ASSESSMENT OF RIVER WATER HEALTH OF LATA JANGGUT, KELANTAN USING BACTERIAL MORPHOLOGICAL AND 16S rDNA ANALYSIS

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ABSTRACT

Lata Janggut, Kelantan is famous among visitors for its cascading waterfall and the natural surroundings. As there is limited information on the water health of the Lata Janggut, the study was conducted to investigate the presence of microbes in order to gauge the freshwater ecosystem health status. The diversity of microbes was determined based on bacterial morphology and molecular data. A total of 15 pure colonies were isolated from 60 water samples from Lata Janggut river following one year of monthly water sampling. Gram staining and microscopic method were used for morphological identification. Eleven colonies were gram-positive and the other four are gram-negative bacteria. Bacillus shaped bacteria form the largest constituent of the isolates, followed by cocci, coccobacillus and dipplobacilli shaped bacteria. Four gram-negative rod-shaped bacteria were chosen for amplification of the 16S rDNA region and the purified products were sent for sequencing. The NCBI BLAST results of these sequences indicated that the dominating bacteria found in Lata Janggut water belonged to the *Aeromonas* genus. This finding was confirmed by the phylogeny trees constructed based on the 16S rDNA gene. A thorough survey for *Aeromonas* spp. was suggested for Lata Janggut river water to facilitate proper river water mitigation planning in the future.

Keywords: 16S rDNA, Lata Janggut, water microbial diversity, *Aeromonas*

Received (01-March-2018); Accepted (18-July-2018); Available online (26-July-19)

Citation: Vivian, T., Hassan, K.N.A.K., Ramli, N.S., Jayaraj, V. K. & Appalasamy, S. (2019). Assessment of river water health of Lata Janggut, Kelantan using bacterial morphological and 16S rDNA Analysis. *Journal of Wildlife and Parks,* **34**: *In press*.

INTRODUCTION

Jeli is an area in the Kelantan state which possesses many spectacular geological landscapes, unique geological phenomena, and treasured earth materials. In this district, a few geoheritage sites have been discovered such as Gunung Reng, Jeli Hot Spring, Pergau Lake, gold deposits in Kampung Kalai, Lata Janggut, Lata Renyok, Lata Chenai, Sungai Rual, and Setir Cave complex (Adriansyah *et al*., 2015).

There are a total of seven waterfalls in Kelantan area such as Jeram Mak Nenek, Jeram Linang, Jeram Pasu, Lata Janggut, Lata Berangin, Lata Kertas, and Lata Rek. Lata Janggut is a recreational area visited by many tourists from Kelantan and other states of Malaysia.

Previously, there is no research done on Lata Janggut waterfall and river water or the microorganisms that are present in the river water. Hence, this study was conducted to investigate the health status of the river using morphological and 16S rDNA data to confirm the microbe species found in Lata Janggut. Proper mitigation measures could be suggested and planned by relevant parties using the data obtained from this study.

MATERIALS AND METHODS

Lata Janggut Water Sampling

The study was carried out at Lata Janggut, Jeli, Kelantan (Figure 1). Lata Janggut (coordinates: $5^0$40'9" N, $101^0$46'12.5" E) is situated at the western part of Kelantan. The river passes through Kampung Lata Janggut in the Batu Melintang sub-district, approximately 15 kilometers away from Jeli, Kelantan.

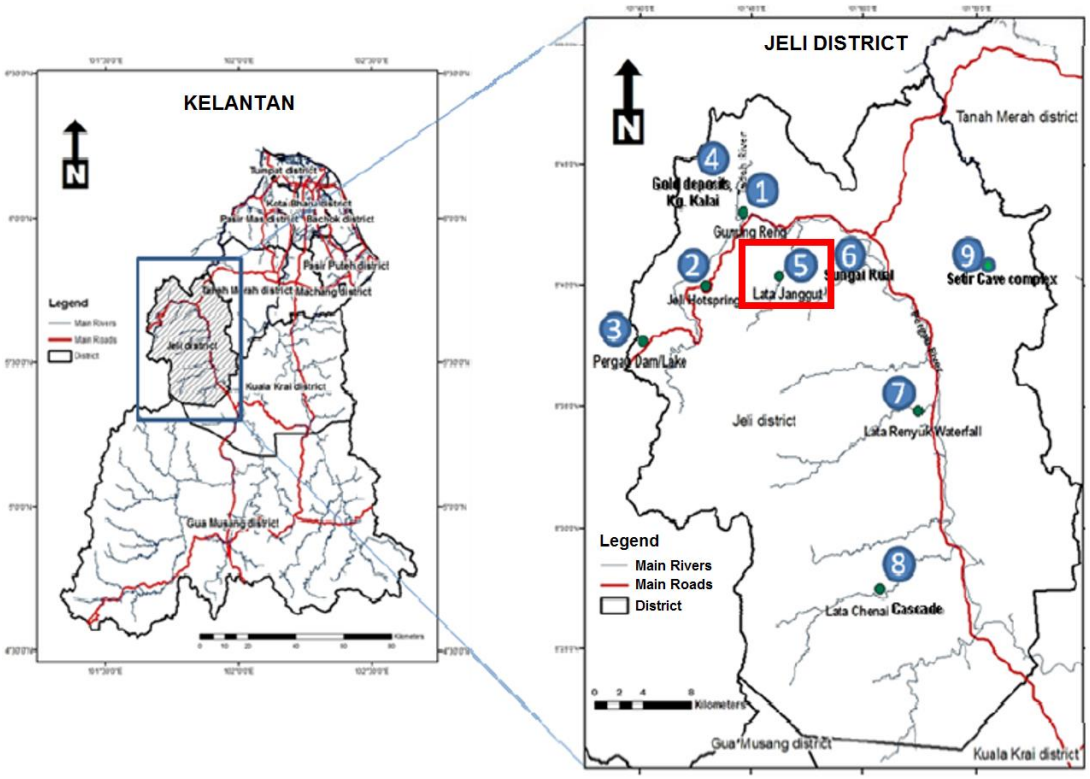

Figure 1 Location of Lata Janggut, Kelantan marked with red box (Adriansyah *et al*., 2015)

River water samples were taken at the depth of 10 cm by using 50 ml sterile Falcon tube. The water sampling was taken randomly at five points along the riverbank of Lata Janggut. The falcon tubes were labeled with name, date, and location. The water samples were stored in a cooler box with sufficient amount of ice and were brought back to the laboratory within 24 hours for storage at 4°C before continuing to bacterial isolation step the next day. Water sampling was repeatedly done every month for 12 months (one year). A sunny day with no rain for the last 24 hours was chosen every month as sampling day.

Isolation of Bacteria

Bacterial isolation from the water sample and culture plates were prepared following the method reported by Bahrin *et al.* (2017). The isolated bacteria were cultured on nutrient agar and the culture plates were then incubated at 30°C for 24-48 hours. Pure bacterial cultures obtained from the monthly river water samples were maintained on nutrient agar at 4°C for further investigation.

Cell Morphology Observation of Pure Bacterial Culture

The morphology identification was done following the method described by Robert (2009). The shape and Gram staining of bacteria were observed by using a compound microscope.

Bacterial DNA Isolation

The bacterial DNA was isolated from pure cultures using the method published by Baharin *et al.* (2017). The pellet of extracted DNA was suspended in 50 µL of sterile TE buffer (pH8.0) and was kept at -20°C freezer.

Quality Analysis of Bacterial DNA

One microliter of extracted bacterial DNA was used to assess DNA quality at A260 and A280 using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, EUA).

Agarose Gel Electrophoresis of Extracted Genomic Bacterial DNA

The extracted bacterial genomic DNA were electrophoresed using 1% agarose gel (Sigma-Aldrich, St. Louis, Missouri, USA) at 80 volts for 30 minutes. The agarose gel with the bacterial DNA was stained with three µL of ethidium bromide (10 mg/mL) in a dark room of Microbiology Laboratory, Universiti Malaysia Kelantan, Jeli Campus. The stained DNA was viewed and documented using Gel Doc XR System (Bio-Rad Laboratories, Berkeley, USA).

Polymerase Chain Reaction (PCR) For the Amplification of the Bacterial 16S rDNA Gene

The amplification of 16 rDNA region from the bacterial genomic DNA was performed using the method reported by Hassan (2017). Four bacterial genomic DNA samples were chosen for this amplification based on the frequency of these four bacteria isolated from the water sample of Lata Janggut. The sequences of 16S rDNA universal primers that were used in this study were (i) 16S-F 5'-GAG TTT GAT CCT GGC TCA G-3', and (ii) 16S-R 5'-AGA GAG GTG ATC CAG CC-3'. The presence of amplicons for each bacterial DNA was detected using agarose gel electrophoresis at 1% and the products were purified using a spin column (Intron Biotechnology, South Korea) protocol.

DNA Sequencing and Analysis of 16S rDNA Amplicons

The purified PCR products were sequenced by a commercial sequencing service provider (First Base Laboratory Sdn. Bhd. Malaysia). The forward and reverse sequences for each of the four DNA sequences were aligned using Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/index.html) to obtain a composite sequence. The quality

of the sequence trace was assessed and trimmed. These combined sequences were then compared using Basic Local Alignment Search Tool (BLAST) and the National Centre for Biotechnology Information (NCBI) server at https://blast.ncbi.nlm.nih.gov/ database based on the percentage of the homology sharing for bacteria identification (Ng, 2011; Chong, 2016). For species-level identification, ≥ 99% of similarity rate was required while for genus-level identification, ≥ 97% of similarity rate was required (Drancourt *et al.,* 2000). Phylogeny tree was constructed using MEGA version 6 software (http://www.megasoftware.net/home). Neighbour-joining (NJ) method based on 1000 replications bootstrapping value was applied to estimate the confidence of tree topologies (Saitou & Nei, 1987; Ng, 2011).

RESULTS AND DISCUSSION

Morphology Identification of Water Microbes

The pure bacterial isolates from Lata Janggut water were characterised based on morphology characteristics (i.e., shape and the Gram stain). Eleven colonies are gram-positive and the other four are gram-negative bacteria. The shapes consist of bacillus was found to be the large constituent shape (6 cultures), followed by cocci (4 cultures), coccobacillus (3 cultures) and dipplobacilli (2 cultures) shaped bacteria.

Cabral (2010) reported that Gram-positive bacteria with bacillus shape found in the upper layer of freshwater are commonly lactobacillus, clostridia, bifidobacterium, and enterococci. Enterococcus are Gram-positive and normally occurs as a single colony, in pairs or short-chain (Zapun *et al.,* 2008). Due to the persistence of enterococci in the environment and ubiquity in the human feces, presence of enterococci in river water is deemed as an indicator to human fecal contamination into river water (Boehm & Sassoubre, 2014).

16S rDNA Analysis of Bacteria DNA Isolated from Lata Janggut

The classification of bacterial species has traditionally been based on morphological and growth characteristics, antibiotic resistance, and biochemical testing. However, these methods are known to be not exclusive, laborious, and time-consuming (Brown-Elliott *et al.,* 2006). Hence, molecular methods have been reported to be able to provide crucial insights into the identification and classification of the bacteria. The DNA region commonly used for taxonomic purposes is 16S rDNA gene sequence analysis which can be used for species identification for bacteria that are poorly described, rarely isolated and studied, novel pathogens and phenotypically aberrant strains (Palys *et al.,* 1997; Kolbert & Persing, 1999). Previous studies have shown that the quality of 16S rDNA sequences is imperative for accurate phylogenetic placement and identification determination (Schloss, 2010). The A260/A280 ratio for the bacterial genomic DNA isolated was determined to be in the range of 1.8 to 2.0, suggesting a high quality of the DNA preparations (Table 1). This observation indicated that the protocol reported by Bahrin *et al.* (2017) could be adapted for isolation of river water bacterium DNA. Figure 2 showed the agarose gel electrophoresis of bacterial genomic DNA which conformed to the spectrophotometer reading in Table 1. There was no DNA sample exhibiting purity ratio higher than 2.0, indicating the absence of RNA contaminations. Pure nucleic acids normally produce a A260/A280 ratio of ~1.8 for DNA and a A260/A280 ratio of ~2.0 for RNA (Desjardins & Conklin, 2010). Thus, the success of the extraction method in isolating bacterial genomic DNA from water samples were determined by agarose gel electrophoresis. Figure 2 showed the agarose gel electrophoresis results for 15 DNA samples from bacteria isolated from

Lata Janggut water. The expected size of bacterial genomic DNA was 10 kbp. The agarose gel electrophoresis result in Figure 2 showed that Lane 1 to 15 contained visible bands above 10 kbp as compared to the one kb ladder (Promega Corp., United States) which indicates the success of bacterial DNA isolation from the water sample.

Table 1 UV-Spectrophotometer analysis of bacterial DNA isolated from Lata Janggut water sample

Bacterial DNA	**Purity (A_{260}/ A_{280})**
UMK_SLLG_1_DNA	1.93
UMK_SLLG_3_DNA	1.82
UMK_SLLG_4_DNA	1.82
UMK_SLLG_5_DNA	1.91
UMK_SLLG_6_DNA	1.84
UMK_SLLG_7_DNA	1.87
UMK_SLLG_8_DNA	1.80
UMK_SLLG_9_DNA	1.85
UMK_SLLG_10_DNA	1.87
UMK_SLLG_11_DNA	1.83
UMK_SLLG_12_DNA	1.88
UMK_SLLG_13_DNA	1.89
UMK_SLLG_14_DNA	1.80
UMK_SLLG_15_DNA	1.81

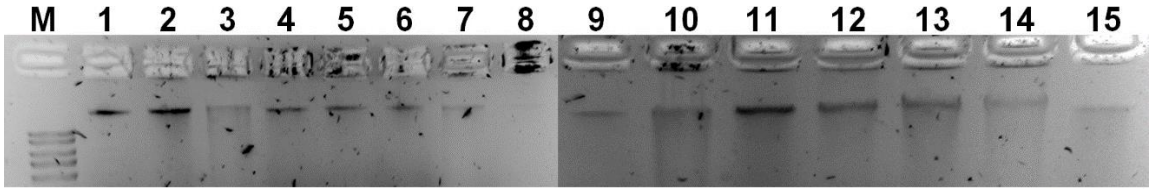

Figure 2 Agarose gel electrophoresis of bacterial DNA isolated from Lata Janggut water samples. Lane 1 to 15 show the presence of bacterial DNA isolated from water sample visualised under UV transilluminator, run with 1% agarose gel at 90V for 60 minutes. Lane 1: UMKSLLG_1_DNA, Lane 2: UMKSLLG_2_DNA, Lane 3: UMKSLLG_3_DNA, Lane 4: UMKSLLG_4_DNA, Lane 5: UMKSLLG_5_DNA, Lane 6: UMKSLLG_6_DNA, Lane 7: UMKSLLG_7_DNA, Lane 8: UMKSLLG_8_DNA, Lane 9: UMKSLLG_9_DNA. Lane 10: UMKSLLG_10_DNA, Lane 11: UMKSLLG_11_DNA, Lane 12: UMKSLLG_12_DNA, Lane 13: UMKSLLG_13_DNA, Lane 14: UMKSLLG_14_DNA, Lane 15: UMKSLLG_145_DNA. Lane M: 1kb DNA ladder (Promega, United States)

Four bacteria with bacillus shape were chosen based on the highest occurring of these bacteria in the sampled river sample every month. Figure 3 shows the amplification of the 16s rDNA sequence in the four bacterial DNA isolated from Lata Janggut water sample by PCR. All four species of bacteria were identified to be from the *Aeromonas* genus. The BLAST results for all sequences results were summarised in Table 2. The sequences were found to be 98%, 96%, 98% and 96% similar to *Aeromonas cavernicola, Aeromonas hydrophilla,* and *Aeromonas*

salmonicida. However, 16S rRNA gene has different resolving power among bacterial taxa and in this study, 16S rRNA gene was unable to resolve species difference in *Aeromonas* genus. The bacterial species could not be deduced based on the BLAST results alone.

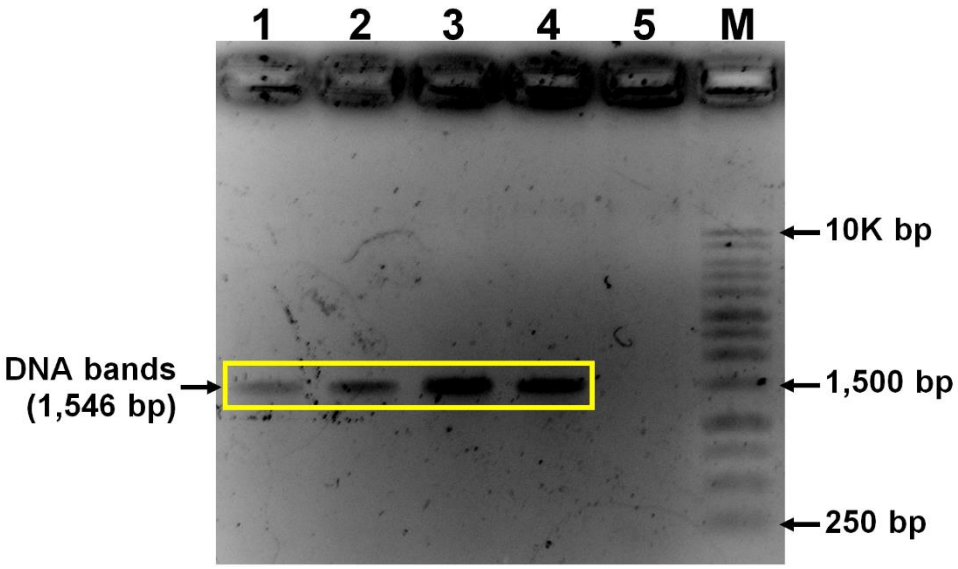

Figure 3 Amplification of 16S rDNA gene for four bacterial DNA isolated from Lata Janggut water sample. Lane 1 to 4 contained 5µl of PCR products of bacterial DNA, electrophoresed on 1% agarose gel at 90V for 60 minutes. Lane 1: UMK_SLLG_3_ DNA; Lane 2: UMK_SLLG_4_ DNA; Lane 3: UMK_SLLG_5_ DNA; Lane 4: UMK_SLLG_8_ DNA; Lane 5: Negative control (no DNA). Lane M: 1kb DNA ladder (Promega, United States)

Table 2 Identification of water sample isolates from Lata Janggut, Kelantan by comparing the percentage of similarity with the closest match from Nucleotide BLAST

DNA samples	Closest match from Nucleotide BLAST	Accession number	% Similarity
UMK_SLLG_3_ DNA	*Aeromonas cavernicola*	NR 132718.1	98%
UMK_SLLG_4_ DNA	*Aeromonas hydrophilla*	NR 119190.1	96%
UMK_SLLG_5_ DNA	*Aeromonas cavernicola*	NR 132718.1	98%
UMK_SLLG_8_ DNA	*Aeromonas salmonicida*	NR 118547.1	96%

The phylogenetic trees were constructed as shown in Figure 4 to 7. There are four types of phylogenetic trees construction which are NJ, Unweighted Pair Group Method with Arithmetic Mean (UPGMA), Maximum Parsimony (MP) and Maximum Likelihood (ML) (Bíró, 2015). In this study, the phylogenetic trees were constructed by using NJ method. This method was selected for this study as it is based on minimum evolution principle (Saitou & Nei, 1987) which has the highest accuracy compared to the other three methods (Kim *et al*., 1993). The constructed phylogenetic trees for the four sequences of bacteria DNA from Lata Janggut water sample showed that the identity of the culture UMK_SLLG_3_DNA was not conclusive but only confirming the sequences belong to the genus *Aeromonas.*

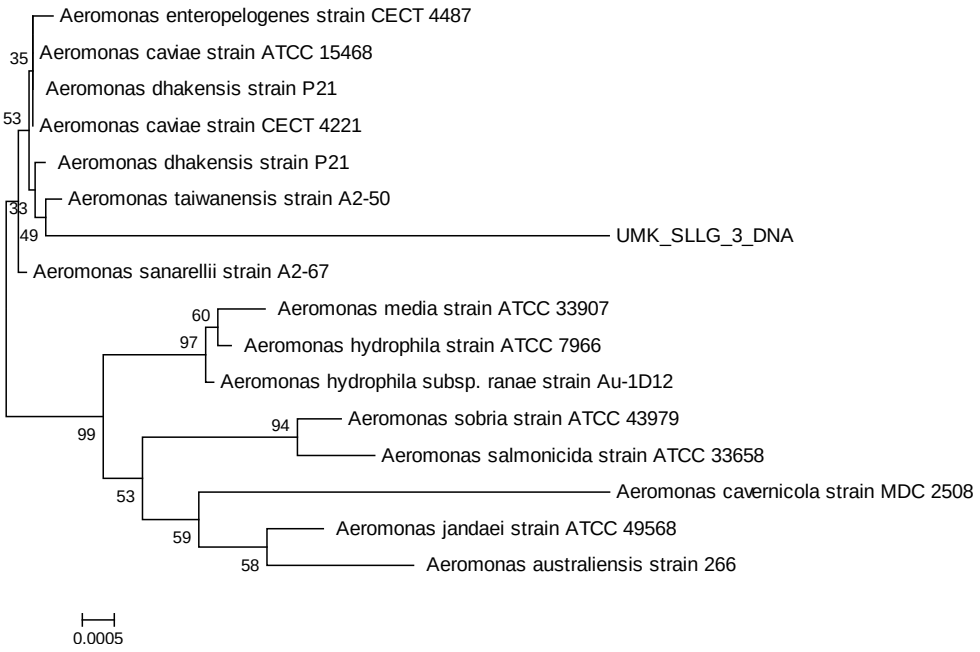

Figure 4 The phylogenetic relationship between UMK_SLLG_3_DNA with the closest sequences found in Nucleotide BLAST. The percentage of similarity that had taken for comparing with UMK_SLLG_3_DNA were 98% and 96%. The phylogenetic relationship is constructed using MEGA6

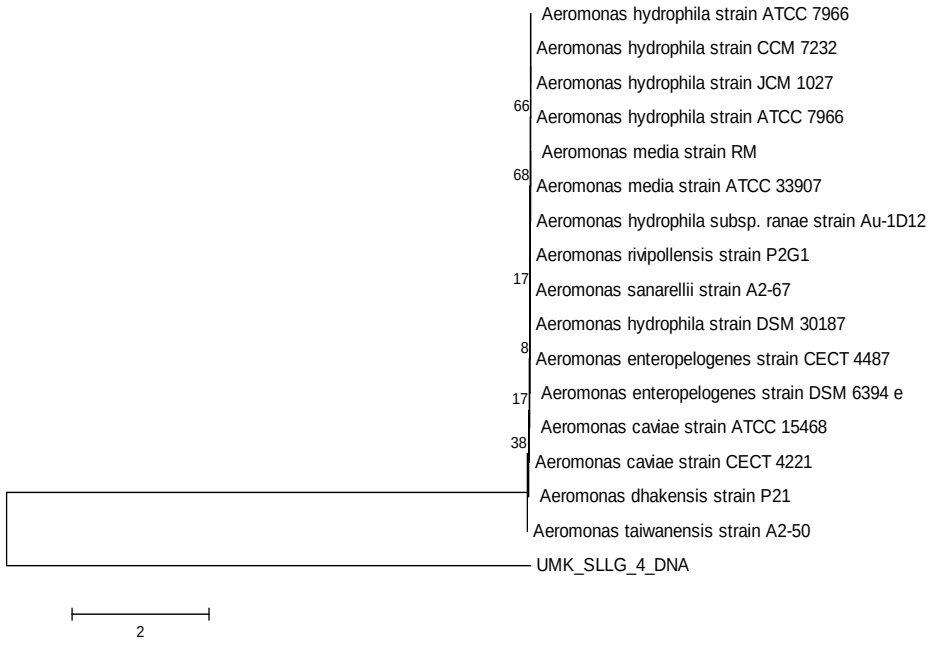

Figure 5 The phylogenetic relationship between UMK_SLLG_4_DNA with the closest sequences found in Nucleotide BLAST. The percentages of similarity that had taken for comparing with UMK_SLLG_4_DNA were 96% and 95%. The phylogenetic relationship is constructed using MEGA6

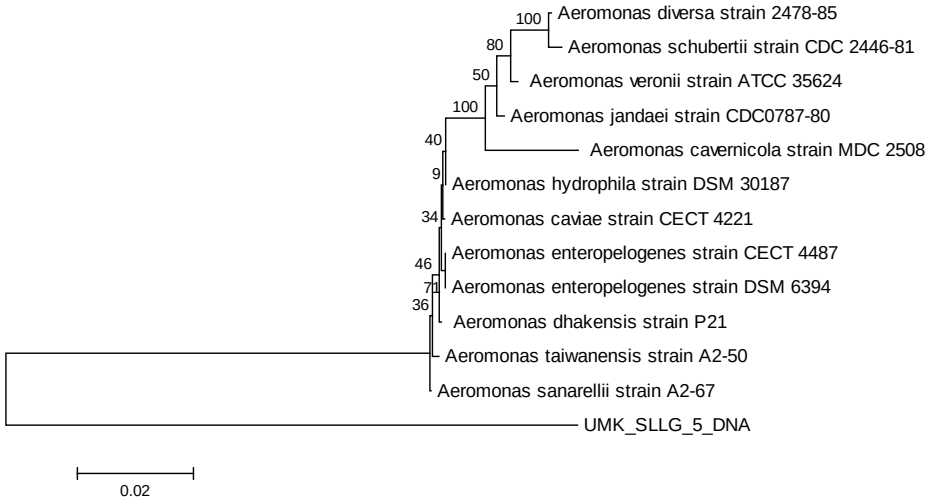

Figure 6 The phylogenetic relationship between UMK_SLLG_5_DNA with the closest sequences found in Nucleotide BLAST. The percentages of similarity that had taken for comparing with UMK_SLLG_5_DNA were 98%, 97%, and 95%. The phylogenetic relationship is constructed using MEGA6

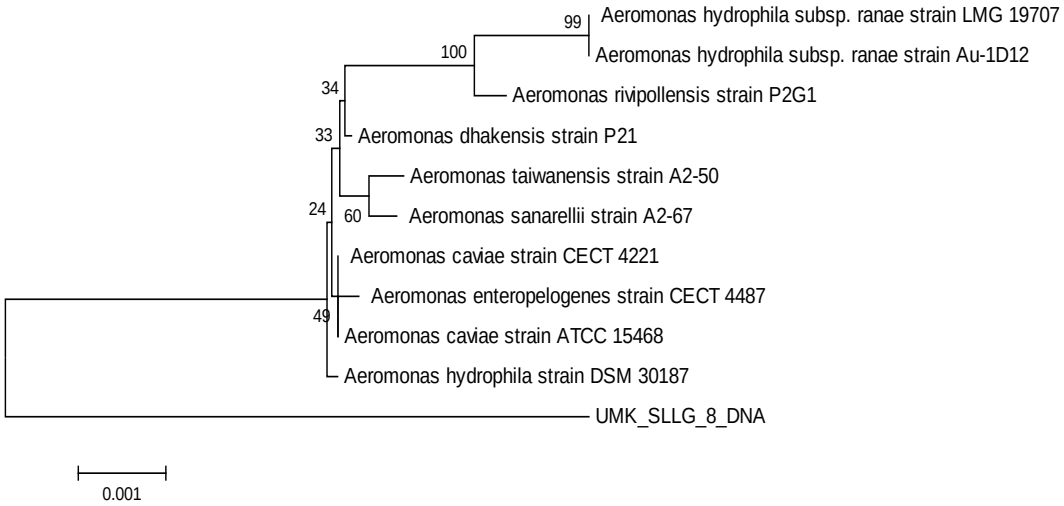

Figure 7 The phylogenetic relationship between UMK_SLLG_8_DNA with the closest sequences found in Nucleotide BLAST. The percentages of similarity that had taken for comparing with UMK_SLLG_8_DNA were 96% and 95%. The phylogenetic relationship is constructed using MEGA6

In the future, bacteria genome-level comparison study and species-specific DNA markers could be used to further distinguish bacteria species from each other and also for more accurate species assignment of the *Aeromonas* spp. *Aeromonas* spp. are common aquatic bacteria found in river water, freshwater, groundwater and even in bottled mineral water (Dumontet *et al.*, 2000). In recent years, the detection of *Aeromonas* spp. in aquatic ecosystem has increased due to the emerging human pathogenic properties caused by this bacteria group (Miyagi *et al.*, 2016). *Aeromonas* spp. has been reported to cause septicaemia, wound infections, and

diarrhoeal illness in people who consumed contaminated water or exposed open wound to contaminated river water (Gavriel *et al.*, 1998; Fewtrell & Bartram, 2001; Pianetti *et al.*, 2005). In Malaysia, 4% of diarrhea among children was reported due to *Aeromonas* species (Lee & Puthucheary, 2002). Therefore, the findings of this study should be used as preliminary data for further Lata Janggut water mitigation to avoid the spread of human-related disease among recreational visitors.

CONCLUSION

Through this study, four rod-shaped bacteria isolated from Lata Janggut river water was classified under the *Aeromonas* genus which was confirmed with molecular data. These rod-shaped bacteria were found to be commonly isolated from Lata Janggut river water. Hence, a thorough survey for the *Aeromonas* spp. in this river was suggested for proper mitigation planning of the river water.

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