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Molecular Characterization of 16S rRNA and Internal Transcribed Spacer (ITS) Regions of *Aeromonas* spp. Isolated from Cultured Freshwater Fishes in Malaysia

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A B S T R A C T

The *Aeromonas* spp., which are found mostly in freshwater fishes are known to cause uncontrolled disease outbreak in aquaculture system. In the present study, twenty-two of *Aeromonas* spp. strains isolated from diseased freshwater fishes were identified using the 16S ribosomal RNA (rRNA) and internal transcribed spacer (ITS) regions gene amplification where they produced a 1500 bp and 1000-1200 bp amplicon each. Phylogenetic analysis with BLASTn and 16S rRNA neighbor-joining bootstrapping methods revealed that all strains were identified up to genus level with 15 strains were classified as *Aeromonas hydrophila* (*A. hydrophila*) and the rest were *Aeromonas veronii* (*A. veronii*). Further ITS gene analysis disclosed strains identification up to species level; *A. hydrophila* (14 strains) and *A. veronii* (8 strains). These rapid DNA-based detection methods are simple, easy to perform and faster in identifying *Aeromonas* spp., thus are absolutely efficient for regular monitoring of *Aeromonas* spp. in a potential outbreak situation.

Introduction

Aeromonas are divided into three species that are phenotypically and genetically differed namely *Aeromonas hydrophila*, *A.caviae and A.sobria* (Corry *et al*., 2003). The presence of *Aeromonas* spp. in aquaculture system has been evidenced to induce outbreaks with high mortality rates in cultured fish, where the group that commonly causes infection and diseases is associated to a psychrophilic and non-motile strain known as *Aeromonas salmonicida* (Janda and Abbott, 2010).

Development of DNA-based molecular techniques in identifying bacterial species has ignited a great influence on prokaryotes identification based on 16S rRNA gene analysis (Rossello-Mora and Amann, 2001). Moreover, the 16S rRNA gene classification has been widely accepted and used for the

detection and delineation of *Aeromonas* spp. in aquaculture field. The 16S rRNA is very useful in classifying *Aeromonas* spp. up to genus level (Ludwig *et al*., 1998). Previous studies demonstrated that more than 70 % of genomic similarities were discovered in organisms that shared 97 % of their 16S rRNA sequences (Stackebrandt and Goebel, 1994; Garcia-Martinez *et al*., 1999).

To date, an internal transcribed spacer-PCR (ITS-PCR) method based on the different distances separating conserved functional RNA genes is applied to classify species within genus (Magni, 2010). According to Singh *et al*. (2012), amplification of ITS region which flanking between 16S-23S rDNA regions will produce distinctive DNA banding patterns that could recognize microorganism up to species level (Jensen *et al*., 1993; Gurtler and Stanisich,1996).

In our study, we aimed to characterize molecularly local of *Aeromonas* isolated by using two genes, 16S Rrna and ITS that can be used as phylomarker genes combination in diagnosis *Aeromonas* infected fish.

Materials and Methods

Bacterial Cultures, Morphological and Biochemical Characterization

Twenty-two strains of *Aeromonas* spp. (*Clarias* spp., *Pangasius* spp., and *Oreochromis* spp.) culture isolated from diseased freshwater fishes in different places in Malaysia were provided by Malaysian National Fish Health Research Centre (Nafish), Batu Maung, Penang, Malaysia (Table 1). These cultures were maintained on tryptic soy agar (TSA; Difco, USA.) plates at 37^{o}C for 18-24 hours. Single colonies from TSA plates were retrieved in tryptic soy broth (TSB; Difco, USA) by incubation for 18 hours at 200 rpm.

All *Aeromonas* spp. strains were then subjected to biochemical identification including Gram-staining, catalase and oxidase tests (Abbott *et al*., 2003).

DNA Extraction

A single colony from culture was grown overnight in 5 ml TSB at 37^{o}C, 200 rpm. Total genomic DNA of each strain was extracted with GeneJet Genomic DNA Purification Kit (*Thermo*Fischer *Scientific* Inc, USA) according to the manufacturer's instructions. The concentration of purified DNA was measured by using BioPhotometer Plus (Eppendorf, Germany).

Polymerase Chain Reaction Amplification and DNA Sequencing

All extracted total genomic DNA from the respective *Aeromonas* spp. strains was used as templates in the polymerase chain reaction (PCR) amplification to amplify the 16S rRNA and ITS genes.

The primer pairs, Forward (5'-GAGTTTGATCCTGGCTCAG-3') and Reverse (5'-GGT TACCTTGTTACGACT-3') were used for amplifying the 16S rDNA region of the *Aeromonas* spp. These primers were expected to amplify a 1500 bp amplicon. PCR was carried out in a 25 µl reaction volume containing 1 µL of 100ng genomic DNA template, 5 µL of 5X PCR Buffer, 2 µL of 2mM $MgCl_2$, 2 µL of 200 µM dNTP, 0.5 µL Taq Polymerase (Promega, USA), 12.5 µl of sterile distilled water and 1 µL of 20 pmol of each primers (First Base Laboratories, Malaysia). PCR amplification was performed with the following protocol; 94 °C for 10 min; 35 cycles of 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 90 sec; and a final extension of 72 °C for 10 min.

For ITS gene PCR amplification, primers user in this study were the ITS Forward (5'-CGGTGAATACGTTCCCGGGYCTTG-3') and the ITS Reverse (5'-TTTCRCCT TTCCCTCACGGTA-3').

These primers were expected to amplify a 1000-1200 bp amplicon. PCR was performed as follow; 94 °C for 3 min; 35 cycles of 94 °C for30 sec, 50 °C for 1 min, and 72 °C for 90 sec; and a final extension of 72 °C for 5 min. All PCR amplification was performed using Mastercycler Gradient PCR system (Eppendorf, Germany). Negative control without template was included in every set of reactions.

Detection of PCR products was carried out with 1 % agarose gel electrophoresis for 60 min at 70 volts. All PCR products were purified using GeneJET Gel Extraction Kit according to the manufacturer's instructions (*Thermo* Fischer *Scientific* Inc, USA). The purified DNAs were then sent for sequencing (First Base Laboratories Sdn. Bhd., Malaysia).

Sequence Alignment and Phylogenetic Tree Analysis

DNA sequences were compared to other known sequences from GenBank database using BLASTn (Nucleotide Basic Local Alignment Search Tool) searches of National Center for Biotechnology Institute (http://blast.st-va.ncbi.nlm.nih.gov/Blast.
cgi). All sequences were tanalyzed using BioEdit version 7.0 multiple alignment program (Hall, 2005).

Phylogenetic analysis of the aligned sequences was constructed using a neighbour-joining (NJ) method with 1000 replications utilizing MEGA 5.0 software (Tamura *et al*., 2011).

Results and Discussion

Morphological and Biochemical Characterization

Results from biochemical identification showed that all strains produced typical characteristics of *Aeromonas* spp., which were Gram-negative, motile and rod shaped in morphology. These strains were verified up to species level as *A. hydrophila, A. sobria, and A. hydrophila/caviae* using the API 20E system (Table 1) which in line with report by, Soler *et al*., 2003; Ormen *et al*., 2005).

16S rRNA Gene Amplification and Phylogeny Analysis

Amplification of 16S rRNA gene in all *Aeromonas* spp. strains each produced a 1500 bp amplicon as expected (Figure 1) as reported by Martinez-Murcia *et al*., 1992. Our BLASTn results of 16S Rrna gene showed all *Aeromonas* spp. strains revealed that 15 strains were classified as *A. hydrophila* and 7 strains were identical to *A. veronii*, all with 98-100 % similarities to GenBank nucleotide sequences database.

The neighbor-joining (NJ) 16S rRNA sequence phylogenetic tree showed a branching pattern that clearly separates all our *Aeromonas* spp. and reference strains into 2 major clusters, G1 and G2 (Figure 2). Based on the phylogenetic tree, *Aeromonas* spp. strains PK53S, PK60S and PK60L were grouped together with *A. hydrophila* reference strains from GenBank (accession Nos. GU563992, AB610604, FN997617 and GQ70996) with 98 % to 100 % bootstrap values (sub-group G1 (i)). The second cluster, G2 consisted of 4 minor sub-groups that are G2 (i), G2 (ii), G2 (iii) and G2 (iv). Sub-group G2 (i) comprised of the *Aeromonas* spp. strains K1B and PSM545,

while sub-group G2 (iii) showed 8 *Aeromonas* spp. strains which were clustered together with the reference strain, *Aeromonas jandeii* (accession No. JF713702). The last sub-group, G2 (iv) consisted most of our *Aeromonas* spp. strains that were clustered together (TPK5K, TTG6E, TTG11B, TPK2K, TTG11E, KTG3Sb, TTG9K, PK159S and TFT3B).

ITS Gene Amplification And Phylogeny Analysis

In this study, amplification of the 16S-23S ITS region on all *Aeromonas* spp. strains each yielded an amplicon of 1000-1200 bp (Figure 3). The results were in line with previous published findings that reported these diverse pattern bands (1000-1200 bp) are due to the tRNA number encoding gene and the number of copy ribosomal unit contained by the ITS (Singh *et al*., 2012).

The neighbor-joining phylogenetic tree of *Aeromonas* species based on the ITS had shown that the neighbor-joining tree has formed branches that labeled as G1 and G2 (Figure 4). The group G1 has formed the branch that consists of two samples (PSM545 and TPK6K). These two samples have been cluster into as one group (G1) due to the similarity of gene sequences. Based on G2 have formed two branches which are the G2 (i) and G2 (ii). The G2 (i) was formed a cluster known as G2 (a) consist of six samples clustered together. G2 (a) are clustered together with references strain of *Aeromonas hydrophila* (HM856362.1). The G2 (e) shown a sample of TTG6E was similar to *Aeromonas caviae* (AF198382.1).

The largest cluster group was G2 (f) where this cluster formed two branches which is known as G2 (f) and G2 (f) b. The variation in gene sequences are very high (mutation) but there were some regions for conserved gene sequences (the point shows similarities of sequences). According to previous study by Kong *et al*. (1999), the heterogeneities (sequence and length polymorphisms) were observed based on the alignment in the central region of the spacers and mention that this 16S-23S (ITS) that the profiling of rDNA has potential as a quick rDNA typing and strain detection tool for *Aeromonas* species.

Kong *et al*., (1999) also have stated that in his study that, the highest similarity was observed amongst three *Aeromonas hydrophila* (95 % - 99 %). This can be refer to the Berridge *et al*. (2001) and Hassan *et al*. (2001), the ITS region has been useful to identified the species level as in instance relative genus analysis of the *Streptococcus* and *Staphylococcus.*

Comparison between biochemical tests, 16S rRNA and ITS gene analysis for identification and characterization of *Aeromonas* spp. Strains

The comparison of phenotypic and genetic identification of 22 *Aeromonas* strains isolates from freshwater fishes species revealed in Table 2. Biochemical test results towards the samples mostly detected the 59.1 % (13/22) of *Aeromonas sobria* and 36.4 % (8/22) of *Aeromonas hydrophila*/ *caviae*. *B*ased on 16S rRNA gene sequence results, showed that most of the detection were 63.6 % (14/22) from *Aeromonas hydrophila*, 18.2 % (4/22) from *Aeromonas hydrophila*/*veronii* and 18.2 % (4/22) from *Aeromonas veronii.*

ITS gene sequences showed that most of the detection comes from 63.6 % (14/22) of *Aeromonas hydrophila* and 36.4 % (8/22) of *Aeromonas veronii*.

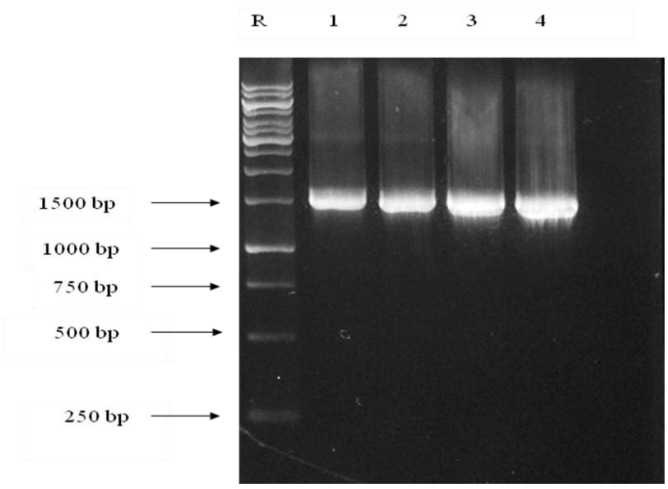
Table.1 *Aeromonas* spp. cultures used in this study (2010-2013).

No.	Strain reference	Source	Location	Morphological and Biochemical Characterization				
				Gram Stain	Catalase	Oxidase	API 20E system	%
1	KTG 3S(b)	*Clarias* spp.	Tasek Gelugor	-ve (rod)	+ve	+ve	*A. sobria*	99.2
2	K1B	*Clarias* spp.	Fri Gelami-Lemi	-ve (rod)	-ve	+ve	*A.hydrophila*	-
3	PHE 578L	*Pangasius* spp.	Pahang	-ve	-ve	+ve	*A. sobria*	99.4
4	PK 159S	*Pangasius* spp.	Pahang	-ve (short, rod)	-ve	+ve	*A. sobria*	99.2
5	PK 60L	*Pangasius* spp.	Pahang	-ve (rod)	+ve	+ve	*A.hrdrophila/caviae*	99.7
6	PK 60S	*Pangasius* spp.	Pahang	-ve (rod)	+ve	+ve	*A.hrdrophila/caviae*	99.7
7	PK 53S	*Pangasius* spp.	Pahang	-ve (rod)	+ve	+ve	*A.hrdrophila/caviae*	99.7
8	PSM 545	*Pangasius* spp.	Pahang	-ve (short, rod)	+ve	+ve	*A. sobria*	83.8
9	TPK2B	*Oreochromis* spp.	Pantai Kamloon	-	-	-	*A.sobria*	99.2
10	TPK4B	*Oreochromis* spp.	Pantai Kamloon	-	-	-	*A.sobria*	99.2
11	TPK5B	*Oreochromis* spp.	Pantai Kamloon	-	-	-	*A.sobria*	94.9
12	TTG9K	*Oreochromis* spp.	Tasik Gelugor	-ve	+ve	+ve	*A.hydrophila/caviae*	95.7
13	TFT 3B	*Oreochromis* spp.	Felda Titi	-ve	+ve	+ve	*A.sobria*	-
14	TPK 5K	*Oreochromis* spp.	Pantai Kamloon	-	-	-	*A.sobria*	94.9
15	TTG 11K	*Oreochromis* spp.	Tasik Gelugor	-ve	+ve	+ve	*A.hydrophila/caviae*	99.7
16	TFT 3K	*Oreochromis* spp.	Felda Titi	-ve	+ve	+ve	*A.sobria*	-
17	TFT 3E	*Oreochromis* spp.	Felda Titi	-ve	+ve	+ve	*A.sobria*	-
18	TPK 2K	*Oreochromis* spp.	Pantai Kamloon	-ve	+ve	+ve	*A.sobria*	94.9
19	TTG11E	*Oreochromis* spp.	Tasik Gelugor	-ve	+ve	+ve	*A.hydrophila/caviae*	95.7
20	TTG6E	*Oreochromis* spp.	Tasik Gelugor	-ve	+ve	+ve	*A.hydrophila/caviae*	95.7
21	TTG11B	*Oreochromis* spp.	Tasik Gelugor	-ve	-ve	+ve	*A.hydrophila/caviae*	99.7
22	TPK 6K	*Oreochromis* spp.	Pantai Kamloon	-	-	-	*A.sobria*	82.2

Table.2 Comparison of phenotypic and genetic identification of 22 *Aeromonas* spp. strains isolates from freshwater fish species.

No.	Strain	Table 3: Taxonomic identification (species name) based on		
		Biochemical Test	**16S rRNA gene sequences**	**ITS gene sequences**
1	KTG 3S(b)	A. *sobria*	*A. hydrophila/veronii*	*A. veronii*
2	K 1 B	A. *hydrophila*	*A. hydrophila*	*A. hydrophila*
3	PHE 578L	*A. sobria*	*A. hydrophila*	*A. veronii*
4	PK159S	*A. sobria*	*A. hydophila*	*A. veronii*
5	PK60L	*A. hrdrophila/caviae*	*A. hyrophila*	*A. hydrophila*
6	PK60S	*A. hrdrophila/caviae*	*A. hydrophila*	*A. hydrophila*
7	PK53S	*A. hrdrophila/caviae*	*A. hydrophila*	*A. hydrophila*
8	PSM545	*A. sobria*	*A. hydrophila*	*A. hydrophila* subsp. *hydrophila*
9	TPK2B	*A. sobria*	*A. hydrophila*	*A. veronii*
10	TPK4B	*A. sobria*	*A. hydrophila*	*A. veronii*
11	TPK5B	*A. sobria*	*A. hydrophila*	*A. hydrophila*
12	TTG9K	*A. hydrophila/caviae*	*A. veronii*	*A. hydrophila* subsp. *hydrophila*
13	TFT3B	*A. sobria*	*A. hydrophila*	*A. hydrophila* subsp. *hydrophila*
14	TPK5K	*A. sobria*	*A. hydrophila*	*A. veronii*
15	TTG11K	*A. hydrophila/caviae*	*A. hydrophila/veronii*	*A. hydrophila* subsp. h*ydrophila*
16	TFT3K	*A. sobria*	*A. veronii*	*A. hydrophila* subsp. *hydrophila*
17	TFT3E	*A. sobria*	*A. veronii*	*A. hydrophila* subsp. *hydrophila*
18	TPK2K	*A. sobria*	*A. veronii*	*A. veronii*
19	TTG11E	*A. hydrophila/caviae*	*A. hydrophila/veronii*	*A. veronii*
20	TTG6E	*A. hydrophila/caviae*	*A. hydrophila*	*A. hydrophila* subsp. *hydrophila*
21	TTG11B	*A. hydrophila/caviae*	*A. hydrophila/veronii*	*A. hydrophila* subsp. *hydrophila*
22	TPK6K	*A. sobria*	*A. hydrophila*	*A. hydrophila* subsp. *hydrophila*

Fig.1 Agarose gel electrophoresis analysis of PCR amplification of the 16S rRNA gene of *Aeromonas* spp. strains.



Lane R: Gene ruler DNA ladder 1kb (Fermentas)
Lane 1: *Aeromonas* spp. K1B
Lane 2: *Aeromonas* spp. TPK 6K
Lane 3: *Aeromonas* spp. TTG11B
Lane 4: *Aeromonas* spp. TTG6E
Lane 5: Control

Fig.2 Neighbor-joining phylogenetic tree of *Aeromonas* spp. from freshwater fishes and reference strains based on the 16S gene sequences. Number on each node is the bootstrap value (1000 replicates). The scale bar represents 1 nucleotide substitution per 10 nucleotides.

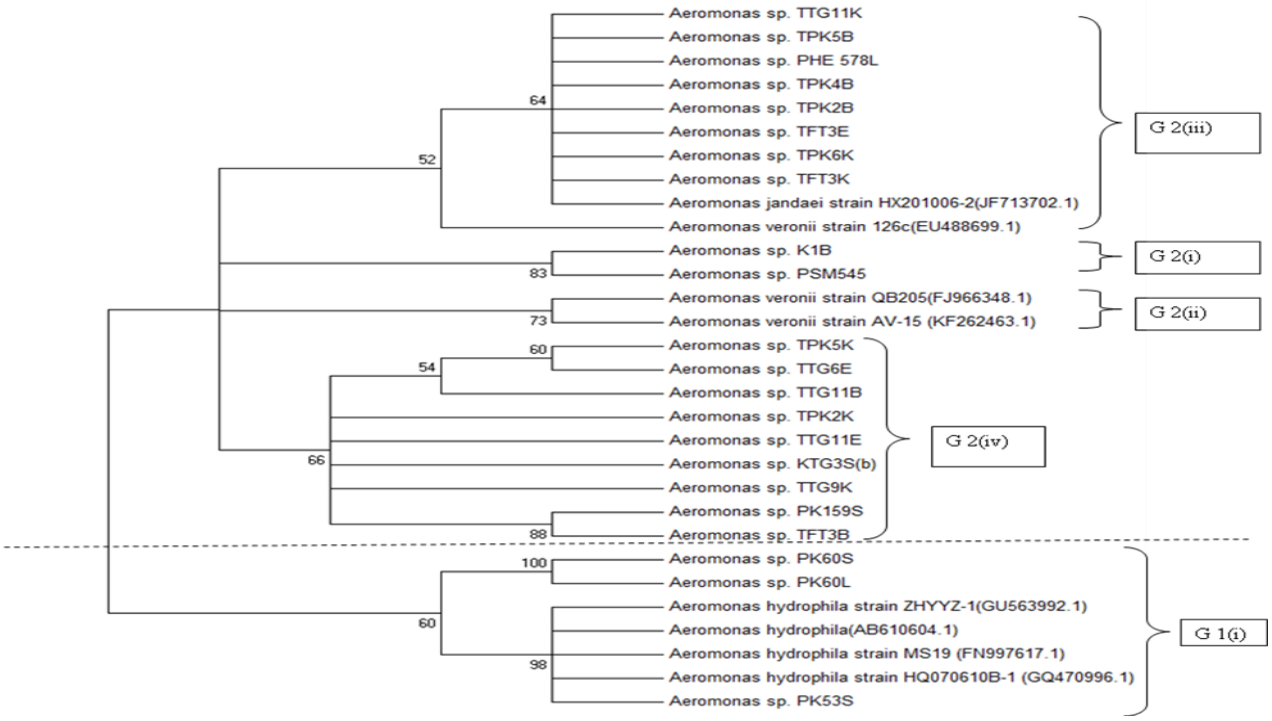
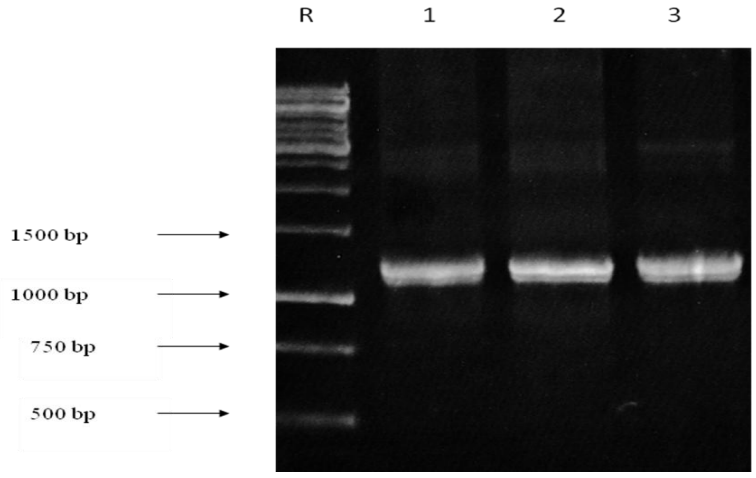
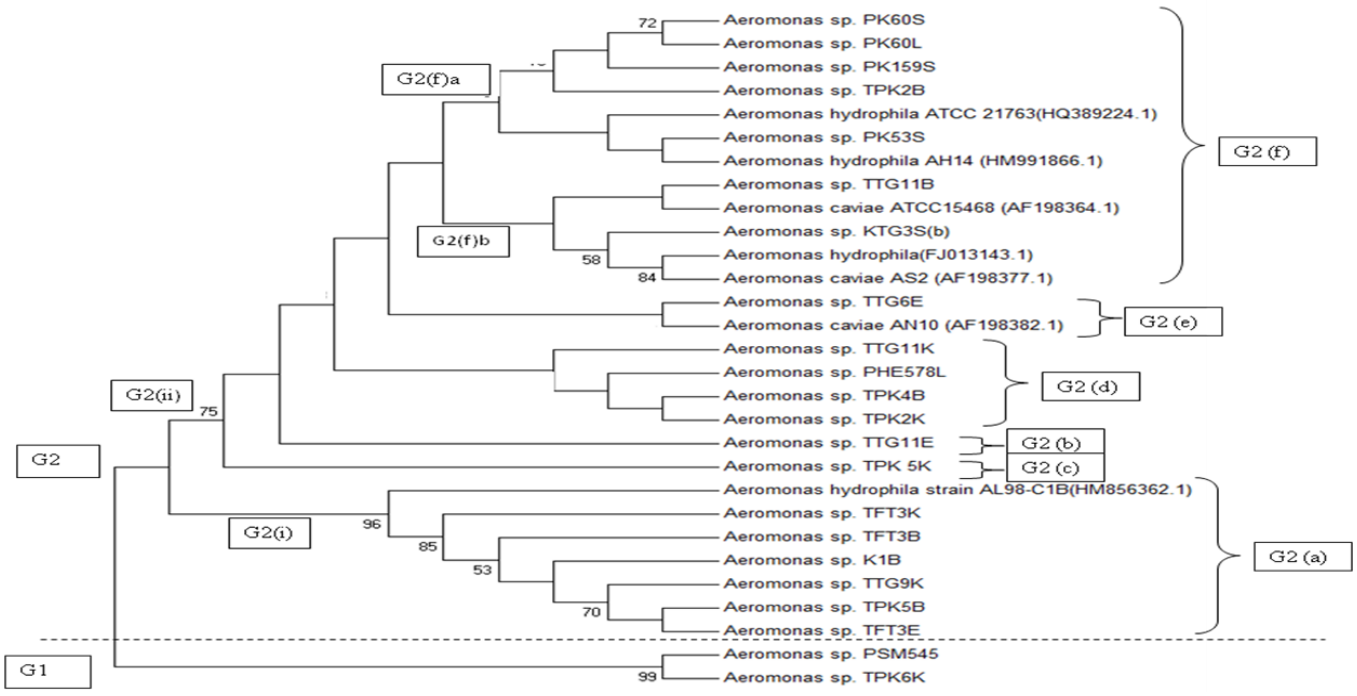


Fig.3 Agarose gel electrophoresis analysis of PCR amplification of the ITS rRNA gene of *Aeromonas* spp. strains.



Lane R: Gene ruler DNA ladder 1kb (Fermentas)
Lane 1: *Aeromonas* spp. KTG3S
Lane 2: *Aeromonas* spp. TPK2B
Lane 3: *Aeromonas* spp. PK159S

Fig.4 Neighbor-joining phylogenetic tree of *Aeromonas* spp. from freshwater fishes and reference strains based on the ITS gene sequences. Number on each node is the bootstrap value (1000 replicates). The scale bar represents 1 nucleotide substitution per 10 nucleotides.



The both molecular techniques used in this study have showed there are discrepancies on the detection results from the 22 isolates compared with biochemical test. As in the previous study was highlighted that there still have discrepancies in data collected based on the molecular techniques and biochemical test (Borrell *et al*., 1997; Castro-Escarpulli *et al*., 2003). The molecular comparison between the 16S rRNA gene sequences with the ITS gene sequences also shows conflict to determine up to the species level

In conclusion, in the present study, this can be conclude that the ITS can be useful tools to identify *Aeromonas* species until to the species level. Besides that, molecular diagnosis of fish pathogen can be analyzed using the ITS – 16S Rrna-PCR based method which proved the most reliable method in determined and identified bacterial species including *Aeromonas* isolates.

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