MICROSATELLITES POLYMORPHISM ASSOCIATED WITH HEPCIDIN/HAMP GENES POTENTIAL FOR SELECTIVE BREEDING OF DISEASE-RESISTANT BY *Streptococcus agalactiae* IN NILE TILAPIA IN VIETNAM

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ABSTRACT

Streptococcosis is considered an infectious disease causing significant economic loss in tilapia aquaculture in the world. Fish hepcidin is known as a hepatic antimicrobial peptide (HAMP), and is associated with innate immunity, which defends against various bacterial infections and viruses. Sixty Phu Ninh tilapia after the challenge test with *Streptococcus agalactia* at a dose of $2{,}2x10^6$ cfu/ml were sampled to analyse microsatellite/SSRs polymorphism in the hepcidin/HAMP genes and the resistance to *S. agalactia*. This study examined the relationship between seventeen microsatellite/SSRs polymorphism in the hepcidin/HAMP genes and the resistance to *S. agalactia* in the Phu Ninh tilapia strain. The results indicated that 13/17 hepcidin/HAMP-related SSRs were polymorphic markers based on the number of alleles, effective alleles, observed heterozygosity, expected heterozygosity, polymorphism information content, and Hardy-Weinberg equilibrium. In this study, there was a significant difference in genotype and allele frequency between two groups (alive fish and dead fish after challenging with *S. agalactiae*) for four SSRs (SSR3, SSR5, SSR14, and SSR16) ($p < 0.05$). These four SSRs are utilized as marker-assisted selection (MAS) in the breeding of tilapia for *S. agalactiae* resistance.

Keywords: *Disease-resistance, hepcidin/HAMP gene, Microsatellite, Phu Ninh tilapia strain, Streptococcus agalactiae*

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1. INTRODUCTION

Tilapia is among the world's most important aquaculture finfish species. Recently, streptococcosis is a bacterial disease caused by *Streptococcus* spp in tilapia culture. This disease has been recognized as one of the most serious bacterial diseases and caused high mortality. It is reported that the antimicrobial peptide hepcidin/HAMP genes were resistant to both gram-negative, gram-positive bacteria, and viruses. Chen *et al.* (2022) [1] identified 18 hepcidin genes in Nile tilapia, of which 12 hepcidin/HAMP genes were in the linkage group 11 (LG11), 6 hepcidin genes in two unplaced contig 825 and contig 1099. 12 hepcidin/HAMP genes in the LG11 encoded 4 HAMPs (eight HAMP1 genes, one HAMP2 gene, one HAMP3 gene, and two HAMP4 genes. Two hepcidin-like antimicrobial peptides (TH1-5 and TH2-3) were obtained in Mozambique tilapia. TH1-5 and TH2-3 were reported to defend against some bacteria. The translated regions of these two peptides contained 88, and 91 amino acids, respectively [2], [3]. The TH1-5 peptide was reported as an antimicrobial peptide against both

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gram-negative and gram-positive bacteria. Transgenic zebrafish with TH1-5 peptide resisted against *Vibrio vulnificus* (gram-negative bacteria) and *S. agalactiae* (gram-positive bacteria) [3]. Gong *et al.* (2016) [4] revealed that three hepatic antimicrobial peptides (HAMP1, HAMP2, HAMP3) were strongly expressed in the liverand the spleen of Nile tilapia in responding to *S. inae* infection.

Microsatellites, also named simple sequence repeats (SSRs), consist of short tandem repeat units of 1-6 base pairs (bp) in length [5]. SSRs are often highly polymorphic, abundant distribution throughout genome, codominant inheritance, and small size. Microsatellites have become a useful tool in population genetic analysis, genetic mapping, and marker-assisted selection. Microsatellites can be used to assess genetic diversity and develop molecular-breeding tools in fish. Microsatellites associated with disease resistance have been reported in several important aquatic species. In Japanese flounder (*Paralichthys olivaceus*), 50 SSRs were used to study lymphocytic disease resistance (LD-R). In which only the Poli9-8TUF locus was reported to be associated with LD-R [6]. In rock bream (*Oplegnathus fasciatus*), three potential microsatellite markers (CA3-05, CA3-06, and CA3-36) were suggested to be used to facilitate the selection of rock bream iridovirus-resistant (RBIV-resistant) [7].

This study aims to determine the relationship between microsatellite/SSRs polymorphisms and *S. agalactiae* disease-resistance in Phu Ninh tilapia strain.

2. MATERIALS AND METHODS

2.1. Materials

The 2nd selection generation on growth performance (Phu Ninh strain) of Nile tilapia (*Oreochromis niloticus*), from the Tilapia Research Centre (TILACEN), Research Institute for Aquaculture No.1 (RIA.1) was used in this study. This tilapia strain was offspring from two full-sib families and one half-sib family produced from November to December 2019. Tilapia (with size 25-30g/fish) was challenged with *Streptococcus agalactiae* 015-RIA1 (GenID: OK047709.1) in LD_{50} dose (2,2 $x10^6$ cfu/ml) after 21 days. The pectoral fin samples of Nile tilapia were collected from the dead and survival groups (30 samples/group) after the challenge. The fin samples were preserved in 96% ethanol and stored at 4°C for long-term storage.

2.2. DNA extraction

The genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's recommendations. The total DNA quantity and quality were measured using spectrophotometer NanoDropĂ 2000 (Thermo Scientific, USA) and analyzed by electrophoresis on a 0,8% agarose gel.

2.3. Microsatellite amplification and genotyping

Seventeen microsatellite-specific primers in hepcidin/HAMP genes reported by Chen *et al.* (2022) [1] were used in this study (shown in Table 1). These microsatellite-specific primers were designed by an online tool (Websat, http://wsmartins.net/websat/accessed on 7 May 2015). The primer length was 22 bp, Tm was 60°C, GC was 60%, and the product length was between 100 and 400 bp. All SSRs were located in intron or 5′- and 3′- flacking regions within/linked to HAMP genes in LG11 (as table 1).

The primer sets (Multiplex) of 17 SSRs were designed by Multiplex Manager software based on the annealing temperature of each primer pair [8]. The primer dimer detection was checked by an online tool (Multiple Primer Analyzer, Thermo Scientific, UK: https://www.thermofisher.com/vn/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html).

A total of four multiplex PCR sets were created, including three multiplex PCR with tetraplex SSRs (multiplex I, III, and III) and one multiplex PCR with pentaplex SSRs (multiplex IV) as shown in table 1.

Table 1. The information on seventeen microsatellite/SSRs used in this study

SSRs	Gen/ID	Primer sequences (5'-3')	Motif	Size (bp)	Dyes
Multiplex I:					
SSR4	HAMP2 3'end 4.3kb (ID: 100698871)	F: CACCACTGTCAACTGGCTAATG R:GTTACCTTCTTGATACCGCAGG	$(CTAC)_7$	359	FAM
SSR5	HAMP2 3'end 4.7kb (ID: 100698871)	F: CTTTGGGTAGAGGAACACTCCA R:TGCAGGTCAATAGCAATACCAC	$(TG)_{21}$	309	HEX
SSR9	HAMP1 5'end 1.3kb (ID: 109204256)	F: GCGCTGTATAAGATTCCCGTTA R:GGAAACACAAGAGACATGAGCA	$(T)_{20}$	370	TAM
SSR14	HAMP1 reverse strand 3'end 6.2kb (ID: 100534415)	F: TCGGAATTGAGCATTAAGACCT R:GGTTCCCCATAGAACTCCTTTT	$(TG)_{22}$	245	ROX
Multiplex II:					
SSR6	HAMP1 5'end 2kb (ID: 109204280)	F: CAGTGGGTGTTTGTTCCTTACA R:TAGTAGGCTTTGTGTGCATTCC	$(GT)_{41}$	315	HEX
SSR8	HAMP1 5'end 1.3kb (ID: 109204256)	F: CCCCATAGCACTCCTTTTATTG R:TCATTTGGAGGTGTTTTCACAG	$(TG)_{22}$	322	ROX
SSR10	HAMP1 3'end 7.6kb (ID: 109204256)	F: GTTTGTAGCTTAACCCATTCGC R:TGCCTTTGTTTAGATGAACTGC	$(TG)_{10}$	364	FAM
SSR17	HAMP1 3'end 4.2kb (ID: 109204285)	F: GGTTCCCCATAGAACTCCTTTT R:TCGGAATTGAGCATTAAGACCT	$(TG)_{15}$	261	TAM
Multiplex III:					
SSR1	HAMP2 5'end 3.8kb (ID: 100698871)	F: CCACACAATCAACACACTGGTA R:TCAAACAGAAACAGGGACACAC	$(TA)_{11}$	375	HEX
SSR2	HAMP2 5'end 3.8kb (ID: 100698871)	F: GCACAGACACAGTAACACATGC R: ACTCCCTGGTACATGCTTCCTA	$(CAGG)_6$	368	FAM
SSR13	HAMP1 reverse strand 5'end 6.3kb (ID: 109204255)	F: TTAAAATGGGCTCAGGAGAAAG R:ACACACATAGATTCATCCGCAC	$(ATTC)_7$	332	ROX
SSR16	HAMP1 reverse strand intron2 (ID: 100534415)	F: GTGGGAAACACAAGAGACATGA R:CAGGGCTGAGATAGATTTTGGT	$(T)_{23}$	232	TAM

Multiplex IV:					
SSR3	HAMP2 5'end 3.8kb (ID: 100698871)	F: TAGGAAGCATGTACCAGGGAGT R: AAAATCACTCAACCGTGTCCTT	$(GT)_{13}$	348	FAM
SSR7	HAMP1 3'end 4.7kb (ID: 109204280)	F: CCACACGCACTCTCACCA R:CTGGTAGCCTTGACCCAGTTT	$(AC)_{12}$	236	HEX
SSR11	HAMP4 5'end 1kb (ID: 109204092)	F: AAGTGTCGTTCCACCCACAT R:ACAGAGTGTTCTGGCTTTCACA	$(TG)_{29}$	382	TAM
SSR12	HAMP1 reverse strand 5'end 2kb (ID: 109204255)	F: GTGCATTACAGAGTGTTCTGGC R: GTCTGGAGCCAAGTGTCGTT	$(TG)_{27}$	395	HEX
SSR15	HAMP1 reverse strand 3'end 750bp (ID: 100534415)	F: CAACAAGTGAAGCGACCATATT R:TCACACACAAGCAGGTCAATTA	$AC)_{13}$	300	ROX

The PCR was carried out in a 25 µl total reaction using 2× My Taq™ HS Mix (Meridian Bioscience, Germany), 0.32 µM of each primer, 100 ng/µl of gDNA, and ddH_2O. PCR amplification was performed on the Mastercycler proS (Eppendorf, Germany) under the following conditions: Initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 45 s, 72°C for 1 min, and a final extension of 72°C for 2 min. The PCR products were separated by electrophoresis on a 1.5% agarose gel with SafeViewTM Classic (ABM, Canada).

For the genotyping, the PCR products were analyzed fragments on the ABI (FirstBase, Malaysia). After the analysis, GeneMarker software V.2.2.0 was used to analyze allele sizes.

2.4. Determination of microsatellite/SSR polymorphisms

Evaluation of polymorphisms of SSRs is the first step to identifying potential SSRs associated with resistance to *S. agalactiae*. The polymorphism of the SSRs marker was evaluated on 60 individuals of the Phu Ninh strain. The evaluation was based on the following parameters:

- The polymorphism information content (PIC) was calculated following instruction on the Website (https://www.gene-calc.pl/pic). The PIC value of each SSR was determined automatically based on the number of alleles and their distribution frequency.

- Population genetic parameters (number of alleles-N at each microsatellite site, observed heterozygosity-Ho, expected heterozygosity-He and Hardy-Weinberg Equilibrium Assessment-HWE) were analyzed by using the software GenAlex 6.5-Genetic Analysis in Excel [9].

2.5. The identification of potential microsatellite/ SSRs associated with disease resistance

To determine potential SSRs associated with disease resistance by *S. agalactiae*, IBM SPSS Statistics version 20.0 software (SPSS Inc., Chicago, IL, USA) was used. The Chi-square tests ($p<0.05$) were performed on genotype and allele frequencies between two groups of live-dead after challenging test (30 samples/group of fish) for each polymorphic SSR

3. RESULTS AND DISCUSSION

3.1. PCR optimization

PCR production of each microsatellite/SSRs and Multiplex PCR is shown in Figures 1 and 2.

Figure 1. Gel electrophoresis of PCR product of seventeen SSRs

Lane 1 to15 are PCR products of SSRs; lane 16: Negative samples; Ladder 100bp

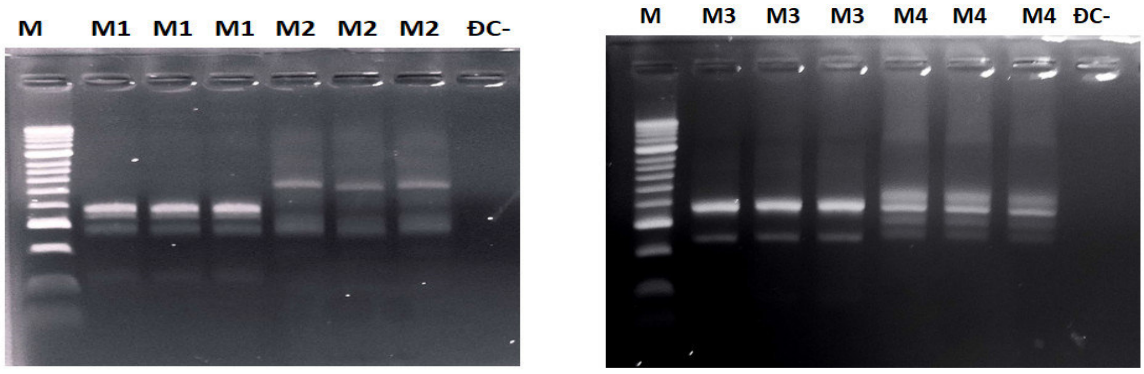

Figure 2. Gel electrophoresis of multiplex PCR product of 17 SSRs

Lane M1, M2, M3, M4 are Multiplex-PCR products of SSRs; lane ĐC: Negative samples; M: Ladder 100 bp.

The PCR was optimized with a clear and unbroken band no by-products, size corresponding to each SSR marker (Figure 1). The initial single-primer PCR optimization results showed fragments sized in the range of 200-500 bp. This result was consistent with previous studies [10], [11]. Additionally, four multiplex PCR sets of seventeen microsatellites were successfully optimized as shown in figure 2.

3.2. The polymorphisms of microsatellite/SSRs

The polymorphism of the seventeen microsatellite markers is shown in table 2.

Table 2. Polymorphism of the seventeen microsatellite markers

SSRs/Microsatellite	N	Na	Ne	Ho	He	PIC	HWE test
SSR 1	60	3	2.67	0.733	0.625	0.555	***
SSR 2	60	2	1.96	0.850	0.489	0.369	***
SSR 3	60	4	3.07	0.767	0.675	0.624	**
SSR 4	60	3	2.69	0.750	0.628	0.557	***
SSR 5	60	4	2.63	1.000	0.620	0.545	***
SSR 6	60	5	4.44	1.000	0.775	0.738	***
SSR 7	60	4	2.11	0.400	0.526	0.481	***
SSR 8	60	2	2.00	1.000	0.500	0.375	***
SSR 9	60	4	2.85	0.800	0.649	0.582	***
SSR 10	60	5	4.19	0.867	0.761	0.721	***
SSR 11	60	4	2.62	0.683	0.618	0.563	***
.SSR 12	60	3	2.57	1.000	0.611	0.533	***
SSR 13	60	2	2.00	1.000	0.500	0.375	***
SSR 14	60	5	4.31	1.000	0.768	0.731	***
SSR 15	60	3	2.64	1.000	0.622	0.550	***
SSR 16	59	5	3.69	0.627	0.729	0.686	***
SSR 17	59	5	3.78	1.000	0.735	0.691	***
Mean	60	4	2,95	0,852	0,637	0,569	

N: samples size; Na: alleles per locus; Ne: effective alleles; Ho and He: observed and expected heterozygosities; PIC: polymorphism information content;

*** Significant difference with Hardy–Weinberg equilibrium at p<0.01 level;*

**** Significant difference with Hardy–Weinberg equilibrium at p<0.001 level.*

A total of 63 alleles with sizes of 200 bp-500 bp were detected at seventeen microsatellite loci in the Phu Ninh tilapia strain (Table 2). The average number of Na and Ne were 4.0 and 2.95, respectively. Five microsatellites - SSR6, SSR10, SSR14, SSR16, and SSR17 have the highest number of alleles with the appearance of five alleles. The second highest group were SSR5, SSR7, SSR9, SSR11 with four alleles. The third group includes SSR1, SSR4, and SSR12 with three alleles. The SSR2, SSR8, and SSR13 loci appeared two alleles.

For polymorphism information content, the PIC value of each locus ranged from 0.369 to 0.738, with an average of 0.569 (Table 2). The PIC value is considered as indicator of genetic polymorphism and the degree of genetic variation. PIC>0.5 is highly polymorphic; 0.25<PIC<0.5 is a reasonable polymorphism and PIC<0.25 is a slight polymorphism [12], [13]. In

this study, thirteen loci were considered highly polymorphic (PIC > 0.5); four loci - SSR2, SSR7, SSR8, and SSR13 were considered to have a moderate degree of polymorphism. Therefore, the above thirteen loci are suitable for use in evaluating population genetic diversity and determining SSRs associated with disease resistance. Sun *et al.* (2015) reported the association between SSR markers and growth traits in Mandarin fish. The study detected 18/120 SSRs with high polymorphism (the average of PIC is 0.50) which were selected for testing correlation with growth (weight, length, and height) [14].

The observed heterozygosity (Ho) and expected heterozygosity (He) ranged from 0.400 to 1.000 and 0.489 to 0.775, respectively. The average Ho and He values were 0.852 (only SSR7 had Ho<0.5) and 0.637 (only SSR2 had Ho<0.5), respectively. In this study, the Ho was higher than the He, indicating that all loci were heterozygous. Heterozygousness within a population can be considered as a "measurement" of the genetic diversity of the population. Kotzé and Muller (1994) [15] reported that if a population is highly heterozygous for a single locus, the levels of genetic diversity at many different loci will also be high. The higher of heterozygosity parameter, the more genetic variation there is, and the opposite. In addition, comparing Ho and He can reveal the influence of foreign gene flows on the genetics of a population. Specifically, if Ho>He, the presence of foreign gene lines has a great influence on increasing heterozygosity in the population [16]. In this study, the Phu Ninh tilapia strain was the 2nd selective breeding generation (G2). The Phu Ninh tilapia strain was selected from intercrossbreeding four different strains of Nile tilapia (Genetically Improved Farmed Tilapia - GIFT strain; a GIFT-derived strain named NOVIT4; Chinese tilapia strain from ProGift Aquaculture Technology Co. Ltd. (ProGift); and a Philippine strain from Freshwater Aquaculture Center of Central Luzon State University) [17]. Therefore, many different genetic sources influenced polymorphism in the population of the Phu Ninh tilapia strain.

The results also show that all SSRs deviated from the Hardy-Weinberg equilibrium (P<0.01). In this study, it may be due to small number of samples, families (three families), and low frequency appear alleles (allele fequencies of allele of 334. in locus SSR5; allele of 231. in locus SSR7; allele of 371. in locus SSR9; allele of 352. in locus SSR10; allele of 375. in locus SSR11; result is not shown in the present study), and thus Hardy-Weinberg may be violated [18]. Crossbreeding between tilapia lines of different origins is an effective solution to help maintain allele diversity and can partially restore lost genetic diversity [19].

The polymorphism of microsatellites in this study was similar to that reported in the study of Chen *et al.* (2022) [1] and more than that reported in the study of Dong *et al.* (2008)[20]. In Nile tilapia, total alleles were identified of 62; 70, and 46 alleles in the A, B, and N2 strains, respectively. The mean number of Na, Ne, and PIC were 6.2; 3.015; and 0.575, respectively [1]. In the Chinese shrimp, the mean number of Na, and PIC were 2.5; and 0.446, respectively [20]. The present study found that 13/17 microsatellite markers had high polymorphism, except for SSR2, SSR7, and SSR13 markers.

3.3. The potential microsatellite/SSRs associated with disease resistance by *S. agalactiae*

The genotype distributions and allele frequencies of the SSRs in the hepcidin/HAMP genes in two tilapia groups (dead and alive) are shown in table 3.

There were significant differences in the SSRs genotype distributions and allele frequencies of four microsatellites - SSR3, SSR5, SSR14, and SSR15 (P<0.05) between alive and dead groups. The remaining nine SSRs had no significant differences between SSR genotypes and allele distributions (P>0.05) (Table 2). These results indicated that four microsatellite loci (SSR3, SSR5, SSR14, and SSR15) linked the hepcidin/HAMP were possibly associated with resistance to *S. agalactiae* in the Phu Ninh tilapia strain.

Table 3. The allele and genotype distributions of thirteen SSR_s in the hepcidin/HAMP genes in two groups of tilapia after challenge with *S. agalactiae*

SSRs	Gen/ID	Primer sequences (5'-3')	Motif	Size (bp)	Dyes
Multiplex I:					
SSR4	HAMP2 3'end 4.3kb (ID: 100698871)	F: CACCACTGTCAACTGGCTAATG R:GTTACCTTCTTGATACCGCAGG	$(CTAC)_7$	359	FAM
SSR5	HAMP2 3'end 4.7kb (ID: 100698871)	F: CTTTGGGTAGAGGAACACTCCA R:TGCAGGTCAATAGCAATACCAC	$(TG)_{21}$	309	HEX
SSR9	HAMP1 5'end 1.3kb (ID: 109204256)	F: GCGCTGTATAAGATTCCCGTTA R:GGAAACACAAGAGACATGAGCA	$(T)_{20}$	370	TAM
SSR14	HAMP1 reverse strand 3'end 6.2kb (ID: 100534415)	F: TCGGAATTGAGCATTAAGACCT R:GGTTCCCCATAGAACTCCTTTT	$(TG)_{22}$	245	ROX
Multiplex II:					
SSR6	HAMP1 5'end 2kb (ID: 109204280)	F: CAGTGGGTGTTTGTTCCTTACA R:TAGTAGGCTTTGTGTGCATTCC	$(GT)_{41}$	315	HEX
SSR8	HAMP1 5'end 1.3kb (ID: 109204256)	F: CCCCATAGCACTCCTTTTATTG R:TCATTTGGAGGTGTTTTCACAG	$(TG)_{22}$	322	ROX
SSR10	HAMP1 3'end 7.6kb (ID: 109204256)	F: GTTTGTAGCTTAACCCATTCGC R:TGCCTTTGTTTAGATGAACTGC	$(TG)_{10}$	364	FAM
SSR17	HAMP1 3'end 4.2kb (ID: 109204285)	F: GGTTCCCCATAGAACTCCTTTT R:TCGGAATTGAGCATTAAGACCT	$(TG)_{15}$	261	TAM
Multiplex III:					
SSR1	HAMP2 5'end 3.8kb (ID: 100698871)	F: CCACACAATCAACACACTGGTA R:TCAAACAGAAACAGGGACACAC	$(TA)_{11}$	375	HEX
SSR2	HAMP2 5'end 3.8kb (ID: 100698871)	F: GCACAGACACAGTAACACATGC R: ACTCCCTGGTACATGCTTCCTA	$(CAGG)_6$	368	FAM

SSR13	HAMP1 reverse strand 5'end 6.3kb (ID: 109204255)	F: TTAAAATGGGCTCAGGAGAAAG R:ACACACATAGATTCATCCGCAC	$(ATTC)_7$	332	ROX
SSR16	HAMP1 reverse strand intron2 (ID: 100534415)	F: GTGGGAAACACAAGAGACATGA R:CAGGGCTGAGATAGATTTTGGT	$(T)_{23}$	232	TAM
Multiplex IV:					
SSR3	HAMP2 5'end 3.8kb (ID: 100698871)	F: TAGGAAGCATGTACCAGGGAGT R: AAAATCACTCAACCGTGTCCTT	$(GT)_{13}$	348	FAM
SSR7	HAMP1 3'end 4.7kb (ID: 109204280)	F: CCACACGCACTCTCACCA R:CTGGTAGCCTTGACCCAGTTT	$(AC)_{12}$	236	HEX
SSR11	HAMP4 5'end 1kb (ID: 109204092)	F: AAGTGTCGTTCCACCCACAT R:ACAGAGTGTTCTGGCTTTCACA	$(TG)_{29}$	382	TAM
SSR12	HAMP1 reverse strand 5'end 2kb (ID: 109204255)	F: GTGCATTACAGAGTGTTCTGGC R: GTCTGGAGCCAAGTGTCGTT	$(TG)_{27}$	395	HEX
SSR15	HAMP1 reverse strand 3'end 750bp (ID: 100534415)	F: CAACAAGTGAAGCGACCATATT R:TCACACACAAGCAGGTCAATTA	$AC)_{13}$	300	ROX

Allele names (A, B, C,D, …) applied only for each locus

SSR3, SSR5, SSR14, and SSR15 are associated with the hepcidin/HAMP1 gene (SR14, SSR15) and the hepcidin/HAMP2 gene (SSR3, SSR5) in 3'-/5'- flanking region (as shown in Table 1). The 5′ flanking region has mainly functions in the regulation of gene transcription. The region contains the promoter and enhancers or other protein binding sites. The 3' flanking region often contains sequences that affect the formation of the 3' end of the Message RNA (mRNA). It may also contain enhancers or other protein binding sites [21-23]. SSRs located in the 5' flanking region of an immune-related susceptibility gene (STAT4-MS1-254), were significantly associated with sarcoidosis in humans [24]. In Nile tilapia, SSRs with (GT)n in the 5' upstream region of prolactin 1 (prl 1) were associated with differences in prl 1 gene expression and the growth response of salt-challenged fishes [23]. In this study, four microsatellites are located on chromosome 11 (LG 11) in the gene map of tilapia. The hepcidin/HAMP1 and hepcidin/HAMP2 genes were structurally similar to the hepcidin/TH1-5 and hepcidin/TH2-3 genes, containing 22 amino acids GIKCRFCCGCCTPGICGVCCRF and 26 amino acids QSHLSLCRWCCNCCRSNKGC in the protein-coding regions, respectively [2], [4]. The hepcidin/HAMP1 and hepcidin/HAMP2 genes were reported to defend against various bacterial pathogens (both gram-negative and gram-positive bacteria) in tilapia, such as *S. iniae, S. aureus, V. vulnificus* [1], [2], [3], [4]. Among 12 hepcidin/HAMP genes on LG11, 7 HAMP1, 1

HAMP2, 1 HAMP3, and 3 HAMP4 were detected [1], [10], [11], [25].

The results of our study are consent with previous studies, suggesting the relationship between SSR markers on the HAMP1 and HAMP2 genes and *Streptococcus sp.* resistance in Nile tilapia. Nhat *et al.* (2021) [26] identified three molecular markers (SSR7, SSR9, SSR16) associated with the hepcidin gene illustrating *S. inae* resistance tilapia cultured in Taiwan. In Nile tilapia, eleven disease-resistance-associated microsatellite markers (three markers in HAMP2 gene; four in HAMP1 gene; one in PGRN2 gene; two in PGRN1 gene; and one in piscidin-4 (TP4) gene) were found in tilapia strains farmed in Taiwan after challenging test with *S. inae* [1].

4. CONCLUSION

This study initially identified four potential SSRs (SSR3, SSR5, SSR14, and SSR15) linked to hepcidin/HAMP gene possibly associated with disease resistance to *S. agalactiae* in Phu Ninh tilapia strain (P<0.05). These potential markers can be applied as) MAS in the selection of tilapia associated with Streptococosis resistance caused by *S. agalactiae* in Vietnam.

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REFERENCES

1. Chen C. C., Huang C. W., Lin C. Y., Ho C. H, Pham H.N, Hsu T. H, ... and Gong H.Y. (2022). Development of Disease-Resistance-Associated Microsatellite DNA Markers for Selective Breeding of Tilapia (*Oreochromis spp.*) Farmed in Taiwan. *Genes*, 13 (1), 99.

2. Huang P. H., Chen J. Y., and Kuo C. M. (2007). Three different hepcidins from tilapia, Oreochromis mossambicus: analysis of their expressions and biological functions. *Mol Immunol*, 44 (8), 1922–1934.

3. Pan C. Y., Peng K. C., Lin C. H., Chen J. Y. (2011). Transgenic expression of tilapia hepcidin 1-5 and shrimp chelonianin in zebrafish and their resistance to bacterial pathogens. *Fish Shellfish Immunol*, 31 (2), 275 – 285.

4. Gong H. Y., Ho C. H., Wu S. H., Lin W. F, Kuo Y.-H, Chang Y.-H. (2016). Three HAMP genes of Nile tilapia were differentially activated in spleen to defend against *Streptococcus iniae* infection. *Fish & Shellfish Immunology*, 53, 122.

5. King D. G. (2012). Evolution of simple sequence repeats as mutable sites. *Adv Exp Med Biol*, 769, 10 – 25.

6. Fuji K., Hasegawa O., Honda K., Kumasaka K., Sakamoto T., Okamoto N. (2007). Marker-assisted breeding of a lymphocystis disease-resistant Japanese flounder (*Paralichthys olivaceus*). *Aquaculture*, 272, 291 – 295.

7. Jung M.-H., and Jung S.-J. (2021). Microsatellite marker distribution pattern in rock bream iridovirus (RBIV) infected rock bream, Oplegnathus fasciatus. *J. Fish Pathol., 34 (1), 009-015.*

8. Holleley G. P. G. (2009). Multiplex Manager 1.0: a cross-platform computer program that plans and optimizes multiplex PCR. *Biotechniques*, 46 (7), 511-517, doi: 10.2144/000113156.

9. Peakall R. and Smouse P. (2006). GENALEX 6: Genetic Analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, 6, 288–295.

10. Gong H. Y., Wu S. H., Chen C. Y., Huang C.-W, Lu J. K, Chou H. Y. (2017). Complete Genome Sequence of *Streptococcus iniae* 89353, a Virulent Strain Isolated from Diseased Tilapia in Taiwan. *Genome Announc*, 5 (4), e01524-16.

11. Pham H. N., Tseng P. C., Kuo Y. H., Wu S. H, Huang C. W, Gong H. Y. (2017). Highly amplification of hepcidin genes and associated microsatellites are potential molecular markers in marker-assisted selection for disease resistance of Nile tilapia. Tokyo University of Marine Science and Technology, Shinagawa, Tokyo, Japan.

12. Shete S., Tiwari H., and Elston R. C. (2000). On estimating the heterozygosity and polymorphism information content value. *Theoretical Population Biology*, 57 (3), 265 - 271.

13. Botstein D., White R., Skolnick M., Davis R.W. (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human genetics*.

14. Sun L. F., Li J., Liang X. F., Yi T. L, Fang L., Sun J.,... and Yang M. (2015). Microsatellite DNA markers and their correlation with growth traits in mandarin fish. *Genetics and Molecular Research*.

15. Kotze A. and Muller G.H. (1994). Genetic relationships between southern African cattle breeds. *Proc 3rd World Congr Genet Appl Livestock Prod, Guelph, Canada*, 20, 201.

16. Gao. W., Cui. W., Wu. F., Chen H., Liu. S., Guan, M., ... and Ma H. (2023). Genetic diversity and differences among three F1 families and two wild populations of genus scylla using microsatellite Markers. *Fishes*, 8 (1), 18.

17. Diep N. H., Sang V. V., Su V. H., Duong N.C, Oanh T. T. K., Tung D. S. (2022). Survival rate and final weight at harvest of hybrid combinations of Nile tilapia (*Oreochromis niloticus*) as influenced by genetic background of parental populations. *Vietnam J Agri*, 20 (5), 626–634.

18. Ferguson M. (1994). The role of molecular genetic markers in the management of cultured fishes. Rev Fish Biol Fisheries 4, 351–373. https://doi.org/10.1007/BF00042909.

19. Goyard E., Goarant C., Ansquer D., Brun P., Decker S.D., Dufour R., ... Patrois J. (2008). Cross breeding of different domesticated lines as a simple way for genetic improvement in small aquaculture industries: Heterosis and inbreeding effects on growth and survival rates of the Pacific blue shrimp *Penaeus* (*Litopenaeus*) *stylirostris*. *Aquaculture*, 278 (1), 43–50.

20. Dong S., Kong J., Meng X., Zhang Q., Zhang T., Wang R. (2008). Microsatellite DNA markers associated with resistance to WSSV in *Penaeus* (*Fenneropenaeus*) *chinensis*. *Aquaculture*, 282, 138-141.

21. Kiwimedia. The 5′ flanking region. Date of access 10.08.2023. Extracted from https://en.wikipedia.org/wiki/5'_flanking_region.

22. Fisher A. J., Beal P. A, (2018). Structural basis for eukaryotic mRNA modification. *Current Opinion in Structural Biology*, 53, 59-68.

23. Streelman J. T., Kocher, T. D., 2002. Microsatellite variation associated with prolactin expression and growth of salt-challenged tilapia. *Physiol. Genomics* 9, 1–4.

24. Tanaka G., Matsushita I., Ohashi J., Tsuchiya N., Ikushima S., Oritsu M., ... and Keicho N. (2005). Evaluation of microsatellite markers in association studies: a search for an immune-related susceptibility gene in sarcoidosis. Immunogenetics, 56, 861-70.

25. Ho C.-H., Wu S. H., Gong H. Y. (2017). Novel hepatic antimicrobial peptide HAMP3 genes are strongly activated not only in the liver but also in gill, head kidney and spleen of Nile tilapia to defend against virulent *Streptococcus iniae*. Tokyo University of Marine Science and Technology, Shinagawa, Tokyo, Japan, Tokyo.

26. Nhat P. H., Ho C. H., Tseng P. C., Gong H. Y. (2021). Microsatellites polymorphism associated with hepcidin/hamp genes potential for selective breeding of disease-resistant by *Streptococcus iniae* in Nile tilapia. *Journal of Agricultural Science & Technology*, 5 (3), 2727–2739.