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MOLECULAR IDENTIFICATION OF DENITRIFYING BACTERIA TO EVALUATE THE SENSITIVITY OF SELECTIVE MEDIA

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ABSTRACT

Eutrophication is the main problem in Rawa Pening that occurs due to the high supply of organic material from various sources. The high level of nitrate in the water can be reduced through denitrification mechanism using some bacteria such as *Pseudomonas* group. This bacteria group was often isolated using Glutamate Starch Phenol (GSP) selective media. This study aims to identify the species from the floating net cage of Rawa Pening that was suspected as Pseudomonas group based on colony morphology in GSP through molecular approach, build the phylogenetic tree, and analyze the genetic distance. The research was conducted from January to May 2020. The molecular approach was carried out using the Polymerase Chain Reaction (PCR) method with 16S rRNA gene amplification. The results of BLAST analysis showed that isolate D1 which obtained from water samples in the Rawa Pening floating net cage area had 99.23% homology with *Acinetobacter junii* strain tu13. The partial sequence of DNA was already deposited to GenBank with accession number LC603787. The phylogenetic tree construction was built using Neighbor-Joining analysis on MEGA X software. The results showed that isolate D1 was related to *Acinetobacter junii* strain tu13 with 0.006 genetic distance while *Pseudomonas fluorescens* with 0.144 genetic distance. The result revealed that isolate D1 closely related to *A. junii* rather than *Pseudomonas* group. This study indicated that GSP medium has a sensitivity range at the Order level, namely Pseudomonadales.

Keywords: *Acinetobacter junii*; denitrifying bacteria; GSP medium; Pseudomonas; Rawa Pening

INTRODUCTION

Eutrophication is characterized by high content of nitrogen and phosphorus in water, resulting in excessive growth of aquatic plants which has become a global environmental issue (Liu *et al.*, 2010). Rawa Pening is one of the lakes in Indonesia which has a major problem with the eutrophication phenomenon. This is caused by various anthropogenic activities around the lake which supply organic matter. Rawa Pening acts as a sink for pollutants released by industrial, urban, and agricultural development which has increased the loads of fertilizers and metal pollutants to the lake. Now, over 70% of the lake is covered with water hyacinth (Soeprobowati, 2015).

Eutrophication in Lake Rawa Pening is affected by the nitrate content in the waters. According to Indonesian Ministry of Environment (2012), total N pollutant load in the Lake Rawa Pening which comes from domestic, agricultural, livestock, and fisheries waste is 28,307.07 kg/day. This pollutants generate a relatively high nitrate content ranging from 0.027 to 1.8 mg/L (Sulastri *et al.*, 2016). Nitrate content of more than 0.2 mg/L is a trigger factore for the rapid growth of aquatic plants such as water hyacinth (Tatangindantu *et al.*, 2013).

Reducing N from wastewater by using biological treatments is the most straightforward action in eutrophication control (Zhang *et al.*, 2019). This action can be done by utilizing denitrifying bacteria (Zhang *et al.*, 2020).

Denitrifying bacteria has been studied because denitrification process can effectively remove excess NO_3- from water system (Zheng *et al.*, 2016). NO_3- is converted by denitrifying bacteria to inert nitrogen gas and the waste product usually contains only biological solids (Kaserru *et al.*, 2003). One of them is the Pseudomonas bacteria group. Pseudomonas denitrificans for aquaculture wastewater denitrification can be effectively reduced 87 – 100 mgTN/L24h of nitrate (Chen *et al.*, 2020). In wastewater experiment, Pseudomonas sp. is the highest nitrate reducer with 11.6 mg (N)/ Lh, P. stutzeri with 8.75 mg (N)/Lh, and P. putida with 2.85 mg(N)/Lh (Xie *et al.*, 2020). Furthermore, a pure culture of P. denitrificans ATCC 17822 was used as a model of nitrite inhibition in denitrification process (Almeida *et al.*, 1995).

Glutamate starch phenol (GSP) agar was formulated by Kielwein (1969). This is a selective medium for Pseudomonas and Aeromonas (Corry *et al.*, 2012). Many studies have used this medium to isolate Pseudomonas. Comorera *et al.* (2016) has used GSP medium to isolating Pseudomonas from water samples which these bacteria were assessed for their ability to form biofilms in vitro. Another study showed that isolates confirmed as Pseudomonas spp. were found to be surrounded by red-violet zone on GSP medium (Hussain & Ahmad, 2018). GSP medium is often used in the calculation of total bacteria claimed as Pseudomonas spp. Triyatmo *et al.* (2020) has used GSP agar medium in the

total calculation of Aeromonas and Pseudomonas bacteria for water quality observations. This was also used by Asril & Lisafitri (2020) on their study to isolate Pseudomonas as a phosphate dissolving potential isolate.

Current molecular approach is highly elaborated for identification due to its high effectivity and specificity (Pertiwi *et al*., 2018). In this study, identification of isolates that have similar morphological characters with Pseudomonas was carried out using the 16S rRNA gene to prove whether the GSP media can filter bacteria to the genus level. Then, the phylogenetic tree is constructed to determine the phylogenetic relationships of the species that have been found. Moreover, the genetic distances from nucleotides dataset are observed to demonstrate an evolutionary history amongs species.

RESEARCH METHODS

Sample Collection and Nitrate Measurement

In this study, total of 3 water samples were collected from different source (Figure 1) at 5-10 cm depth from the surface of Nglonder floating net cage, Rawa Pening, Semarang Regency, Central Java, Indonesia on January 23th, 2020 by sterile flasks. Point 1 was located at 07°16'48.9"S and 110°25'31.7"E. Point 2 was located at 07°16'48.9"S and 110°25'31.7"E. Point 3 was located at 07°16'54.9"S and 110°25'35.9"E Samples were transported to the laboratory in the day of collection by cool box and isolated directly to avoid contamination. Nitrate levels were measured in the laboratory by Hach Program, NitrateVer5 reagent powder pillows (Park *et al*., 2006).

Isolation of Bacteria

Isolated water sample was taken from point which has the highest of nitrate concentration. A 100 µL of water sample was pipette and spread on a GSP agar plate (12 g extract agar, 10 g sodium glutamate, 20 g soluble starch, 2 g potassium dihydrogen phosphate, 0.5 g magnesium sulfate, 0.36 g phenol red) using the spread technique (Comomera *et al*., 2016). The plates are then incubated into the incubator for 48 hours at 35°C. Furthermore, colony which suspected to be Pseudomonas group was selected based on the morphological colony characteristics. It was then purified into new GSP slant agar while determining the Gram staining and oxidase test (Nurhasanah *et al*., 2020).

Molecular Characterization

Molecular identification was carried out in the Tropical Marine Biotechnology Laboratory, Universitas Diponegoro. DNA was extracted using Chelex 20% solution. A loop of fresh colony transferred aseptically to 250 µL Chelex 20% solution. The suspension was then vortexed for 20 seconds then centrifuged at 13,000 rpm for 15 seconds, afterwards heating at 95°C for 45 minutes then re-vortexing for 20 seconds and centrifuged at 13,000 rpm for 2 minutes. The supernatant was assumed as a genomic DNA that was ready to downstream molecular-based application (Sabdaningsih *et al*., 2017).The PCR amplification was processed using 27F as forward primer: 5'- AGA GTT TGA TCC TGG CTC AG-3' and 1492R as reverse primer: 5'-GGT TAC CTT GTT ACG ACT T-3' (Chen *et al*., 2000). PCR cycle conditions consisted of initial denaturation at 95°C for 1 minute, followed by 35 cycles (denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, extension at 72°C for 10 seconds), then post extension at 72°C for 5 minutes. Analysis of PCR product was done by electrophoresis on 0.8% gel agarose to control the accuracy (Maleki *et al*., 2017). After that, the fragment was visualized under UV light in the gel documentation system.

Sequencing process was done by sending the amplicon to PT. Genetika Science Indonesia, Jakarta. The sequencing result was then trimmed and assembled by MEGA X software. Moreover, it was further compared to the database in NCBI (National Center for Biotechnology Information) online at http://www.ncbi.nlm.nih.gov using the BLAST (Basic Local Alignment Search Tool) program to determine closely related species (Nurhasanah *et al*., 2020).

Phylogenetic analysis

Phylogenetic tree was constructed using MEGA X software. Similar nucleotide sequences data were obtained from GenBank, furthermore dataset were aligned using the CLUSTALW program. The sequence was then compared with other relevance species on the phylogenetic tree using the Neighbor-Joining method, with bootstrap analysis setting as 1000 replications (Sabdaningsih *et al*., 2020).

RESULTS AND DISCUSSION

Sample Collection and Nitrate Measurement

The floating net cage (KJA) used in this study is the KJA Nglonder where located in Bejalen Village, Ambarawa District, Central Java, Indonesia. This study site is close to the "Kampoeng Rawa" as a local tourist attraction. KJA Nglonder is one of the floating net cages in Rawa Pening which is a relatively busy with aquaculture and fishing activities. The majority of aquaculture in this place is tilapia (*Oreochromis niloticus*). This KJA can be reached by using a boat rented by the Kampoeng Rawa tourist attraction for 5 minutes from the pier.

Water samples were carried out at three points which were considered to be representative of all Nglonder KJA waters (Figure 1). Point 1 (T1) lies at 07°16'40,4"S and 110°25'27,9"E. This location is the closest observation location to the "Kampoeng Rawa" tourist attraction. This point is chosen with the assumption that there is an effect of waste input from tourism activities which has an impact on the physicochemical conditions of the waters and the abundance of bacteria. The KJA where the samples were taken at T1 was used for tilapia cultivation. Point 2 (T2) lies at 07°16'48,9"S and 110°25'31,7" E. This location was a location in the middle of the KJA area which was used for tilapia cultivation and fishing. Point 2 was selected with the assumption that the highest nitrate content come from this point which was the main center of the KJA area. Thus, the abundance of Pseudomonas as denitrifying bacteria was also high. Point 3 (T3) lies at 07°16'54,9"S and 110°25'35,9"E. This location was the closest location to the Sungai Panjang inlet, where domestic wastes run off KJA area, thus affecting the chemical physics of the

waters as well as bacterial abundance. Overall, the condition of the Rawa Pening waters when sampling was receding due to the prolonged dry season. Nitrate concentration in the sampling sites was measured in two replicates (Table 1).

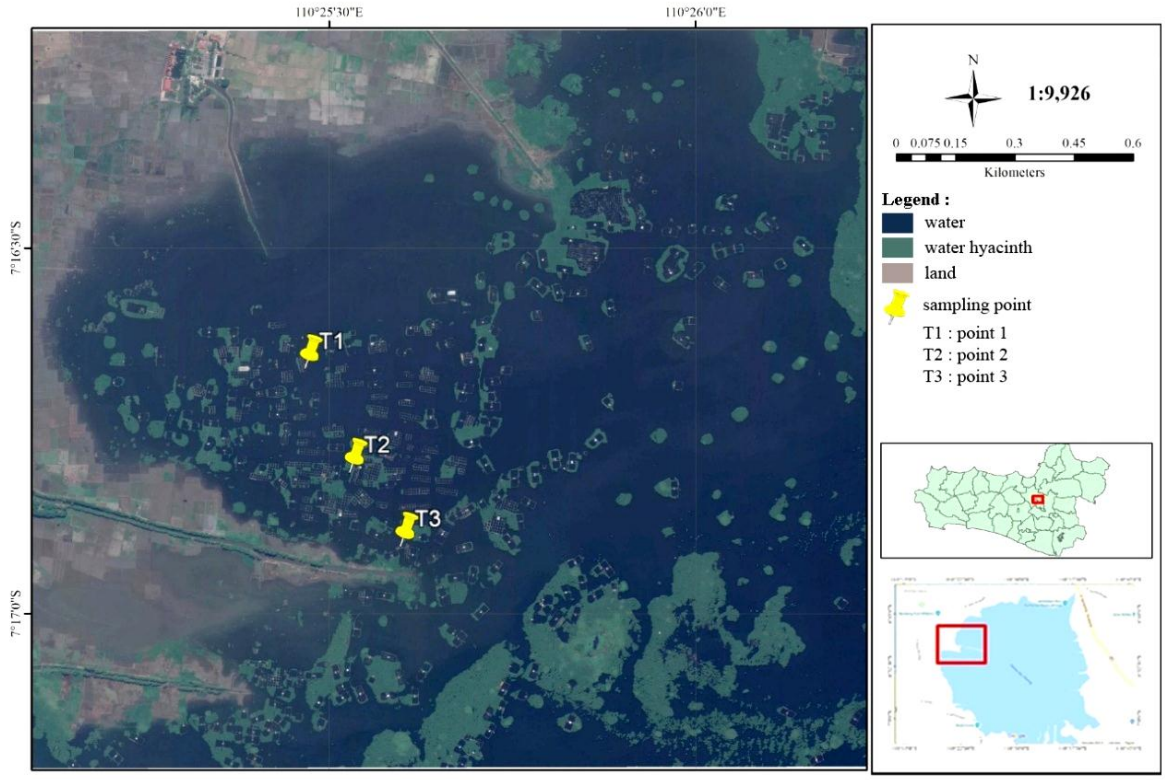

Figure 1. Sampling Point of Water Collection in Rawa Pening Lake (KJA Nglonder) on January 2020.

Table 1. Nitrate Concentration from Sampling Sites in KJA Nglonder

Point	Concentration (mg/L)		Average (mg/L)	Standard*) (mg/L)
	I	II		
1	1.90	1.20	1.55	
2	1.50	1.80	1.65	10
3	1.60	1.40	1.50	

*) = according to PP No. 82 of 2001

The results of nitrate measurements at the study site showed a range between 1.20 to 1.90 mg/L. The highest average was obtained at point 2, while the lowest average was obtained at point 3. The measurement result was then compared with a value standard from Government Regulation (PP) No. 82 of 2001 concerning Management of Water Quality and Control of Water Pollution. The concentration of nitrate did not exceed for quality standard in freshwater aquaculture (class II), that was 10 mg / L. However, nitrate levels of more than 0.2 mg/L can lead eutrophication in the waters, which in turn can cause blooming as well as a trigger factor for the rapid growth of aquatic plants such as water hyacinth (Tatangindatu ***et al***., 2013).

Isolation of Bacteria

All the water samples were inoculated in GSP medium for gaining the pure isolates. Meanwhile, a selected pure isolate was then investigated its molecular characteristic. The molecular characterization was initially started with observations of bacterium morphological and physiological characteristics.

Morphological observations showed that isolate D1 had a round colony shape, cream color, with a flat elevation angle. Furthermore, physiological observations through two biochemical tests showed that isolate D1 was Gram-negative bacterium and oxidase negative. Based on the results of morphological and physiological observations obtained, isolate D1 referred to *Pseudomonas* characteristics. Moreover, the isolate D1 was obtained from water sample with the high concentration of nitrate in the main center of aquaculture activity (T2). According to Rahmadian ***et al***. (2018), *Pseudomonas* bacteria colonies generally appeared in cream-colored, round, with flat elevations. In addition, Yuanita ***et al***. (2019) stated that almost all species in the genus *Pseudomonas* were motile, aerobic, oxidase can be positive or negative, and catalase positive.

Molecular Characterization

The isolate D1 was then extracted its genomic DNA using Chelex 20%. Chelex method is a simple technique to extract the DNA from various of biological samples consist of chelating resin (Walsh ***et al***., 2013). This method was high successful, cheap, easy, and quick step to isolate bacteria DNA. The genomic DNA was subsequently amplified using universal primer, 16S ribosomal RNA (16S rRNA). This molecular approach was conducted to proof whether colony that grow in GSP

medium was *Pseudomonas* sp. or other species. The band DNA of amplicon was demonstrated in Figure 2. The visualization result of isolate D1 using the Gel Documentation System (Geldoc) showed that the amplicon amplified using 27F and 1492R primers had a size about 1500 bp. The amplicon band result was thick and did not smear (scatter). This means that the PCR amplification process carried out was running well. Mardiana ***et al***. (2020) stated that thick DNA bands indicate high DNA concentrations while clumping (not scattered) bands indicate intact total extracted DNA.

Table 2. Homology Analysis of Isolate D1 from KJA Nglonder

Isolate Code	Close Relative	Homology	Identities	Acc. Number NCBI
D1	*Acinetobacter junii*	99.23%	1420/1431	FJ544395.1

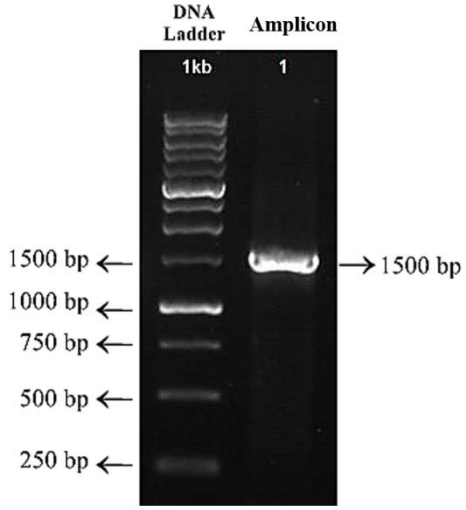

Figure 2. Visualization of the Isolate D1 Amplicon Under Gel-doc Systems.

The amplicon was further sequenced using 1st Base protocol. Sequencing is a process to determine the sequence of nucleotide bases in a DNA molecule. The sequencing process obtained the base sequences of adenine (A), guanine (G), cytosine (C), and thymine (T). The result from this process was received in AB1 file for each primer forward and reverses. This file can be opened using Genetic Software, one of them is MEGA X. The nucleotide sequences from primer forward and reverse was analyzed whether there was N on it. N is a code for unknown nucleotide base, N can appear if the peak of electropherogram was double or overlaps each other. Only nucleotide base of A, G, C, T can be read in the further analysis. Therefore, N should be deleted or replaced with the highest peak of nucleotide base (A, G, C, and T) in electropherogram result. The good result of sequencing process from 16S rRNA gene should contain approximately 1,500 bp. In this study, we obtained a total of 1,437 bp from isolate D1.

The nucleotide sequences of isolate D1 was then compared with GenBank databases using BLAST. The homology table was performed in Table 2. Based on molecular approach, the isolate D1 was closely related to *Acinetobacter junii* strain tu13 FJ544395.1 with 99.23% of homology percentage. The comparison between base sequence from isolate D1 and *A. junii* strain tu13 FJ544395.1 has the same as 1420 nucleotides (99% query cover) with 4 gaps (4/1431).

According to the hierarchy of taxonomy, *A. junii* is a member of Family Moraxellaceae, Order Pseudomonadales, Class Gammaproteobacteria, Phylum Proteobacteria, and Domain Bacteria. Therefore, isolate D1 has the same Order with Pseudomonas group. This species has been reported as a causative agent of fish disease outbreaks in wetlands aquaculture West Bengal, India (Malick *et al*., 2020). The information from World Health Organization (WHO), *Acinetobacter* spp. usually find in soil, water and sewage environments. In addition, *Acinetobacter* has been collected from 97% of natural surface water samples. *Acinetobacter* spp. was also detected as many 38% from untreated groundwater supplies (Bartram *et al*., 2013). Thus, it is clear if isolate D1 that isolated from water samples in the KJA Nglonder belong to *A. junii.* So that, isolation bacteria with GSP medium as selective media for *Pseudomonas* and *Aeromonas* could not always guarantee the obtaining species. We suggest conducting more research regarding selective medium to evaluate the growing species and minimize the generalization of media usage. Consequently, the misidentified of species could be reduced using molecular approach.

Phylogenetic analysis

The phylogenetic tree was constructed to analyze the relationship among isolate D1 and other species. This was built by comparing several sequences of species, including order Enterobacterales, Aeromonadales, and Pseudomonadales. Phylogenetic tree was constructed using the Neighbor-Joining analysis method using MEGA X software. According to Zein & Prawiradilaga (2013), Neighbor-Joining is a method used to distinguish DNA sequences based on genetic distance. In this case several sequences with the closest genetic distance will be combined in the same cluster. The measurement of genetic distance uses a nucleotide substitution model (Kimura2-Parameter Model), which is by comparing a DNA sequence of one nucleotide with another nucleotide. The alignment process is carried out with the CLUSTALW program with the aim of determining the level of homology of the analyzed DNA base sequence.

Based on the results of the molecular phylogenetic analysis, a phylogenetic tree was obtained as shown in Figure 3. The results of the analysis showed that D1 isolate was related to the bacterial species *Acinetobacter junii* with a bootstrap value of 100 rather than other species.The phylogenetic test used the bootstrap method with a bootstrap replication value of 1000. According to Abdullah *et al*. (2018), the bootstrap method was used to evaluate the stability of phylogenetic tree branches. The bootstrap value in a phylogenetic tree is stable if it has a value above 95% and it is unstable if the bootstrap value is below 70%. The phylogenetic tree of isolate D1 has a bootstrap value within 83 – 100%, therefore the branches structure of tree was stable.

According to Fahlevi *et al*. (2018), outgroup is needed in making phylogenetic tree due to the role becoming the last ancestors of a more modern group. Therefore, the outgroup selected from this study were different species at the genus level, that is *Streptococcus pygenes.*

In order to observe the history of evolutionary among species, genetic distance was generated using Kimura-2 parameter model (Table 3). Genetic distance showed relationship within species. The lower the value, the closer species are. The result indicated that isolate D1 was closer to *A. junii* with genetic distance value 0.006, while *Pseudomonas* and *Aeromonas* as a targeted group in GSP medium have genetic distance higher within 0.144 to 0.175.

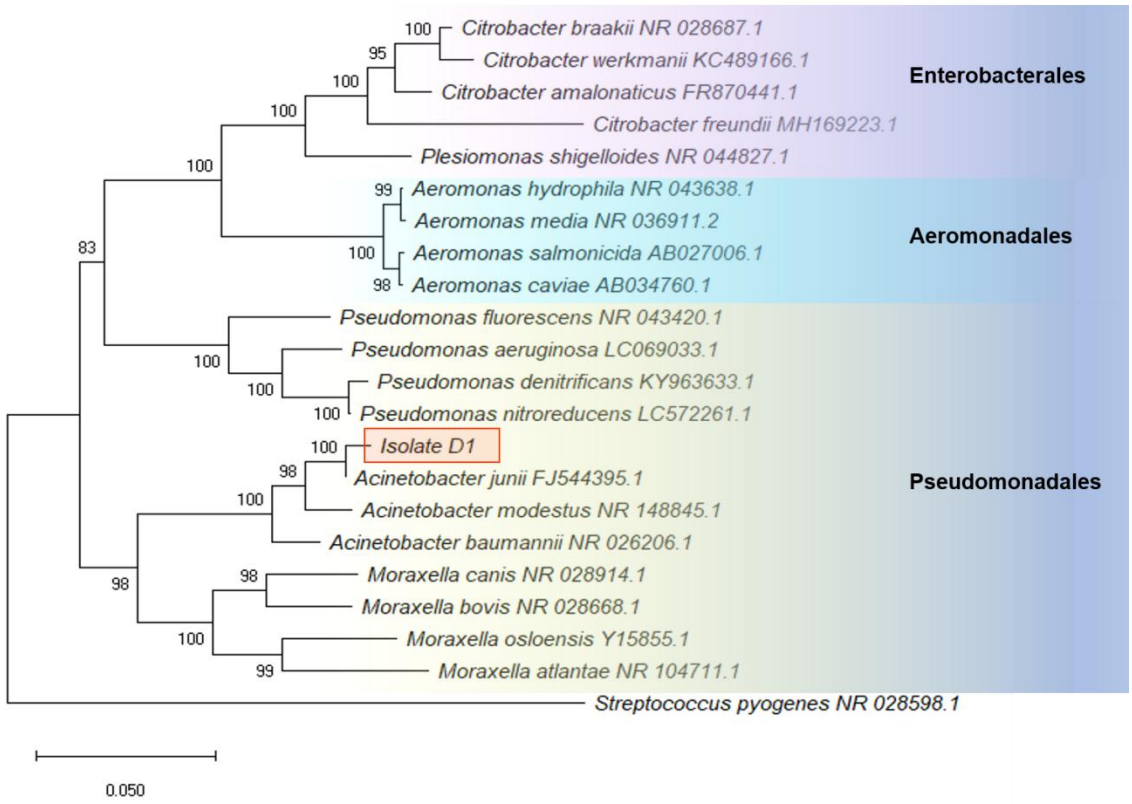

Figure 3. Phylogenetic Tree of Isolate D1 (Red Box) from KJA Nglonder Compared to Three Order Such as Enterobacterales, Aeromonadales, and Pseudomonadales.

Table 3. Estimates of Evolutionary Divergence between Sequences

No	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	Isolate_D1																						
2	*Acinetobacter_junii* _FJ544395.1	0,006																					
3	*Acinetobacter_modestus* _NR_148845.1	0,032	0,023																				
4	*Acinetobacter_baumannii* _NR_026206.1	0,045	0,036	0,033																			
5	*Moraxella_bovis* _NR_028668.1	0,122	0,114	0,122	0,113																		
6	*Moraxella_canis* _NR_028914.1	0,127	0,118	0,124	0,110	0,050																	
7	*Moraxella_osloensis* _Y15855.1	0,138	0,130	0,136	0,122	0,088	0,081																
8	*Moraxella_atlantae* _NR_104711.1	0,158	0,149	0,146	0,131	0,116	0,102	0,074															
9	*Pseudomonas_fluorescens* _NR_043420.1	0,144	0,137	0,136	0,137	0,148	0,155	0,172	0,174														
10	*Pseudomonas_aeruginosa* _LC069033.1	0,146	0,140	0,141	0,130	0,155	0,154	0,167	0,175	0,063													
11	*Pseudomonas_nitroreducens* _LC572261.1	0,153	0,146	0,142	0,137	0,158	0,155	0,170	0,180	0,061	0,035												
12	*Pseudomonas_denitrificans* _KY963633.1	0,155	0,153	0,148	0,145	0,161	0,158	0,173	0,183	0,065	0,043	0,006											
13	*Plesiomonas_shigelloides* _NR_044827.1	0,171	0,164	0,166	0,163	0,177	0,178	0,183	0,185	0,152	0,156	0,174	0,184										
14	*Aeromonas_caviae* _AB034760.1	0,172	0,165	0,164	0,165	0,184	0,164	0,184	0,181	0,135	0,153	0,137	0,140	0,102									
15	*Aeromonas_salmonicida* _AB027006.1	0,172	0,165	0,164	0,165	0,182	0,164	0,188	0,182	0,134	0,153	0,139	0,142	0,102	0,003								
16	*Aeromonas_hydrophila* _NR_043638.1	0,174	0,166	0,164	0,165	0,184	0,165	0,182	0,177	0,134	0,157	0,139	0,143	0,104	0,010	0,012							
17	Aeromonas_media_NR_036911.2	0,175	0,168	0,166	0,168	0,183	0,164	0,181	0,176	0,135	0,158	0,140	0,143	0,104	0,012	0,014	0,003						
18	*Citrobacter_amalonaticus* _FR870441.1	0,178	0,171	0,175	0,179	0,176	0,180	0,186	0,185	0,157	0,167	0,178	0,186	0,065	0,117	0,117	0,113	0,114					
19	*Citrobacter_braakii* _NR_028687.1	0,179	0,172	0,178	0,177	0,182	0,189	0,192	0,190	0,175	0,169	0,182	0,191	0,059	0,120	0,120	0,127	0,120	0,026				
20	*Citrobacter_werkmanii* _KC489166.1	0,179	0,174	0,187	0,184	0,189	0,191	0,196	0,199	0,181	0,177	0,189	0,190	0,067	0,130	0,130	0,130	0,129	0,033	0,013			
21	*Citrobacter_freundii* _MH169223.1	0,216	0,210	0,224	0,218	0,218	0,218	0,222	0,225	0,202	0,204	0,210	0,217	0,113	0,149	0,150	0,156	0,153	0,077	0,086	0,092		
22	*Streptococcus_pyogenes* _NR_028598.1	0,260	0,252	0,262	0,257	0,240	0,262	0,279	0,260	0,247	0,261	0,264	0,267	0,264	0,274	0,274	0,273	0,276	0,265	0,271	0,275	0,311	

CONCLUSION

This study has revealed that isolate D1 was related to Acinetobacter junii strain tu13 with homology 99.23%. The genetic distance between A. junii and P. fluorescens was 0.006 and 0.144, respectively. It showed that isolate D1 closely related to A. junii rather than Pseudomonas group. Therefore, GSP medium has a sensitivity range at the Order level, namely Pseudomonadales.

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