

Identification and determination of toxin genes of *Vibrio* strains causing hemorrhagic disease on red drum (*Sciaenops ocellatus*) using PCR method

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Abstract

Data were isolated from 30 strains of *Vibrio* and sampled on different organs (brain, hemorrhagic site and digestive tract) of *Sciaenops ocellatus* infection. The results showed that nucleotide sequences 16S rRNA region are highly, similar to those of *V. alginolyticus*, *V. azureus*, *V. fluvialis* and *V. orientalis* published on Genebank, ranging from 98.05 to 100 %. The digestive tract has the most common *Vibrio* strains (*V. alginolyticus*, *V. azureus* and *V. fluvialis*). Thereout, 25 of 30 strains of *Vibrio* contained 1 to 3 toxin genes, excepted *V. parahaemolyticus*. Six parameters were used to measure the DNA polymorphism of 33 homologous DNA sequences in this *Vibrio* bacteria population. The results indicated number of separate polymorphic sites (S), total number of mutant sites (Eta), number of haplotype (h), haplotype diversity (Hd), average number of nucleotide differences (k), nucleotide diversity (Pi) were 98 (S) 103 (Eta), 9 (h), 0.887 ± 0.032 (Hd), 25.789 (k) and $17.980 \times 10^{-3} \pm 0.003$ (Pi), respectively ($P < 0,05$). The G+C content above 1434 sites positions of nucleotide sequences accounts for 0.542. The phylogenetic tree showed that these strains are divided into six groups. As observed, the appearance of isolated *Vibrio* on 3 organs of fish (*S. ocellatus*) hemorrhagic are *V. azureus* (27,67 %), *V. alginolyticus* (50 %), *V. orientalis* (6,67 %) and *V. fluvialis* (16,67 %). Through this result, we found that the diversity of *Vibrio* species that appeared on the red drum was used in the 16S rRNA region and the presence of toxin genes in these *Vibrio* species.

Introduction

More than 100 *Vibrio* spp. have been reported and are predominantly associated with a variety of marine, estuarine, or other aquatic habitats (Janda, Newton, & Bopp, 2015). Red drum (*Sciaenops ocellatus*) was discovered originally in the Atlantic Ocean and the Gulf of Mexico; it was introduced into China in 1991 and since then it has been cultured extensively in several provinces in China (Zhang & Sun, 2011). In recent years, red drum (*S. ocellatus*) mortalities associated with *Streptococcus iniae* infection (Eldar, Perl, Frelier, & Bercovier, 1999), (Mmanda et al., 2014). There were seven *Vibrio* strains (included *V. vulnificus* HM-TA-D2-L2-V2; *V. vulnificus* HM-TA-G2-V1-D2; *V. brasiliensis* HM-X-13/6; *V. cholerae* V-13/6; *V. parahaemolyticus* HM-17/6; *V. cholerae* HM-V-13/6 and *V. vulnificus* HM-X-13/6) to cause hemorrhagic disease in red drum (*S. ocellatus*) had only *tlh* gene and none of *Vibrio* strains had *tdh* and *trh* genes (Hoang Tan Quang et al., 2020). The research identified this fish (*S. ocellatus*) viperin gene (SoVip) and analyzed its expression in relation to bacterial challenge. The complete gene of SoVip is 2570 bp in length and contains six exons and five introns. The open reading frame of 1065 bp, which is flanked by a 50 untranslated region (UTR) of 34 bp and a 30 UTR of 350 bp and the fish pathogen *Edwardsiella tarda* but down regulated by the fish pathogens *Listonella anguillarum* and *Streptococcus iniae* (Dang, Zhang, Hu, & Sun, 2010). Toxin genes neutrality were tested by three methods (Tajima's D test, Fu and Li's D* and F* test, Fu's Fs), (Tajima, 1989), (Fu & Li, 1993) and (Fu, 1995), they were indicated an excess of low frequency polymorphisms relating to expectation, evidence for a deficiency of alleles, as expected from a recent population bottleneck and the evolution of the studied 30 strains bacteria *Vibrio*, was balancing selection, sudden contraction, rare alleles appeared in populations with low frequency. The studied

population had a few individuals showing large differences in comparison with other individuals. The study aims in identification and determination of toxin genes of *Vibrio* infected on red drum, hence understanding of *Vibrio* spp. infected on fish to cause *Vibriosis* for aquatic animals in brackish and marine water.

Materials And Methods

Collection of fish disease

In this study, we used thirty strains of bacteria with different morphologies isolated from three different organs in the fish (*S. ocellatus*) that have hemorrhagic disease (Fig 1) in Thua Thien Hue province, Vietnam, basing on the medium TCBS (Thiosulphate Citrate Bile Salt Sucrose).

Total DNA extraction method

The DNA extraction method presented in this paper is an improved method of phenol/chloroform according to method of (Neumann, Pospiech, & Schairer, 1992). We eliminated step to use SDS/lysozyme or proteinase K and extraction of cells directly by phenol. To extract the DNA from bacteria isolated from hemorrhagic disease in fish, 1 mL cell suspension was centrifuged at 8000 rpm for 2 minutes, for the collection of pellet cells. After removing the supernatant, the cells were washed with 400 μ l STE Buffer (100 mM NaCl, 10 mM Tris/ HCl, 1 mM EDTA, pH 8.0) twice, then centrifuged at 8000 rpm for 2 min. The pellets were resuspended in 200 μ l TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). After this, 100 μ l Tris-saturated phenol (pH 8.0) was added to these tubes, followed by a vortex-mixing step of 60 s. The samples were subsequently centrifuged at 13000 rpm for 5 min at 4°C to separate the aqueous phase from the organic phase. 160 μ l upper aqueous phase was transferred to a clean 1.5 ml tube. 40 μ l TE buffer was added to make 200 μ l and mixed with 100 μ l chloroform and centrifuged for 5 min at 13000 rpm at 4°C. Lysate was purified by chloroform extraction until a white interface was no longer present; this procedure might have to be repeated two to three times. 160 μ l upper aqueous phase was transferred to a clean 1.5 ml tube. 40 μ l TE and 5 μ l RNase (at 10 mg/ml) were added and incubated at 37°C for 10 min to digest RNA. Then 100 μ l chloroform was added to the tube, mixed well and centrifuged for 5 min at 13000 rpm at 4 °C. 150 μ l upper aqueous phase was transferred to a clean 1.5 ml tube. The aqueous phase contained purified DNA and was directly used for the subsequent experiments or stored at 20°C. The purity and yield of the DNA were assessed spectrophotometrically by calculating the A_{260}/A_{280} ratios and the A_{260} values to determine protein impurities and DNA concentrations according to (Neumann et al., 1992).

Determination of toxin gene

The presence of toxin genes in *Vibrio* spp., strains were determined through the presence of genes encoding toxic proteins (*tlh*, *tdh*, *trh* and *toxR*) which is based on specific primers for these genes (Table 1). PCR procedure: 50 ng of total DNA, 10 pmol of each primer, 25 μ l PCR master mix 2 \times (2.4 mM dNTP each, 0.3 units Taq DNA polymerase, Promega, USA), and sterile distilled water (total volume of 50 μ l).

PCR amplification was performed in MJ Mini™ Thermal Cycler (Bio-Rad, USA) as follows: 94 °C for 3 minutes; followed by 30 cycles at 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute; the last cycle of 72°C for 7 minutes. PCR products were used for electrophoresis on 1% agarose gel, using standard electrophoresis procedures in TAE 1X buffer with Ethidium bromide dye and read electrophoresis images by direct UV reading system (UV-transilluminator, Model: DyNa Light).

16S rRNA Gene Amplification and Sequencing

Performing PCR reaction to amplify the 16S rRNA region, originating from genome with a pair of 16S primers: 27F: AGAGTTTGATCMTGGCTCAG and 1492R: TACGGYTACCTTGTTACGACTT (Jeremy A Frank et al., 2008). The PCR reaction is performed on the Applied Biosystems – Life Technologies – Thermo Fisher Scientific – USA with a reaction component of 25 µl PCR master mix 2 × (2.4 mM dNTP each, 0.3 units Taq DNA polymerase), 10 pmol of 27F primer, 10 pmol of 1492 primer, 1 µl of total DNA (50 ng/µl) and sterile distilled water to a final volume of 50 µl. The 16S rRNA gene region is amplified with the following thermal cycle: 95°C/5 minutes; 30 cycles x (95°C/60 seconds; 57°C/50 seconds; 72°C/60 seconds); 72°C/10 minutes. Aliquots (10 µl) of PCR products were electrophoresed and visualized in 1% agarose gels using standard electrophoresis procedures in TAE 1X buffer with Ethidium bromide dye and read electrophoresis images by direct UV reading system (UV-transilluminator, Model: DyNa Light). Partial 16S rRNA genes of selected isolates in each site were sequenced by MACROGEN, Republic of Korea (dna.macrogen.com). Finally, 16S rRNA sequence of the isolation was compared with that of other microorganisms using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi>).

Table 1 Sequence of primers

Genes	Primer names	Nucleotide sequences 5'→3'	Size (bp)	References
<i>toxR</i>	toxR-F	GTCTTCTGACGCAATCGTTG	367	Luan <i>et al.</i> , 2007; Marlina <i>et al.</i> , 2007
	toxR-R	ATACGAGTGTTGCTGTCATG		
<i>tdh</i>	tdh-F	GTAAAGGTCTCTGACTTTTGGAC	500	Luan <i>et al.</i> , 2007; Marlina <i>et al.</i> , 2007
	tdh-R	TGGAATAGAACCTTCATCTTCACC		
<i>trh</i>	trh-F	TTGGCTTCGATATTTTCAGTATCT	269	Luan <i>et al.</i> , 2007; Marlina <i>et al.</i> , 2007
	trh-R	CATAACAAACATATGCCCATTTCC		
<i>tlh</i>	tlh-F	AAAGCGGATTATGCAGAAGCACTG	450	Luan <i>et al.</i> , 2007; Marlina <i>et al.</i> , 2007
	tlh-R	GCTACTTTCTAGCATTTTCTCTGC		

tdh=Thermostable direct hemolysin, *trh*=TDH-related hemolysin, *tlh*=Thermolabile hemolysin, *toxR*=Toxin operon (Luan et al., 2007), (Marlina et al., 2007).

Sequencing and analyzing genetic relationships

The PCR products of the 16S rRNA region are purified with Isolate II PCR and Gel (Bioline) kits. Then, they are sequenced directly by the dideoxy termination method on the ABI PRISM[®] 3100 Avant Genetic Analyzer (Applied Biosystems) at Maccrogen Company, Korea (dna.macrogen.com). The nucleotide sequences are arranged based on the Clustals program (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997) and edited by using BioEdit 7.0.5 software (Hall, 1999). Finally, 16S rRNA sequence of the isolation was compared with that of other microorganisms using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi>). The DNA polymorphism analysis is based on eight parameters including number of separate polymorphic sites (S), total number of mutant sites (Eta), number of haplotypes (h), haplotype diversity (Hd), average number of nucleotide differences (k), nucleotide diversity (Pi) are considered as a polymorphic measurement in the population (J. Rozas & R. Rozas, 2005). Neutrality is tested based on three methods, Tajima's D test (Tajima, 1989), Fu and Li's D* and F* test (Fu & Li, 1993) and Fu's (Fu, 1995) using DNASP 6.0 software.

Phylogenetic tree showing genetic relationship will be built by MEGA X software (The Molecular Evolution Genetics Analysis), based on methods of UPGMA method (Sneath & Sokal, 1973). The optimal tree with the sum of branch length equal to 0.08795656 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (J, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Koichiro Tamura, Masatoshi Nei, & Kumar, 2004) and are in the units of the number of base substitutions per site. This analysis involved 48 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1434 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar S., Stecher G., Li M., Knyaz C., & K., 2018).

Results

PCR result

The PCR products of the 16S rRNA region are purified with Isolate II PCR and Gel (Bioline) kits. Then, they are sequenced directly by the dideoxy terminator method on the ABI PRISM[®] 3100 Avant Genetic Analyzer (Applied Biosystems) at Maccrogen Company, Korea (dna.macrogen.com). The results of the 16S rRNA region were about 1450 bp for the remaining 30 isolated strain bacteria based on medium TCBS. The BLAST result on NCBI was used to verify and compare with the sequences of the *Vibrio* spp. with accession number Genebank (Table 2) showed that the nucleotide sequences obtained were highly similar to those of the *V. alginolyticus*, *V. azureus*, *V. fluvialis* and *V. orientalis*, ranging from 98,05 to 100 % (Table 2).

The results indicated that all PCR products of the 16S rRNA region in the studied of 30 isolated strain bacteria based on medium TCBS showed a single band with 100% amplification rate. All samples gave

high DNA concentration and are clearly seen. The obtained size was approximately 1500 bp, which goes in line with the initial expected size (Fig. 2).

Determination of toxin gene

The agarose gel electrophoresis of PCR products determined the presence of *trh*, *tdh*, *tlh* and *toxR* genes at bands 269 bp, 500 bp, 450 bp and 367 bp, respectively (Fig. 2). We found 25/30 strains of *Vibrio* containing at least 1 toxic gene whereas 5 isolates carried out 3 toxin genes. However, none of these isolates consisted of all virulence toxins genes (Table 2). The results clearly indicate the presence of virulence toxins (*trh*, *tdh* and *tlh*) and a regulator toxin (*toxR*). Among them, 18 isolates presented *tlh* while only 2 isolates were found to be carried out *tdh* gene

Sequencing and analyzing genetic relationships

Table 2 Phylogenetic affiliation of isolates on the basis of 16S rRNA genes sequences by using BLAST programme in the GenBank database based on sequence similarity and Determination of toxin genes

No	Sample codes	Closest species relative	GenBank accession number	Similarity (%)	Genes			
					<i>toxR</i>	<i>tdh</i>	<i>trh</i>	<i>tlh</i>
1	YN14	<i>Vibrio alginolyticus</i> strain Va-F10	MH298564.1	98,05	+	-	-	+
2	YN19	<i>Vibrio alginolyticus</i> strain Va-F10	MH298564.1	98,05	+	-	-	+
3	YN34	<i>Vibrio alginolyticus</i> strain 4-14	MN938360.1	99,65	-	-	+	+
4	YN38	<i>Vibrio alginolyticus</i> strain Va-F10	MH298564.1	98,05	+	-	-	+
5	YN43	<i>Vibrio alginolyticus</i> strain 4-14	MN938360.1	99,65	+	-	-	-
6	YHTH6	<i>Vibrio azureus</i> strain Xmb005	KT986135.1	100	+	-	-	+
7	YHTH7	<i>Vibrio alginolyticus</i> strain 1-37	MN874162.1	98,06	-	-	+	-
8	YHTH12	<i>Vibrio azureus</i> strain Xmb005	KT986135.1	100	-	-	-	-
9	YHTH16	<i>Vibrio fluvialis</i> strain 2013V-1044	CP051126.1	100	+	-	-	+
10	YHTH18	<i>Vibrio fluvialis</i> strain 2013V-1044	CP051126.1	100	+	-	+	-
11	YHTH35	<i>Vibrio azureus</i> strain Xmb005	KT986135.1	100	-	-	-	+
12	YHTH37	<i>Vibrio fluvialis</i> strain 2013V-1044	CP051126.1	100	-	-	-	+
13	YHTH44	<i>Vibrio alginolyticus</i> strain 1-37	MN874162.1	98,06	+	-	+	+
14	YHTH46	<i>Vibrio azureus</i> strain Xmb005	KT986135.1	100	-	-	+	-
15	YHTH47	<i>Vibrio fluvialis</i> strain 2013V-1044	CP051126.1	100	-	-	-	-
16	YVL5	<i>Vibrio azureus</i> strain Xmb005	KT986135.1	100	-	-	+	-
17	YVL11	<i>Vibrio azureus</i> strain Xmb005	KT986135.1	100	-	-	-	-
18	YVL22	<i>Vibrio alginolyticus</i> strain 3-31	MN843961.1	99,72	+	-	+	+

19	YVL24	<i>Vibrio alginolyticus</i> strain 3-5	MN938185.1	99,86	+	+	-	+
20	YVL26	<i>Vibrio alginolyticus</i> strain 2015AW-0011	CP051109.1	99,59	-	-	-	-
21	YVL27	<i>Vibrio orientalis</i> strain 5-13	MN945276.1	100	-	-	-	+
22	YVL29	<i>Vibrio alginolyticus</i> strain 3-31	MN843961.1	99,72	-	-	-	+
23	YVL31	<i>Vibrio alginolyticus</i> strain 3-5	MN938185.1	99,86	-	-	+	+
24	YVL33	<i>Vibrio azureus</i> strain Xmb005	KT986135.1	100	+	+	-	-
25	YVL40	<i>Vibrio alginolyticus</i> strain 2015AW-0011	CP051109.1	99,59	+	-	-	+
26	YVL42	<i>Vibrio orientalis</i> strain 5-13	MN945276.1	100	+	-	+	+
27	YVL45	<i>Vibrio azureus</i> strain Xmb005	KT986135.1	100	+	-	-	+
28	YVL84	<i>Vibrio alginolyticus</i> strain 3-31	MN843961.1	99,72	-	-	-	-
29	YVL85	<i>Vibrio alginolyticus</i> strain 3-5	MN938185.1	99,86	-	-	-	+
30	YVL86	<i>Vibrio alginolyticus</i> strain 2015AW-0011	CP051109.1	99,59	+	-	-	-

tdh=Thermostable direct hemolysin, *trh*=TDH-related hemolysin, *tih*=Thermolabile hemolysin, *toxR*=Toxin operon

Genbank registration Code No: MT953948[ACCN] : MT953977[ACCN];
<https://submit.ncbi.nlm.nih.gov/subs/?search=SUB8088865>

Table 3 DNA diversity based on 16S rRNA region of strain bacteria *Vibrio* population using the programme DNASp 5.0 (J. Rozas & R. Rozas, 2005)

Genetic region	S	Eta	H	G+C content (1434 sites)	Hd	k	Pi (x10 ³)
16S rRNA	98	103	9	0.542	0.887 ± 0.032	25.789	17.980 ± 0.003

Number of variable sites (S); Total number of mutations (Eta); Number of Haplotypes (H); Haplotype (gene) diversity (Hd); Nucleotide diversity (per site) (Pi); Average number of nucleotide differences (k)

Six parameters including number of polymorphic sites (S), total number of mutant sites (Eta), number of haplotypes (h), haplotype diversity (Hd), average number of nucleotide differences (k), nucleotide diversity (Pi) were used to evaluate the diversity of 30 studied *Vibrio* strains. As shown in table 4, ninety eight separate polymorphic positions (S) created 103 mutant positions (Eta) shown in 30 studied strain bacteria *Vibrio* were classified into nine types of haplotype (h) with haplotype diversity coefficient accounting for 0.887 ± 0.032 (Hd), the average number of nucleotide differences is 25.789 (k), the nucleotide diversity coefficient accounts for $17.980 \times 10^{-3} \pm 0.003$ (Pi). All indicators were processed with statistical significance $p < 0,05$. The G+C content above 1434 sites positions of nucleotide sequences account for 0.542 (Table 3).

Table 4 Neutrality Tests results based on 16S rRNA region of strain bacteria *Vibrio* population

Genetic region	Tajima's D test	Fu and Li's D* test	Fu and Li's F* test*	Fu's Fs
16S rRNA	-0.03099	1.91401	1.49643	13.659
	<i>Statistical significance: Not significant, P > 0.10</i>	<i>Statistical significance: **, P < 0.02</i>	<i>Statistical significance: Not significant, 0.10 > P > 0.05</i>	<i>Strobeck's S statistic: 0.000</i>

Three methods namely (Tajima's D test, Fu and Li's D* and F* test, Fu's Fs) were used to test neutrality. The results in Table 4 indicated that with A negative Tajima's D signifies an excess of low frequency polymorphisms compared with initial expectation (*Statistical significance: Not significant, p>0.10*). Meanwhile, a positive value of F_s (13.659) is the evidence for a deficiency of alleles, as would be expected from a recent population bottleneck (*Strobeck's S statistic: 0.000*). In addition, The Fu and Li's F* (*statistical significance 0.10 > p > 0.05*) and value of Fu and Li's D* (*Statistical significance: **, P < 0.02*) both yield positive ones, which showed that the evolution of the studied 30 strain bacteria *Vibrio* population was balancing selection, sudden contraction or in other words, rare alleles appeared in populations with low frequency, the studied population had very few individuals showing large differences in comparison with other individuals in the population (Table 4). The phylogenetic tree shows the genetic relationship of thirty *Vibrio* strains which are isolated from various three different parts of the fish (*S. ocellatus*) using UPGMA method. Fig. 3, these strains are divided into six groups. Among these, group I includes the strains of isolated *Vibrio* which are closely related to *V. azureus*. These strains mainly concentrate in the digestive system and hemorrhagic. Groups II, III and V consist of *Vibrio* strains, isolated

in 3 different parts (brain, hemorrhagic and digestive system). They are closely related to *V. alginolyticus*. Group 4 includes two strains, isolated from the ulcer which are closely related to *Vibrio orientalis*. Group VI consists of 4 strains, concentrating in digestive system and having a close genetic relationship with *V. fluvialis* (Fig. 3). As observed, the appearance of isolated *Vibrio* on 3 organs of red drum fish showing signs of bleeding hemorrhagic are *V. azureus* (27,67 %), *V. alginolyticus* (50 %), *V. orientalis* (6,67 %) and *V. fluvialis* (16,67 %).

Discussion

In this study, we isolated 30 strains of *Vibrio* from three different organs (brain, hemorrhagic site and digestive tract) of *S. ocellatus*. The results showed that nucleotide sequences 16S rRNA region are highly similar to those of *V. alginolyticus*, *V. azureus*, *V. fluvialis* and *V. orientalis* published on Genbank, ranging from 98.05 to 100%. The digestive system has the most common *Vibrio* species (*V. alginolyticus*, *V. azureus* and *V. fluvialis*). None of *V. parahaemolyticus* presented, the same reported and identified that *Vibrio* species was isolated from cultured olive flounder (*Paralichthys olivaceus*) in Jeju Island, South Korea that none of *V. parahaemolyticus* presence too (Sohn, Kim, Jin, & Lee, 2019). In which *V. parahaemolyticus* strain was a pathogen causing in shrimp disease, (Dao, Linh, & Khanh, 2014), (Khanh et al., 2019).

The present of toxic genes related to the hemolysin of fish are found in various *Vibrio* sp.. Meanwhile, approximately 50% of isolates consist of toxin operon gene. All *V. parahaemolyticus* isolates contained the *toxR* genes but the *trh* gene did not existence in clam (*Corbicula moltkiana*) (Marlina et al., 2007). Our data confirmed three isolates carried both *toxR* and *trh* genes including isolates exhibited highly similarity to *V. fluvialis*, *V. alginolyticus*, and *V. orientalis*. TDH is an enzyme that lyses human red blood cells on Wagatsuma blood agar plates, which is referred to as the Kanagawa phenomenon positive. Another toxin produced by Kanagawa phenomenon negative *Vibrio* strains is the *tdh*-related hemolysin (*trh*) toxin encoded by *trh* gene (Al-Othubi, Alfizah, Son, Humin, & Rahaman, 2011). Thermolabile hemolysin (*tlh*) is an another *Vibrio* enterotoxin that cause blood cell lysis in infected fish, *tlh* is encoded by *tlh* gene (Hasrimi, Budiharjo, & Jannah, 2018). Among 4 toxin genes (*toxR*, *tdh*, *trh* and *tlh*) were investigated from *Vibrio* spp. causing hemorrhagic disease in *S. ocellatus*, the results showed that the frequency of the *toxR* gene was detected in the 15 isolates using PCR assay, lowest of *tdh* gene was 2 isolates, *trh* gene was 9 isolates and the highest of *tlh* was 18 isolates using PCR assay. In addition the frequency of occurrence of toxin gene also showed that there were 5/30 *Vibrio* strains none carried the toxin gene (code number: YHTH12; YHTH47; YVL11; YVL26 and YVL84), 10/30 strains had only 1 toxin gene, 11/30 strains had 2 toxin genes and 4 strains carried 3 toxin genes including (*V. alginolyticus* strain 3-31, code number YHTH44 (*toxR*, *trh* and *tlh*); *V. alginolyticus* strain 3-31, code number YVL22 (*toxR*, *trh* and *tlh*); *V. alginolyticus* strain 3-5, code number YVL24 (*toxR*, *tdh* and *tlh*) and *V. orientalis* strain 5-13, code number YVL42 (*toxR*, *trh* and *tlh*). None of *Vibrio* carry all of 4 toxin genes. All *Vibrio* strains isolated from three marine fish species (*S. ocellatus*, *Lates calcarifer* and *Epinephelus fuscoguttatus*) was only carried one *tlh* gene present (Hoang Tan Quang et al., 2020). According Long et al, 2019 we isolated and identified the *V. parahaemolyticus* 01 strain in Thua Thien Hue province, Vietnam causing ulcer disease in *S.*

ocellatus. The full-length of thermolabile hemolysin (tlh) gene (1257 bp), encoding antigen thermolabile hemolysin toxin (tlh) of the *Vibrio* sp. was cloned and sequenced successfully. The sequence analysis of gene cloned shows a complete similarity to the *V. parahaemolyticus* strain (Genbank: AY289609.1) (Long et al., 2019). We further examined the presence of virulence genes homologous to those in *V. cholerae* (*toxR*, *toxS*, *VPI* and *ace*); *toxR* was found in 16 *V. alginolyticus* strains and *toxS* in 17 strains out of 34. Indicated in two species (*Dicentrarchus labrax*) and (*Sparus aurata*). A positive amplification for the virulence pathogenicity island (VPI) was produced by 12 *V. alginolyticus* strains. Finally, the expected amplification fragment was found in 7 *V. alginolyticus* isolates. Thus, the pathogenicity of *V. alginolyticus* may be the result of a combination of all these factors (Kahla-Nakbi, Chaieb, & Bakhrouf, 2009).

Six parameters were used to evaluate the diversity of 30 studied *Vibrio* bacteria strains. The result show that, ninety eight separate polymorphic positions (S) created 103 mutant positions (Eta) shown in 30 studied *Vibrio* strains classified into nine types of haplotype (h) with haplotype diversity coefficient accounting for 0.887 ± 0.032 (Hd), the average number of nucleotide differences is 25.789 (k), the nucleotide diversity coefficient accounts for $17.980 \times 10^{-3} \pm 0.003$ (Pi). All indicators were processed with statistical significance $p < 0.05$. The G+C content above 1434 sites positions of nucleotide sequences account for 0.542. Neutrality was tested based on three methods (Tajima's D test, Fu and Li's D* and F* test, Fu's Fs) showing that there was an excess of low frequency polymorphisms relative to expectation, evidence for a deficiency of alleles, as would be expected from a recent population bottleneck and the evolution of the studied 30 *Vibrio* bacteria population was balancing selection, sudden contraction or in other words, rare alleles appeared in populations with low frequency, the studied population had very few individuals showing large differences in comparison with other individuals in the population. The phylogenetic tree shows the genetic relationship of 30 *Vibrio* strains using UPGMA method (bootstrap = 1000) showed that these strains are divided into six groups. As observed, the appearance of isolated *Vibrio* on 3 organs of fish (*S. ocellatus*) hemorrhagic are *V. azureus* (27,67 %), *V. alginolyticus* (50 %), *V. orientalis* (6,67 %) and *V. fluvialis* (16,67 %).

Abbreviations

PCR: Polymerase chain reaction; TCBS: Thiosulphate Citrate Bile Salt Sucrose; tlh: Thermolabile hemolysin toxin; trh: tdh-related hemolysin; tdh: Thermostable direct hemolysin; toxR: Toxin operon

Declarations

Ethics approval and consent to participate

Not applicable

Consent for application

Not applicable

Availability of data and materials

All the data were presented in the main paper

Competing interests

The authors declare that they have no competing interests

Author's contributions

Pham Thi Hai Yen collected samples, isolated the bacterial strains, PCR method, DNA extraction and wrote the manuscript. Nguyen Duy Quynh Tram collected samples, analyzed the data and Nguyen Quang Linh analyzed the data, supervisor and wrote the paper. All authors read and approved the final manuscript.

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References

1. Al-Othubi, S. M., Alfizah, H., Son, R., Humin, N., & Rahaman, J. (2011). Rapid detection and E-test antimicrobial susceptibility testing of *Vibrio parahaemolyticus* isolated from seafood and environmental sources in Malaysia. *Saudi Med. J*, 32, 400-406. doi:<https://pubmed.ncbi.nlm.nih.gov/21484001/>
2. Dang, W., Zhang, M., Hu, Y.-h., & Sun, L. (2010). Differential regulation of *Sciaenops ocellatus* viperin expression by intracellular and extracellular bacterial pathogens. *Fish & shellfish immunology*, 29(2), 264-270. doi:<https://doi.org/10.1016/j.fsi.2010.04.015>
3. Dao, N. T. B., Linh, N. Q., & Khanh, N. (2014). Research on some characteristics of *Vibrio parahaemolyticus* which causes EMS disease on juvenile shrimp at Dien Huong community, Phong Dien district, Thua Thien Hue, Vietnam. *Hue University of journal of Science*, 13-22.

4. Eldar, A., Perl, S., Frelier, P., & Bercovier, H. (1999). Red drum *Sciaenops ocellatus* mortalities associated with *Streptococcus iniae* infection. *Diseases of aquatic organisms*, 36(2), 121-127. doi:<http://dx.doi.org/10.3354/dao036121>
5. Fu, Y.-X. (1995). Statistical properties of segregating sites. *Theoretical population biology*, 48(2), 172-197. doi:<https://doi.org/10.1006/tpbi.1995.1025>
6. Fu, Y.-X., & Li, W.-H. (1993). Statistical tests of neutrality of mutations. *Genetics*, 133(3), 693-709. doi:<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1205353/pdf/ge1333693.pdf>
7. Hall, T. A. (1999). *BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT*. Paper presented at the Nucleic acids symposium series. https://www.academia.edu/2034992/BioEdit_a_user-friendly_biological_sequence_alignment_editor_and_analysis_program_for_Windows_95_98_NT
8. Hasrimi, A. N., Budiharjo, A., & Jannah, S. N. (2018). Detection of tlh and tdh genes in *Vibrio parahaemolyticus* inhabiting farmed water ecosystem used for *L. Vannamei* aquaculture. *Journal of Physics: Conference Series*, 1025, 1-9. doi:<https://doi.org/10.1088/1742-6596/1025/1/012058>
9. Hoang Tan Quang, Tran Thuy Lan, Truong Thi Hong Hai, Pham Thi Hai Yen, Tran Quang Khanh Van, Ho Thi Tung, . . . Tram, N. D. Q. (2020). Genetic diversity and toxic genes analysis of *Vibrio* spp. isolated from white leg shrimp and marine fishes cultured in Tam Giang lagoon in Thua Thien Hue province, Vietnam. *INDIAN JOURNAL OF SCIENCE AND TECHNOLOGY*, 13(13), 1412–1422. doi:<https://doi.org/10.17485/IJST/v13i13.161>
10. J, F. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, 39, 783-791. doi: <https://doi.org/10.1111/j.1558-5646.1985.tb00420.x>
11. Rozas, & R. Rozas. (2005). DnaSP version 4.1: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics*, 15, 174-175. doi:<https://doi.org/10.1093/bioinformatics/15.2.174>
12. Janda, J. M., Newton, A. E., & Bopp, C. A. (2015). Vibriosis. *Clinics in laboratory medicine*, 35(2), 273-288.
13. Jeremy A Frank, Claudia I Reich, Shobha Sharma, Jon S Weisbaum, renda A Wilson, & Olsen, G. J. (2008). Critical Evaluation of Two Primers Commonly Used for Amplification of Bacterial 16S rRNA Genes. *Applied and Environmental Microbiology*, 74(8), 2461-2470. doi:<http://doi.org/10.1128/AEM.02272-07>
14. Kahla-Nakbi, A. B., Chaieb, K., & Bakhrouf, A. (2009). Investigation of several virulence properties among *Vibrio alginolyticus* strains isolated from diseased cultured fish in Tunisia. *Diseases of aquatic organisms*, 86(1), 21-28. doi: <https://doi.org/10.3354/dao02091>
15. Khanh, N. V., Linh, N. n. Q., Lan, T. n. T. y., Vân, T. n. Q. K. n., Cờ, N. n. T. K., & Dung, T. n. Q. c. (2019). Isolation and screening of *Vibrio parahaemolyticus* strains to cause acute hepatopancreatic necrosis disease in white-leg shrimps cultured in Phong Dien, Thua Thien Hue, Vietnam using 16S rRNA marker. *Hue University Journal of Science: Natural Science*, 128(1E), 47-58. doi: <http://dx.doi.org/10.26459/hueuni-jns.v128i1E.5443>

16. Koichiro Tamura, Masatoshi Nei, & Kumar, S. (2004). Prospects for Inferring Very Large Phylogenies by Using the Neighbor-Joining Method. *Proc Natl Acad Sci U S A*, 101(30), 11030–11035. doi:<https://doi.org/10.1073/pnas.0404206101>
17. Kumar S., Stecher G., Li M., Knyaz C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution*, 35, 1547-1549. doi:<https://doi.org/10.1093/molbev/msy096>
18. Long, D. T., Hoang, N. T., Hong, H. T. K., Yen, P. T. H., Chuong, H. V., Trang, N. T. Q., & Hiep, N. V. (2019). Isolation and DNA cloning of thermolabile hemolysin gene of *Vibrio* bacteria from *Sciaenops ocellatus* in Thua Thien Hue, Vietnam. *Hue University Journal of Science: Natural Science*, 128(1E), 5-14. doi:<http://dx.doi.org/10.26459/hueuni-jns.v128i1E.5373>
19. Luan, X. Y., Chen, J. X., Zhang, X. H., Jia, J. T., Sun, F. R., & Li, Y. (2007). Comparison of different primers for rapid detection of *Vibrio parahaemolyticus* using the polymerase chain reaction. *Letters in applied microbiology*, 44(3), 242-247. doi: <https://doi.org/10.1111/j.1472-765X.2006.02074.x>
20. Marlina, R. S., Kqueen, C. Y., Napis, S., Zakaria, Z., Mutalib, S. A., & Nishibuchi, M. (2007). Detection of *tdh* and *trh* genes in *Vibrio parahaemolyticus* isolated from *Corbicula molitkiana* prime in West Sumatera, Indonesia. *Southeast Asian J Trop Med Public Health*, 38, 349-355. doi:<https://pubmed.ncbi.nlm.nih.gov/17539286/>
21. Mmanda, F. P., Zhou, S., Zhang, J., Zheng, X., An, S., & Wang, G. (2014). Massive mortality associated with *Streptococcus iniae* infection in cage-cultured red drum (*Sciaenops ocellatus*) in Eastern China. *African Journal of Microbiology Research*, 8(16), 1722-1729. doi:<https://doi.org/10.5897/AJMR2014.6659>
22. Neumann, B. r., Pospiech, A., & Schairer, H. U. (1992). Rapid isolation of genomic DNA from gram-negative bacteria. *Trends in genetics: TIG*, 8(10), 332-333. doi:[https://doi.org/10.1016/0168-9525\(92\)90269-A](https://doi.org/10.1016/0168-9525(92)90269-A)
23. Sneath, P., & Sokal, R. (1973). Numerical Taxonomy. The principle and practice of numerical classification. WH Freeman and Co. *San Francisco*, 263-268. doi:<https://www.jstor.org/stable/2412767?seq=1>
24. Sohn, H., Kim, J., Jin, C., & Lee, J. (2019). Identification of *Vibrio* species isolated from cultured olive flounder (*Paralichthys olivaceus*) in Jeju Island, South Korea. *Fisheries and Aquatic Sciences*, 22(1), 14. doi:<https://doi.org/10.1186/s41240-019-0129-0>
25. Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, 123(3), 585-595. doi:<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1203831/pdf/ge1233585.pdf>
26. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic acids research*, 25(24), 4876-4882. doi:<https://doi.org/10.1093/nar/25.24.4876>
27. Zhang, M., & Sun, L. (2011). The tissue factor pathway inhibitor 1 of *Sciaenops ocellatus* possesses antimicrobial activity and is involved in the immune response against bacterial infection.

Figures

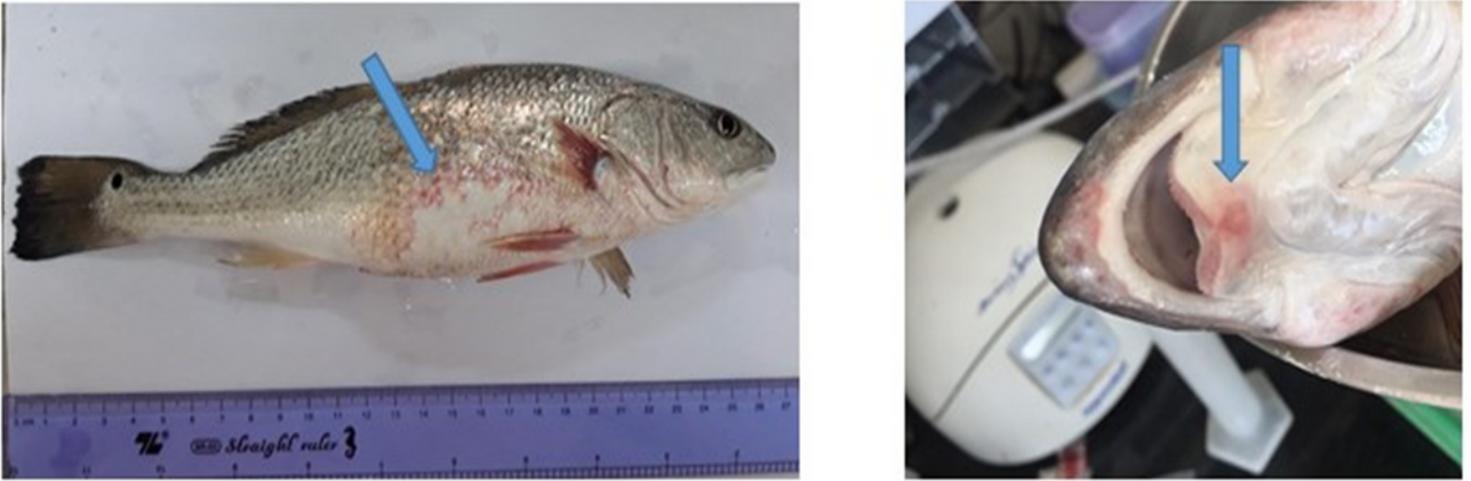


Figure 1

Sample of *Sciaenops ocellatus* hemorrhagic signal

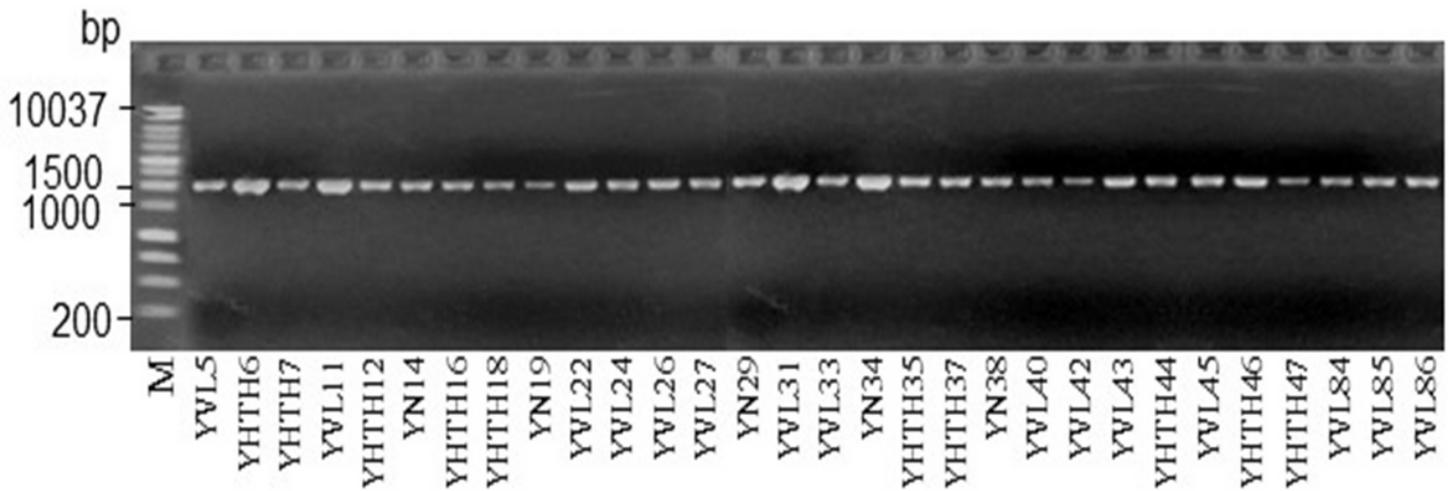


Figure 2

Electrophoresis of PCR product. M: DNA mass scale (HyperLadder™ 1kb (200 bp to 10037 bp), Bionline, Meridian Bioscience)

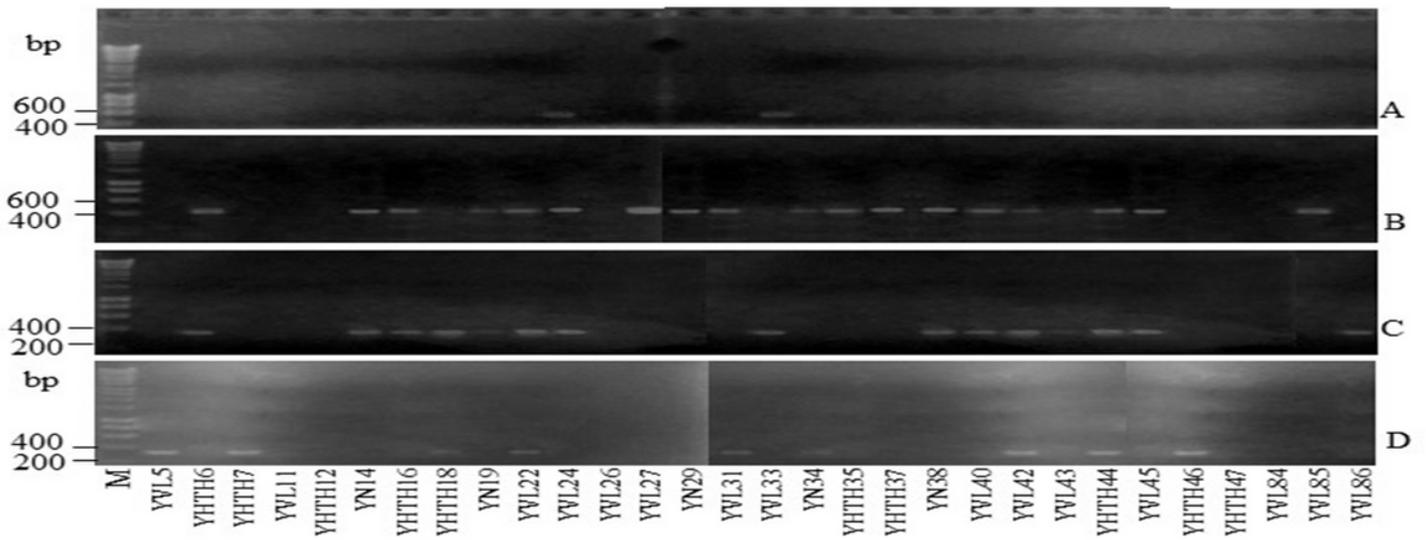


Figure 3

Electrophoresis determination of toxin gene. M: DNA mass scale (HyperLadder™ 1kb (200 bp to 10037 bp), Bioline, Meridian Bioscience). Fig A: PCR product of gene *tdh*. Fig B: PCR product of gene *tlh*. Fig C: PCR product of gene *toxR* and *hnh*. Fig D: PCR product of gene *trh*.

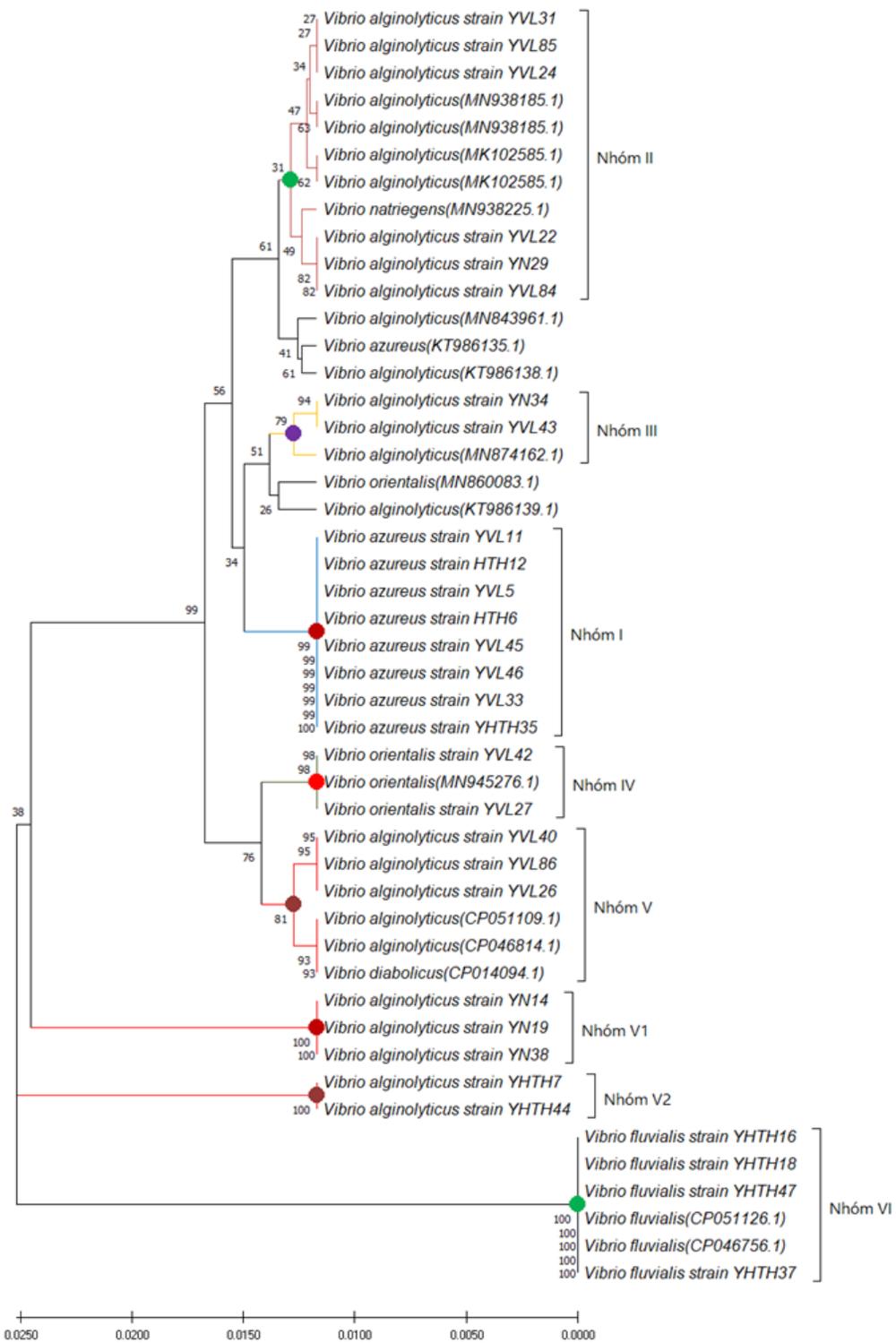


Figure 4

Phylogenetic tree of 30 strain bacteria *Vibrio* varieties collected based on the 16S rRNA region by the UPGMA method