 dph with different genotypes at SNP1 and SNP2 in the *ctsb* gene.

SNP1 Genotype			
	CC	CT	TT
No. of fish	159	120	19
frequencies (%)	53 %	40 %	6 %
BW (g) (mean ± Se)	44.96 ± 3.78	59.35 ± 4.26	77.53 ± 12.83
P value		0.00340	
SNP2 Genotype			
	TT	TC	CC
No. of fish	103	165	30
frequencies (%)	35 %	55 %	10 %
BW (g) (mean ± Se)	48.43 ± 4.44	53.88 ± 3.88	62.16 ± 9.71
P value		0.364	

Despite the interest in the genetic basis of growth in Asian seabass (Xia et al., 2013), studies specifically investigating the relationship between polymorphisms in the *ctsb* gene and growth performance, as well as the role of *cstb* in growth are limited. Addressing this gap is crucial for increasing our understanding of growth regulation in Asian seabass and for developing DNA markers associated with growth for marker-assisted breeding to accelerate genetic improvement for growth.

This study investigates the association between polymorphisms in the *ctsb* gene and body weight in Asian seabass, as well as the potential role of *ctsb* in growth. Using genetic analysis and phenotypic evaluation, we aim to decipher the role of *ctsb* polymorphisms in growth performance and to identify potential molecular markers for selective breeding in Asian seabass aquaculture. Our findings may contribute to accelerating the genetic improvement of Asian seabass growth and deepen our understanding of the role of ***cstb*** in the growth of farmed fish.

2. Materials and methods

2.1. Animals and IACUC approval

Asian seabass from our breeding program (Yue et al., 2024) were utilized in this study. The research complied with Singapore's guidelines of the National Advisory Committee for Laboratory Animal Research (NACLAR). All protocols of using vertebrate animals were reviewed and approved by IACUC of our institute, under approval number TLL (F)-17-001. No human participants were involved in the study.

2.2. Characterising the ctsb gene in Asian seabass

The full-length Asian seabass *ctsb* cDNA sequence (XM_018666941.2) from our previous studies (Vij et al., 2016; Xia et al., 2011) was retrieved from GenBank. RNA was extracted from the muscle tissue of six randomly picked Asian seabass juveniles at the age of six months post hatch. The RNA was converted into cDNA using the). RNA was converted to cDNA using M-MLV reverse transcriptase (Promega Corporation, Madison, WI). The cDNA was used as a template to amplify the *ctsb* gene using PCR. Each 25 μL reaction contains 30 ng of cDNA, 5 × Q5 reaction buffer, each primer (1.25 μL of Lca-ctsb-ORF-F1 and Lca-ctsb-ORF-R1, 10 μM), dNTPs (0.5 μL, 10 nM), and 1.25 U of Q5 high-fidelity DNA polymerase (New England Biolabs, MA, USA). The PCR program started with an initial denaturation at 98 °C for 30 s, followed by 34 cycles of denaturation of DNA at 98 °C for 10 s, annealing of primers to temperate at 58 °C for 30 s, and DNA synthesis at 72 °C for 1 min, with a final extension at 72 °C for 2 min. The PCR product was sequenced using primers Lca-ctsb-ORF-F1 or Lca-ctsb-ORF-R1 on a sequencer (ABI3730xl, Applied Biosystems, CA, USA) as previously described (Fu et al., 2013).

2.3. Analysing the evolution of ctsb genes

Asian seabass *ctsb* protein sequence predicted from the above sequenced coding sequences and its homologs in six fish species, one bird and four mammals, were derived from GenBank database (). MEGA 11 (Tamura et al., 2021) was used to align the homologs and generate a neighbour-joining (NJ) phylogenetic tree with 1000 bootstrap replicates. The protein sequence Asian seabass *ctsb* and its homologs were aligned using MegAlign software (DNASTAR. Madison, WI).

2.4. Mapping the ctsb gene in the genome of Lates calcarifer

To locate *ctsb* within the genome of *Lates calcarifer*, the full-length *ctsb* cDNA sequence was aligned against the genome of Lates calcarifer (Vij et al., 2016) using BLAST. The genomic DNA sequences spanning the *ctsb* gene was downloaded. 3′ and 5' UTRs, exons and introns were detected by aligning the cDNA and gDNA with software Sequencher 5.4.6 (GeneCodes, MI, USA).

2.5. Detecting polymorphisms in ctsb and their association with body weight

To identify the most variable regions of *ctsb* in Asian seabass, genomic DNA sequences of *ctsb* of Asian seabass available in Genbank were downloaded and aligned with Sequencer 5.4.6 (Genecodes, CA, USA). Polymorphisms were detected in the two most variable genomic regions in the *ctsb* gene in the Asian seabass. Primer pairs (See Supplementary Table 1) Lca-*ctsb*-F3&R3 covering exon 1 to exon 4 and Lca-*ctsb*-F1&R1 spanning from exon 8 to part of 3' UTR (See Supplementary Table 1) were designed. DNA of eight Asian seabass from different geographic populations were used initially in PCR and sequencing to confirm the two SNPs (Yue et al., 2009). The 25 μL of PCR reaction contained 20 ng of DNA, 2.5 μL of 10 × reaction buffer, 0.2 μL of each primer (10 μM) (Lca-*ctsb*-F1&R1 or Lca-*ctsb*-F3&R3), 0.2 μL dNTPs (5 nM), and 2 units of Taq polymerase (Fermentas, USA). The PCR program comprised of an initial denaturation at 94 °C for 3 min; followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min 15 s; and a final extension at 72 °C for 5 min. The PCR products were analysed using 2 % gel electrophoresis. The *ctsb* PCR products were sequenced using the primers *Lca-ctsb-F1* and *Lca-ctsb-F3* and automated DNA sequencer (ABI3730xl) as described previous (Fu et al., 2013). DNA variants (SNPS) in the *ctsb* gene were detected by aligning the sequenced genotypes using Sequencher V4.9 (Genecodes, CA, USA).

The association between polymorphisms in *ctsb* and body weight were assessed using a total of 298 samples generated by crossing between 30 males and 30 females in an Asian seabass breeding program (Yue et al., 2024). The culture of brooders and offspring followed a standard protocol described in our previous papers (Wang, et al., 2006, 2008). All offspring were cultured in a 7-ton tank. Body weight of offspring was measured at 90 days post-hatch. Fin clips were collected from each and stored in 75 % ethanol for later DNA isolation. DNA was extracted from fin clips of each fish. SNP genotyping was performed through DNA amplification using primers Lca-ctsb-F3&R3 and Lca-ctsb-F1&R1, followed by Sanger sequencing as described (Wong et al., 2024) and analysis according to the protocol described above. Genotyping with 19 microsatellite markers indicated no evidence of population stratification in the offspring (Wong et al., 2025). Genotype and allele frequencies at SNP1 and SNP2 were calculated in Microsoft Excel. One-way ANOVA test was used to examine the significance of associations between the polymorphisms and the body weight of the Asian seabass, where $P < 0.05$ was regarded to be statistically significant.

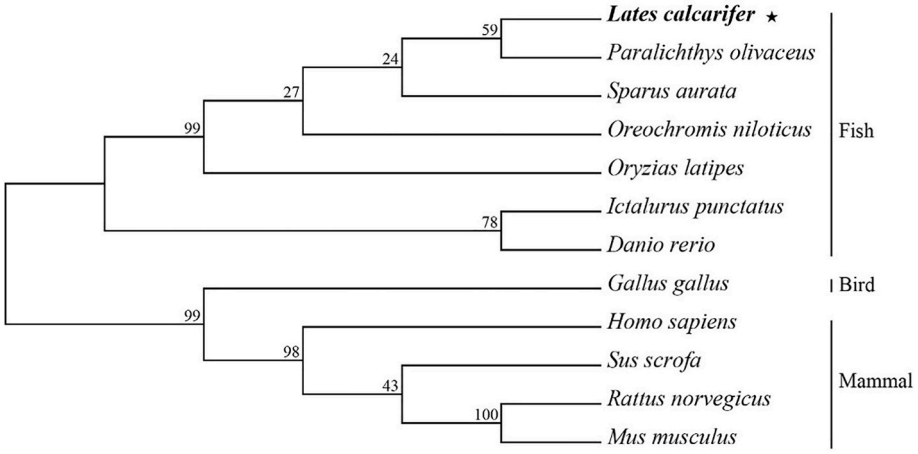

Fig. 1. Phylogenetic relationships among *ctsb* genes in different vertebrate species according to amino acid sequences. The species compared were *Lates calcarifer* (XP_018522457.1), *Paralichthys olivaceus* (XP_019935873.1), *Sparus aurata* (XP_030271106.1), *Oreochromis niloticus* (XP_003454569.1), *Oryzias latipes* (XP_020555295.2), *Ictalurus punctatus* (NP_001316238.1), *Danio rerio* (NP_998501.1), *Gallus gallus* (XP_046769249.1), *Homo sapiens* (NP_001899.1), *Sus scrofa* (XP_005657322.3), *Rattus norvegicus* (NP_072119.2) and *Mus musculus* (NP_031824.1).

2.6. Examining ctsb expression in tissues of developing Asian seabass

Ctsb expression was analysed using tissues from 11 organs (skin, eye, muscle, brain, liver, gills, fin, heart, gut, spleen and kidney) collected from three individuals at 28 days post hatch (dph) in a previous study (Yang et al., 2020). In addition, muscle tissues from six fast-growing (higher body weight) and six slow-growing (lower body weight) Asian seabass at the age of 180 dph from a previous study (Wang et al., 2015) were used to compare *ctsb* expression levels.

Total RNA was isolated from the samples using TRIzol reagent (Thermo Fisher Scientific, CA, USA) according to the manufacturer's protocol. Briefly, genomic DNA was removed by treating the RNA samples with DNase I recombinant Rnase-Free (Roche, Switzerland). cDNA was synthesised from RNA using M-MLV reverse transcriptase (Promega Corporation, Madison, WI) according to the manufacturer's protocol. Quantitative RT-PCR (qRT-PCR) was performed to analyse *ctsb* gene expression in the different Asian seabass organ samples using Lca-ctsb-RTF1 and Lca-ctsb-RTR1 primers (Supplementary Table 1) as previously described (Liu et al., 2016; Wong et al., 2024; Yang et al., 2020). All samples were run in triplicate. The *ef1a* gene was used as the internal control (Yang et al., 2020). The expressions of *ctsb* across 11 tissues were quantified using the $2^{\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Statistical significance was assessed using a two-tailed *t*-test.

2.7. Locating the CSTB protein in Asian seabass cells

The cell line (SB cell line) of *Lates calcarifer* used in our previous study (Wong et al., 2024), was cultured in Leibovitz's L-15 medium (Thermo Fisher Scientific, CA, USA) supplemented with 10 % fetal bovine serum and maintained at 28 °C, following previously described protocols (Liu et al., 2016; Wong et al., 2024; Yang et al., 2020).

Primers Lca-*ctsb*-GFP-*Eco*RI-F1 and Lca-*ctsb*-GFP-*Xma*I-R1 (Supplementary Table 1) were designed to amplify *ctsb* from the ORF to the added stop codon. To obtain the *ctsb* construct, cloning was done via restriction enzyme digestion using the amplified PCR product and pEGFP-C1. The restriction enzymes *Eco*RI and *Xma*I were used to cut the PCR product and pEGFP-C1 at their respective restriction sites. PCR products were inserted to the pEGFP-C1 vector. Sequencing was done for the generated *ctsb*-EGFP construct to validate the sequence.

To investigate the role of *ctsb* in vitro, SB cells were transfected with the empty pEGFP-C1 vector and the ctsb-EGFP construct, respectively, using Lipofectamine 3000 Reagent (Thermo Fisher Scientific, CA, USA) following the manufacturer's instructions. Briefly, SB cells were plated in 6-well plates and allowed to grow to 80 % confluence before transfection. A volume of 4 μL of Lipofectamine™ 3000 was mixed delicately with 125 μL of Opti-MEM™ Medium (Thermo Fisher Scientific, CA, USA). Separately, a solution containing 125 μL of Opti-MEM™ Medium, 0.5 μg of genomic DNA, and five μL of Lipofectamine™ 3000 Reagent, was prepared by gentle mixing. Following this, equal volumes of the DNA solution were introduced to the Lipofectamine™ 3000 preparation and left to stand at ambient temperature for a duration of 15 min. The resulting DNA-lipid complexes were subsequently applied to the SB cells. After 48 h, transfected SB cells were selected using 500 μg/mL G418 for five days. The isolated SB cells were placed into glass-bottomed 35 mm culture dishes (MatTek Corporation, MA, USA). Subsequently, the cells underwent staining with Hoechst 33342 (Sigma-Aldrich, MO, USA), and the resulting blue and green-fluorescent emissions were examined using the Zeiss Axio Observer 7 Microscope (Carl Zeiss, NY, USA).

2.8. Investigating the impact of knockdown and overexpression of ctsb on the growth of Asian seabass cells

RNA interference (RNAi) technology was employed to silence ctsb in SB cells to assess its impact on cell size. shRNA (short hairpin RNA) technique was utilized for the RNAi assay, using the pSilencer™ 4.1-CMV Neo Kit (Thermo Fisher Scientific, CA, USA). Three shRNAs (shRNA-*ctsb*-1, shRNA-*ctsb*-2, shRNA-*ctsb*-3) were designed with ThermoFisher's shRNA designer (https://rnaidesigner.thermofisher.com) for the knockdown of *ctsb*. Primers (shRNA-*ctsb*-1-F/R, shRNA-*ctsb*-2-F/R, shRNA-*ctsb*-3-F/R) with *Hin*dIII and *Bam*HI restriction sites flanking the respective shRNAs were designed and used with the pSilencer-4.1-CMV neo vector (ThermoFisher, CA, USA) to clone the three shRNAs. DNA were sequenced and cut with a restriction enzyme to check for the presence and integrity of the cloned vector.

To achieve overexpression of *ctsb*, the *ctsb* ORF was amplified from cDNA using primers ctsbOE-*Eco*RI-F and ctsbOE-Xbal-R (detailed in Supplementary Table 1). The resulting PCR product was then inserted into the pcDNA-3.1 vector, creating the ctsb-pcDNA construct. The successful insertion and correct sequence of the cloned vector were verified through sequencing and enzyme cutting. The pSilencer empty vector, pSilencer-shRNA1/2/3-ctsb, pcDNA-3.1 empty vector, and the ctsb-pcDNA-3.1 construct were transinfected into cells as described (Liu et al., 2016; Wong et al., 2024; Yang et al., 2020). After 48 h of transfection, the modified SB cells were maintained in complete growth medium supplemented with 10 % FBS and 500 μg/mL G418 for a period

of five days to isolate successfully transfected cells. To assess the efficacy of each transfection, qRT-PCR was performed using primers Lca-ctsb-RTF1 and Lca-ctsb-RTR1 (Supplementary Table 1), with the housekeeping gene ef1a serving as an internal control. The effects of ctsb expression on cell size were examined in the overexpressed and knockdown cell lines selected by G418. These cell lines were seeded in triplicate 6-well plates, and images were captured using the Zeiss Axio Observer 7 Microscope. Cell size was measured using ImageJ (Schneider, Rasband, & Eliceiri, 2012), and the perimeter and area of 10 randomly selected cells from each triplicate were recorded. Statistical significance of ctsb expression in relation to cell size was determined using a two-tailed *t*-test in Microsoft Excel. To determine the relationship of *ctsb* expression and cell proliferation, the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay (Gold Biotechnology, St. Louis, MO, USA) was conducted on the overexpressed and knockdown *ctsb* cell lines. Each cell line sample was prepared by plating 5000 cells in three replicate wells of a 12-well plate. Subsequently, each well received 10 μL of MTT solution at a concentration of 5 mg/mL, followed by incubation at 28 °C for 5 h. The culture medium was then aspirated and 100 μL of DMSO was introduced to each well, with a further incubation period of 15 min at 37 °C. Optical density measurements were taken at 570 nm wavelength at four-time intervals: 0, 24, 48, and 72 h. Statistical analysis of the relationship between ctsb and cellular proliferation was conducted using a two-tailed T-test in Microsoft Excel.

3. Results

3.1. The ctsb gene and its location on the genome of Asian seabass

The genomic size of the Asian seabass (*Lates calcarifer*) *ctsb* DNA was 7274 bp, with nine exons and 10 introns, while the cDNA sequence (GenBank accession number: XM_018666941.2) was 1920 bp with a 225 bp 5′ untranslated region (UTR), 993 bp coding sequence (CDS) and 702 bp 3' UTR. The encoded *ctsb* protein was 330 amino acids long, with an estimated molecular weight of 36.17 kDa. In silico mapping revealed that the gene *ctsb* was located on chromosome 2:26695089–26703361.

Multiple protein sequence alignment revealed that the amino acid identity of the Asian seabass CTSB was most similar to the gilt-head bream (*Sparus aurata*) with a score of 89.70 %, followed by 89.09 % with the Nile tilapia (*Oreochromis niloticus*), and 82.37 % with the zebrafish (*Danio rerio*). In contrast, the Asian seabass shares 71.08 % with the chicken (*Gallus gallus*), 70.39 % with humans (*Homo sapiens*) and only 68.79 % with rats (*Rattus norvegicus*) (Supplementary Fig. S1). The constructed NJ tree using CTSB amino acid sequences showed a split into two groups, with fish taxa (including the Asian seabass) in one group, while the mammal and bird taxa in the other (Fig. 1).

3.2. Polymorphisms in ctsb and their association with body weight

The first SNP (SNP1) was located in intron 2 of *cstb* at chromosome two (Chr 2: 26,696,183, C > T), while the second SNP (SNP2) was located in intron 8 of *ctsb* at chromosome two, position 26,697,550 (Chr 2: 26,697,550, T > C). For SNP1, among the 298 samples analysed, the genotype distribution was as follows: nineteen individuals with TT, 120 with CT, and 159 with CC. A statistically significant variation (P = 0.0034) in body weight was detected across these genotypes (CC: 44.96 ± 3.78 g; CT: 59.35 ± 4.26 g; TT: 77.53 ± 12.83 g) (Table 1). Notably, individuals with the TT genotype exhibited higher body weights than those with the CC genotype. Regarding SNP2, the genotypic counts out of the total samples were 103 for TT, 165 for CT, and 30 for CC. In contrast to SNP1, no significant association with body weight was found for SNP2 (P = 0.364) (Table 1).

3.3. Expression of the ctsb gene in tissues and CTSB protein location in cells

Expression of *ctsb* in 11 tissues (skin, eye, muscle, brain, liver, gills, fin, heart, gut, spleen and kidney) were investigated using qRT-PCR. *Ctsb* was expressed in all 11 tissues. Among the 11 tissues, the gills had the highest expression followed by the spleen. The liver showed the lowest expression followed by the muscle (Fig. 2). Analysis of *ctsb* expression in samples from the 180 days post-hatch (dph) F_2 population revealed that fast-growing Asian seabass exhibited significantly greater (P = 0.045) *ctsb* expression in muscle tissue than slow-growing individuals (Fig. 3).

SB control cells transfected with the pEGFP-C1 empty vector showed green fluorescence (GFP) in both the nucleus and cytoplasm. In contrast, cells transfected with the ctsb-EGFP construct exhibited primarily cytoplasmic localization of the GFP signal (Supplementary Fig. S2).

3.4. Impact of ctsb knockdown and overexpression on cell growth in a cell line

To investigate the impact of ctsb on cell size and proliferation, *ctsb* levels were reduced (knockdown) and increased (overexpression) in SB cells. Compared to cells transfected with the control p-silencer vector, a noteworthy decrease in ctsb expression (P = 0.0007) was observed in SB cells receiving the p-silencer-shRNA vector (Supplementary Fig. S3A). Despite this reduction in ctsb, no statistically significant differences in cell perimeter (P = 0.136) or area (P = 0.743) were detected between the two groups (Supplementary Fig. S4A and S4B). The MTT assay revealed no significant difference in cell proliferation between the groups at the initial time point (0 h, P = 0.998). However, at later time points (24, 48, and 72 h), cells with reduced ctsb expression (transfected with the p-silencer-shRNA vector) exhibited a significant elevation in proliferation compared to the control group (24 h: P = 0.036; 48 h: P = 0.013; 72 h: P = 0.013) (Fig. 4A).

The SB cells that were transfected with the *ctsb*-pcDNA-3.1 had an increase in *ctsb* expression as compared to those that were transfected with the empty pcDNA-3.1 vector (P = 0.0005) (Supplementary Fig. S3B). No significant difference in the cell perimeter (P = 0.067) and area (P = 0.813) were found between the two groups (Supplementary Fig. S4C and S4D). The MTT assay showed that there was no significant change in cell proliferation at 0 (P = 1) and 48 h (P = 0.272), but there was a significant decrease in the cell proliferation of the *ctsb*-pcDNA-3.1 group over the empty pcDNA-3.1 group at 24 (P = 0.031) and 72 h (P =

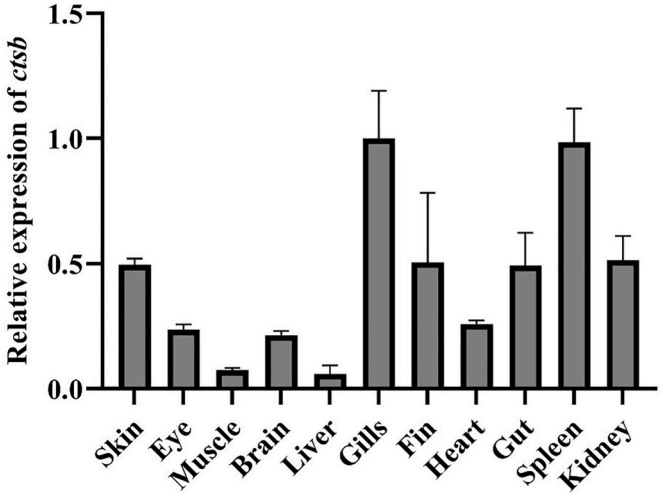

Fig. 2. Relative expression of *ctsb* in 11 tissues in developing juvenile Asian seabass (28 dph). The gene expression of *ctsb* of all organs was normalised to the expression of *ctsb* in gills. Gills have the highest *ctsb* expression while the liver has the lowest.

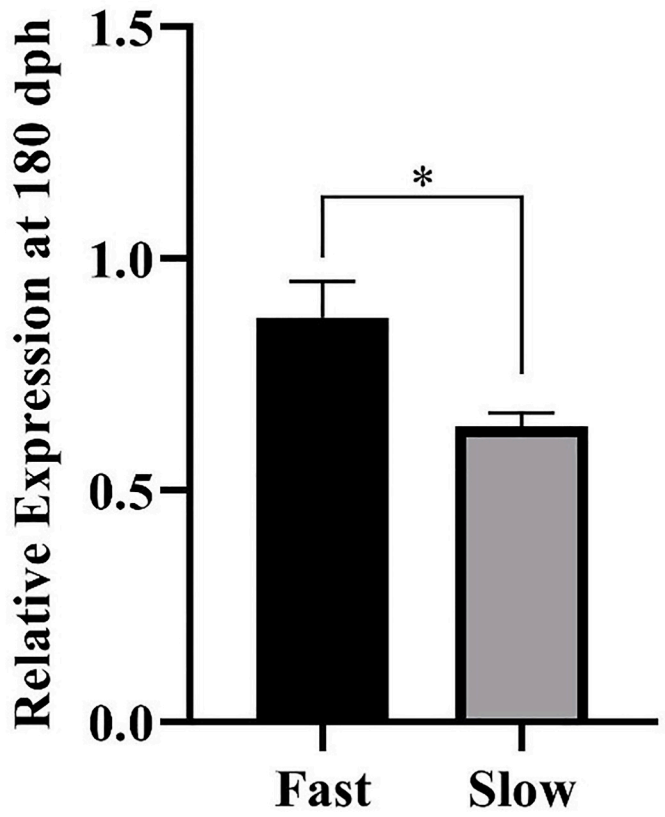

Fig. 3. Differential relative expression of *ctsb* in the muscle tissue of fast-growing and slow-growing Asian seabass (180 dph). The fast-growing Asian seabass had an increased *ctsb* expression compared to their slow-growing counterpart. Statistical significance: "*" represents $P < 0.05$.

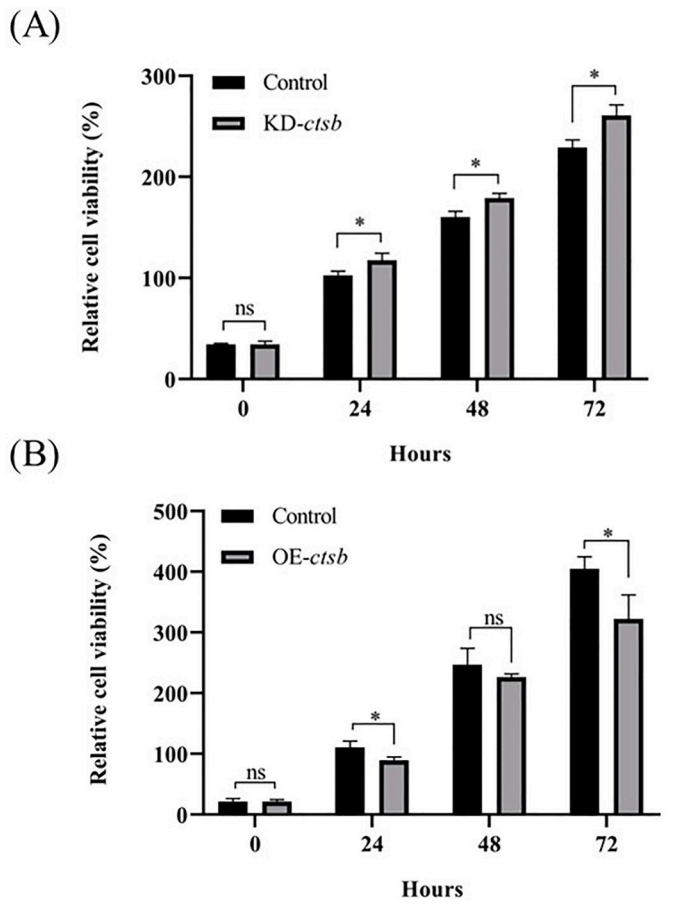

Fig. 4. Effects of knockdown (A) and overexpression (B) on cell growth (MTT assay) at 0, 24, 48 and 72 h. (A) An increased number of cells was observed with the knockdown of *ctsb* as compared to the control at 24, 48 and 72 h. (B) A decreased number of cells was observed with the overexpression of *ctsb* compared to the control at 24 and 72 h. Statistical significance: "ns" represents no significance and "*" represents $P < 0.05$.

0.033) (Fig. 4B).

4. Discussion

4.1. The ctsb gene and its genomic location in Asian seabass

In this study, the full-length cDNA of the *ctsb* gene from Asian seabass cells and fingerlings was successfully characterised. The full-length cDNA was 1920 bp in length, containing an open reading frame (ORF) of 993 bp, along with nine exons and 10 introns. In comparison, the *ctsb* gene consists of 13 exons and 12 introns in humans (*Homo sapiens*) (Ensembl, 2023b), 12 exons and 11 introns in mice (Ensembl, 2023b), and 11 exons and 10 introns in zebrafish (*Danio rerio*) (Ensembl, 2023a). The variability in the genomic structure of *ctsb* across species may arise due to species-specific adaptations, evolutionary divergence, or ecological pressures (Alkan et al., 2011). Understanding these variations provides insights into the complex functions and interactions of genes across different organisms (Mérot et al., 2020) though further studies are required to fully understand the role of these structural differences in *ctsb* genes.

The amino acid sequence of the Asian seabass *ctsb* gene shows the highest similarity to that of gilt-head seabream (*Sparus aurata*) at 89.70 %, followed by Nile tilapia (*Oreochromis niloticus*) at 89.09 %, humans at 70.39 %, and chickens (*Gallus gallus*) at 71.08 %. Phylogenetic analysis demonstrates the clustering of *ctsb* genes into distinct groups, with fish species forming one cluster, while mammals and birds form another. This observation is consistent with previous research on the evolutionary relationships of *ctsb* across species (Yun et al., 2020). Significant differences in amino acid sequences between species often reflect evolutionary divergence, variations in protein function, and adaptations to different environments and selective pressures (Zuckerkandl & Pauling, 1965). These differences are crucial for understanding the unique biological traits and evolutionary history of each species.

In Asian seabass, *ctsb* expression was detected in all examined tissues, with the highest levels in the gills and spleen, and the lowest in the liver and muscle. This pattern of expression is similar to that observed in olive flounder (*Paralichthys olivaceus*), where the highest *ctsb* expression was in the gills and intestine, and the lowest in the liver and muscle (Ahn et al., 2013). In contrast, the highest expression was found in the stomach, followed by the liver and head kidney (Shen et al., 2021) in the golden pompano (*Trachinotus ovatus*). These species-specific expression patterns suggest that *ctsb* may play distinct physiological roles that are potentially driven by unique regulatory mechanisms adapted to the environmental and physiological needs of each species.

The intracellular localization of proteins is crucial for their function, regulation, and interaction with other cellular components (Lundberg & Borner, 2019). In this study, the transfection of a *ctsb*-EGFP construct into Asian seabass cells revealed that the protein localized in spherical aggregates within the cytoplasm. This finding aligns with previous observations in golden pompano (Shen et al., 2021). Functionally, *ctsb* acts primarily as an exopeptidase, although it also exhibits endopeptidase activity and plays a central role in protein degradation within the lysosome (Aggarwal, et al., 2014).

In silico mapping located the *ctsb* gene within a major quantitative trait locus (QTL) for body weight on chromosome 2 (Shen et al., 2016; Wang, et al., 2006, 2008, 2011, 2015). This suggests that *ctsb* may either directly influence growth traits or be closely linked to other genes

associated with growth. Therefore, *ctsb* represents a promising target for future research aimed at understanding growth and other economically important traits in Asian seabass.

4.2. Associations of SNPs in ctsb with growth

Association mapping is a robust method for identifying genes linked to economically significant traits in both livestock and aquaculture species (Weir, 2008; Yue, 2014). In our study, individuals with the TT genotype at SNP1 were significantly heavier compared to those with the CC genotype. This finding is consistent with previous research in pigs, where polymorphisms in the *ctsb* gene have been associated with growth traits (Piorkowska et al., 2012; Russo et al., 2008). These data suggest that the *ctsb* gene may play an important role in influencing growth in Asian seabass, and SNP1 could serve as a valuable genetic marker to enhance the selection of individuals for improved growth performance.

4.3. Potential mechanism underlying the association between ctsb and growth

Cathepsin B is a lysosomal cysteine protease that plays an important role in protein degradation, tissue remodelling, and a variety of cellular processes (Delbarre-Ladrat et al., 2004; Piorkowska et al., 2012). In this study on Asian seabass, *ctsb* expression in muscle tissue was significantly higher in fast-growing individuals than slow-growing ones, suggesting its involvement in muscle development and growth. This observation is consistent with previous studies that link *ctsb* to muscle wasting and reductions in fish meat firmness (Delbarre-Ladrat et al., 2004; Salmerón et al., 2015). Studies on Atlantic halibut and rainbow trout provide additional supporting evidence which reveal increased *ctsb* expression in the skeletal muscle of fasted fish compared to refed individuals (Hagen et al., 2009; Rescan et al., 2007). Moreover, during gilthead sea bream development, muscle growth is regulated by a coordinated mechanism involving *ctsb*, cathepsin D (*ctsb*), and genes from the ubiquitin-proteasome family (Salmerón et al., 2015). The upregulation of *ctsb* in fast-growing fish may reflect an increased demand for cellular remodelling and protein synthesis to support rapid muscle mass accumulation.

Functional studies involving *ctsb* knockdown and overexpression in an Asian seabass cell line revealed that *ctsb* expression does not directly influence cell size, but its knockdown led to increased cell proliferation, while overexpression resulted in reduced proliferation. This aligns with the known role of *ctsb* in regulating cell cycle arrest and apoptosis (Wang et al., 2023). These results, however, contrast with the observation of increased *ctsb* expression in fast-growing seabass, indicating a complex regulatory mechanism and suggesting that the relationship between *ctsb* expression and muscle growth is not linear. The discrepancy between in vivo and in vitro results is not unusual in molecular and cellular biology (Liu et al., 2020; Tiffany-Castiglioni et al., 1999), especially when dealing with complex traits like growth (Glazier et al., 2002). Several factors could explain these differences. (1) *In vivo*, growth is a multi-faceted process involving not just cell proliferation but also tissue development, metabolism, and interactions between various cell types, organs, and systems (Chal & Pourquié, 2017). Cathepsin B may influence growth through systemic effects that are not captured in a single cell culture system. *In vitro*, the cellular environment is artificial and lacks the full complexity of a living organism, such as hormonal regulation, nutrient supply, and tissue-specific signalling pathways (Ravi et al., 2015). Therefore, the effects of gene knockdown or overexpression on growth in cultured cells might be different from those observed in an entire organism where many other factors are involved. (2). In the whole organism, *ctsb* expression might be tightly regulated by growth factors, hormones, and nutritional status, meaning its effects on growth may be more subtle and involve a network of feedback loops. In cultured cells, on the other hand, *ctsb* expression could alter basic cellular processes such as apoptosis, autophagy, or protein turnover in a more isolated manner, leading to different results. The changes in cell size and number observed in vitro may not directly reflect how these processes function in vivo, where tissue-level integration occurs. Anyway, our results suggest that *ctsb* is involved in regulating growth in Asian seabass indirectly. The species-specific effects of ctsb polymorphisms in Asian seabass may stem from unique regulatory or expression patterns of the gene in this species. CTSB is known to play roles in both immune response (Shen et al., 2021) and protein metabolism processes (Qiu et al., 2025) that are closely linked to growth. Variations in ctsb may influence the efficiency of protein turnover or immune energy allocation, thereby indirectly affecting growth performance in Asian seabass under aquaculture conditions. Moving forward, it is essential to study its related signalling pathways, such as pathways for oxidative stress (Bai et al., 2018), inflammation (Okuneva et al., 2016), and cellular homeostasis (Jiang et al., 2022), and identify causal variants in the gene to resolve these contradictions and better understand the gene's role in growth to enhance growth in Asian seabass.

5. Conclusion

The *ctsb* gene is involved in muscle growth, and the SNP1 may serve as an important DNA marker for growth, in combination with other growth-associated DNA markers, for selecting fast-growing Asian seabass at earlier development stages (e.g. fingerling stage). Further investigation into the precise regulatory mechanisms controlling *ctsb* expression in fish, as well as identification of causal variants in *ctsb* associated with faster growth, could provide novel insights for optimising muscle growth in aquaculture.

CRediT authorship contribution statement

Shadame Yeo: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Joey Wong:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Y.X. Tay:** Validation, Investigation, Data curation. **Zituo Yang:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Le Wang:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Fei Sun:** Writing – review & editing, Writing – original draft, Funding acquisition, Data curation. **May Lee:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Yanfei Wen:** Writing – review & editing, Writing – original draft, Investigation. **Gen Hua Yue:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Resources, Project administration, Funding acquisition, Conceptualization.

Ethics statement

The research complied with Singapore's guidelines of the National Advisory Committee for Laboratory Animal Research (NACLAR). All protocols of using vertebrate animals were reviewed and approved by IACUC of our institute, under approval number TLL (F)-17-001. No human participants were involved in the study.

Declaration of generative AI in scientific writing

The authors declare that the manuscript was written without using the help of generative AI.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aaf.2025.05.003.

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