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First record of the bacterium *Pseudomonas putida* on pepper in Iraq

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ABSTRACT

Random samples were collected from the field soil and pepper fruits in different agricultural areas in order to isolate and identify the associated *Pseudomonas putida* (Trevisan 1889) Migula 1895. The diagnosis process was carried out based on the culture, phenotypic and biochemical characteristics. Once isolation on the nutrient agar food media, pure single or double colonies of *P. putida* were appeared, exhibiting the ability to grow in Maconkey medium, negative for gram dye, capable of producing yellowish green Pyouverdin dye under ultraviolet rays, characterizing by an undesirable odor. The molecular diagnosis of bacteria was confirmed by DNA secquencing analysis of the complementary DNA (cDNA) of the double-stranded dsRNA (dsRNA) RNA that forms the genome of bacteria during replication. The extraction was performed using the dsRNA dsRNA extraction method from the studied samples and the results of the sequence analysis were compared with the nucleotide database of the NCBI using BLAST. The isolates of bacteria with similar nucleotide sequences were identified and the Iraqi isolate was placed in the phylogenetic tree diagram to show the relationship between the Iraqi isolate and the global ones. Bacterial isolates were recorded in the National Center for Genetic Bank NCBI, European ENA and the Japanese Information Bank, DNA with Accession Number (s) SUB9666355 AHM MZ209185, which is the first record of *P. putida* on pepper in Iraq.

Keywords: Biological control, GP bacteria, Soft rot.
Article type: Report.

INTRODUCTION

Pseudomonas is a member of the non-lactose fermented gram-negative bacillus from Pseudomonadaceae family (Palleroni 1992). Zumft (1997) reported that the German investigator, Walter Migula was one of the first to describe this genus in 1894. Stanier *et al.* (1966) described *Pseudomonas* as a gram-negative, chemo-trophic, non-sporogenic, straight or curved rod, motile with one or more flagella, positive for the oxidase and catalase test, arranged single or in short chains, widely distributed in nature. It lives in the form of aggressive colonies in the area surrounding the root of many vegetable plants and crops, since it is found within the root tissues between the epidermis and the cortex (Compeau *et al.* 1988; Duijff 1997). These bacteria are on top of the plant growth-promoting rhizobacteria, since they constitute a large part of the bacterial group surrounding the roots of most plants (Yarub *et al.* 2016). *Pseudomonas* can increase the availability of phosphorous and decompose complex organic compounds such as lignin, cellulose, betaine, proteins and urea by the process of dilution, and some types of these bacteria can also be able to fix nitrogen (Alexander 1988). It promotes plant growth as well as their effectiveness in biological control of plant pathogens (Rezzonico *et al.* 2007; Abbas-Zadeh *et al.* 2010).

In general, *Pseudomonas* contributes to the cycling of nutrients, with the efficiency of their use in the treatment of chemical soil pollutants (Segura *et al.* 2009; Segura & Ramos 2012) and also contribute to the recycling of

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xenobiotic materials (Weimer *et al.* 2020). In addition, they were recorded as contaminating red meat and poultry meat, causing spoilage and reducing the shelf life, especially fresh meat preserved in refrigeration (Mohareb *et al.* 2015; Papadopoulou *et al.* 2020).

MATERIALS AND METHODS

Random samples of bill pepper fruits were collected from different local markets, washed thoroughly with running water, then sterilized with 1% sodium hypochlorite for two minutes. Then, the samples were washed with sterile distilled water and dried using sterile filter papers. Five pieces were then placed in a sterile 9-cm petri dish containing NA medium. After incubation for 3 days, a smear was taken from the growth edge with a sterile lube. The bacteria were superficially mapped by streaking onto the NA solid media in new plates and incubated at 25 ± 2 °C for 48 hours. Afterward, a single colony was transferred using a sterile lube and cultured in NA sterile petri dishes, then incubated under the same conditions for 24 hours (Ramos *et al.* 1991; Schaad *et al.* 2001).

Bacterial diagnostic tests

The phenotypic and biochemical physiological tests of the isolated bacteria were carried out based on LOPAT tests (Schaad *et al.* 2001) based on the Sakhrin Encyclopaedia (Goszcynska *et al.* 2000). The bacteria were diagnosed according to the tests KOH, Catalase, Oxidase, H2S, indol, urease, Simmonase, MR, VP, Lipase, Starch, and the fermentation of carbohydrates (sucrose, ribose, lactose, maltose, fructose, galactose, xylose, xylose, and silubicin) as well as dostol, anostol and Levan production. In the case of the molecular diagnosis of *P. putida* by using polymerase chain reaction (PCR), the primers AGAGTTGATCMTGGCTCAG-3'5 and 5'-TACGGYTACCTTGTTTACGACTT-3'3 (Table 1) were used by targeting the 5S 16sRNA gene and amplifying a genetic domain with a length of 1485 base pairs.

Distilled water was added to each primer separately according to the manufacturer's instructions to obtain a concentration of 100 pcmol mL^{-1} (stock solution). The primers were diluted to a concentration of 10 picomole mL^{-1} (adding 10 µL stock to 90 µL sterile distilled water). The reaction was carried out with a mixture of 2 µL extracted DNA, 1 µL of each fore and posterior initiator, 5 µL Pre-mix PCR (Intron / Korea) and 16 µL Free water, with a total volume of 25 µL using reaction tube volume of 50 µL (Table 2). Amplification was performed using a thermocycler (MultiGene "Mini" Labnet Company) according to the amplification program approved by the manufacturer (Table 3).

Table 1. Primers (forward and reverse) used for bacteria diagnosis.

Primer	Sequence	Tm (°C)	GC (%)	Product size
Forward	5'-AGAGTTGATCMTGGCTCAG-'3	52.8	50.0 %	1485
Reverse	5'- TACGGYTACCTTGTTACGACTT-'3	54.6	43.2 %	base pair

Table 2. The specific reaction mixture for bacteria diagnostic gene of *P. putida.*

Components	Concentration
Taq PCR PreMix	5 µL
Forward primer	1 µL
Reverse primer	1 µL
DNA	2 µL
Distill water	16 µL
Final volume	25 µL

Table 3. PCR procedure for determining *P. putida* diagnostic gene.

No.	Phase	T (°C)	Time	No. of cycle
1	Initial Denaturation	96 °C	3 min.	1cycle
2	Denaturation	94 °C	45 sec	
3	Annealing	54.6 °C	1 min	35cycle
4	Extension-1	72 °C	1 min	
5	Extension -2	72 °C	10 min.	1cycle

On agarose gel, electrophoresis was performed for the PCR products of bacterial DNA to determine the DNA fragmentation. The process included PCR result in the presence of the standard DNA produced by Kapa/USA to distinguish the package size.

RESULTS AND DISCUSSION

The results showed that the bacterium isolated from pepper fruits is *Pseadomonas putida* and was diagnosed based on microscopic, morphological, cultural and biochemical characteristics (Table 4). According to the results, the bacterial isolate showed ability to grow in MacConkey agar medium, and it was distinguished upon microscopic examination by its appearance of single or double rod cells, Gram negative, positive for oxidase and catalase test. Phenotypic examination of *P. putida* revealed the formation of small, smooth, circular raised colonies with some large colonies. Glossy under ultraviolet rays for its ability to produce Pyoverdin dye, its colour is creamy yellow with an undesirable smell.

The *P. putida* isolate was characterized by its production of bright (yellowish green) pigments. Biochemical tests showed the inability of bacteria to decompose starch, positive KOH test. In the case of IMViC group of tests, the bacteria gave a negative result for the indole test, urea hydrolysis, Fox Brooks, hydrogen sulfide production H_2S, positive for methyl red, citrate decomposition, and gel production from sucrose. In the case of the sucrose fermentation test, the bacterium showed negative results for inositol, rhamnose, cellobiose, mannitol, maltose, lactose, ribose, sorbitol, sucrose, and positive for dulcitol, trehalose, xylose, galactose and fructose (Table 4).

These confirmatory tests are most important for distinguishing the genus *Pseudomonas* (Franklin *et al.* 1981). Based on previous studies on *Pseadomonas* bacteria, the traits corresponded to the microscopic and morphological characteristics of *P. putida* (Palleroni 1984; Holt *et al.* 1994, Benizri *et al.* 1997). According to the results (Table 4), temperature exhibited an effect on bacterial growth, which did not develop at temperatures 4, 27 and 40 ºC, while bacterial growth was evident at 27, 30 and 37 ºC (Williams & Murray 1974). Mineral salts and medium pH displayed a clear effect on bacterial growth. Treatment with sodium chloride salt showed an effect on bacterial growth, and bacterial growth was observed at concentration of 1%, while the bacteria did not grow at 5%. Bacterial growth did not occur when the medium pH was 4.5 (Duque *et al.* 1993). Millas *et al.* (2006) reported that sodium chloride functions to balance the amount of salt in the bacterial cell, which is important for bacterial metabolism.

Table 4. Pseudomonas putida diagnostic tests used in the study.

Test type	Result	Test type	Results
Oxidase	+	Urea hydrolysis	-
Catalysts	+	Fox Brooks	-
Starch hydrolysis	-	Methyl red	+
Potassium hydroxide	+	Citrate hydrolysis	+
Indole	-	Hydrogen sulfide	+
Sucrose fermentation			
Test type	**Result**	**Test type**	**Results**
Sucrose	-	Rhamnose	-
Sorbitol	-	Inositol	-
Ribose	-	Fructose	+
Lactose	-	Galactose	+
Maltose	-	Xylose	+
Mannitol	-	Trehalose	+
Cellobiose	-	Dulcitol	+
Temperature effect on bacterial growth			
Test type	**Result**	**Test type**	**Results**
4 ºC	**-**	**30 ºC**	**+**
20 ºC	**-**	**37 ºC**	**+**
27 ºC	**+**	**40 ºC**	**-**
Effect of minerals and medium pH on bacterial growth			
Test		**Result**	
NaCl 1%		**+**	
NaCl 5%		**-**	
pH 4.5		**-**	

Molecular diagnosis of bacteria

The results of the polymerase chain reaction (PCR) and migration electrophoresis in agarose gel confirmed the results of the morphological diagnosis by the appearance of the DNA bundle with a size of 1485 bp (Fig. 1). This bundle is expected to be formed when the 1616S ribosomal RNA Gene of bacteria is amplified using primers specialized for the bacteria under study (Table 1). The nucleotide sequence matching was applied for the DNA extracted from bacteria isolated from pepper fruits in Nineveh Governorate, Iraq. The results confirmed that the bacteria is *Pseudomonas putida* (Table 3).

Only matches with values equal to zero that represent the best possible match are included. This value statistically expresses the compatibility between the sample and the NCBI data, through which we note that the highest percentage of nucleotide sequence match for the Iraqi bacteria isolate was with the Egyptian isolate *P. putida* NGB-MS2 gene for 16S ribosomal RNA with a percentage of 98.53%. The bacterial isolate was registered in the National Centre for Genetic Bank NCBI, European ENA and Japanese DNA Data Bank for the first time in Iraq after obtaining the Accession Number (S) linkage numbers with SUB9666355AHM MZ209185 (Fig. 2). The findings showed the Iraqi Query of the Complete Genome of a Bacteria Isolate *P. putida* NGB-MS2 gene for 16S (Fig. 2). It also shows the relationship between the Iraqi isolate and the global isolates (Fig. 3), according to a diagram of the genetic tree that was designed based on matching the DNA sequence of the tested sample with the data base available in the NCBI for the same bacteria.

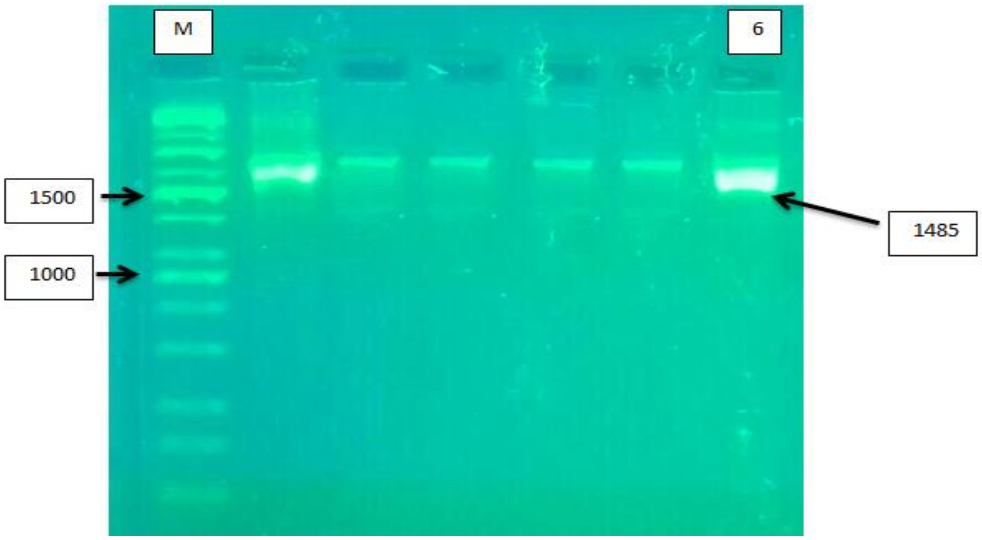
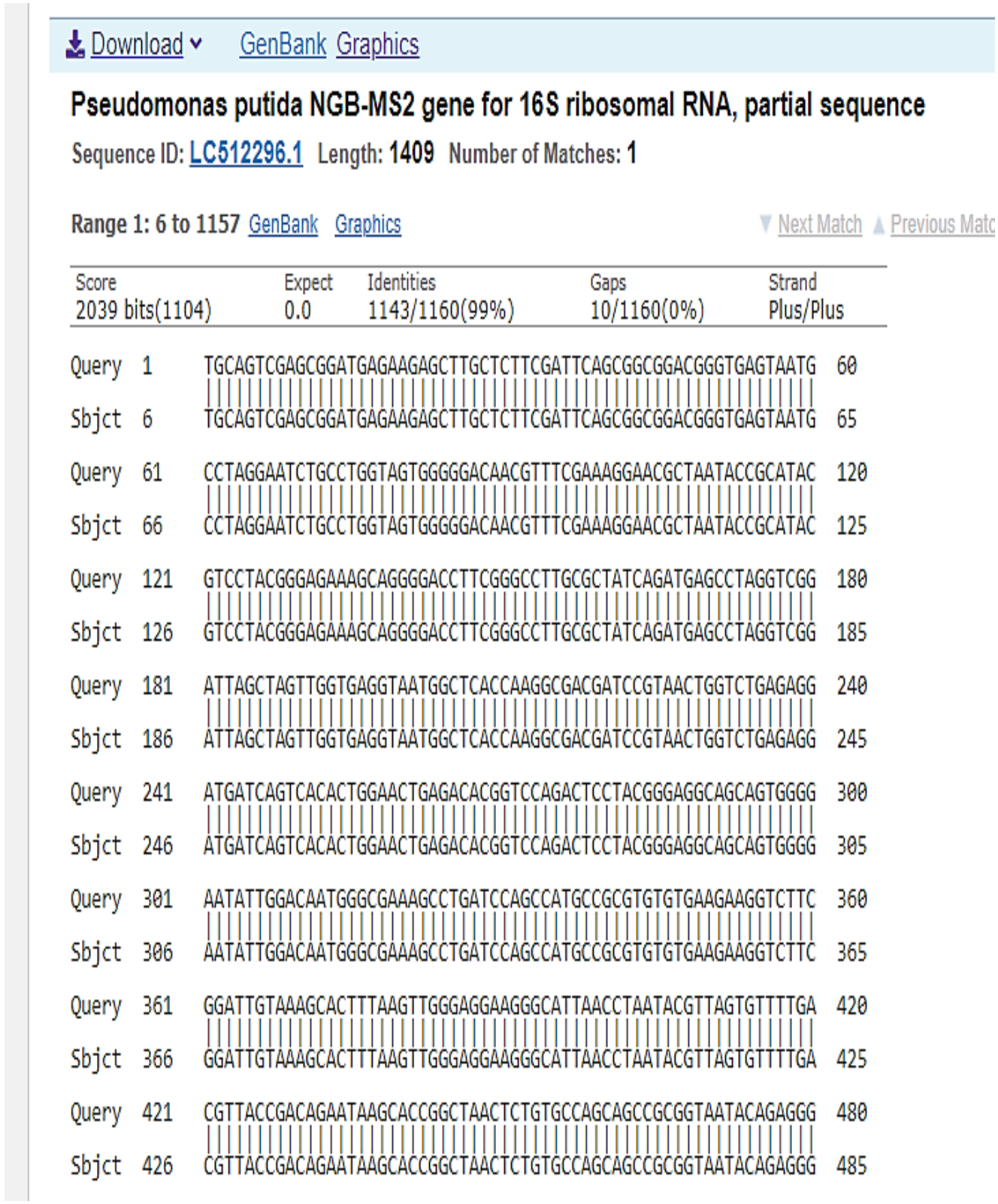


Fig. 2. PCR product electrophoresis showing the 1485 bp pack size (lane 6) resulting from amplification of the 16S ribosomal RNA Gene region of *P. putida* under study.



Download GenBank Graphics

Pseudomonas putida NGB-MS2 gene for 16S ribosomal RNA, partial sequence

Sequence ID: LC512296.1 Length: 1409 Number of Matches: 1

Range 1: 6 to 1157 GenBank Graphics ▼ Next Match ▲ Previous Matc

Score	Expect	Identities	Gaps	Strand
2039 bits(1104)	0.0	1143/1160(99%)	10/1160(0%)	Plus/Plus

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Query  1    TGCAGTCGAGCGGATGAGAAGAGCTTGCTCTTCGATTCAGCGGCGGACGGGTGAGTAATG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6    TGCAGTCGAGCGGATGAGAAGAGCTTGCTCTTCGATTCAGCGGCGGACGGGTGAGTAATG  65

Query  61   CCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATAC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  66   CCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATAC  125

Query  121  GTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  126  GTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGG  185

Query  181  ATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  186  ATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGG  245

Query  241  ATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  246  ATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG  305

Query  301  AATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  306  AATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTC  365

Query  361  GGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATTAACCTAATACGTTAGTGTTTTGA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  366  GGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATTAACCTAATACGTTAGTGTTTTGA  425

Query  421  CGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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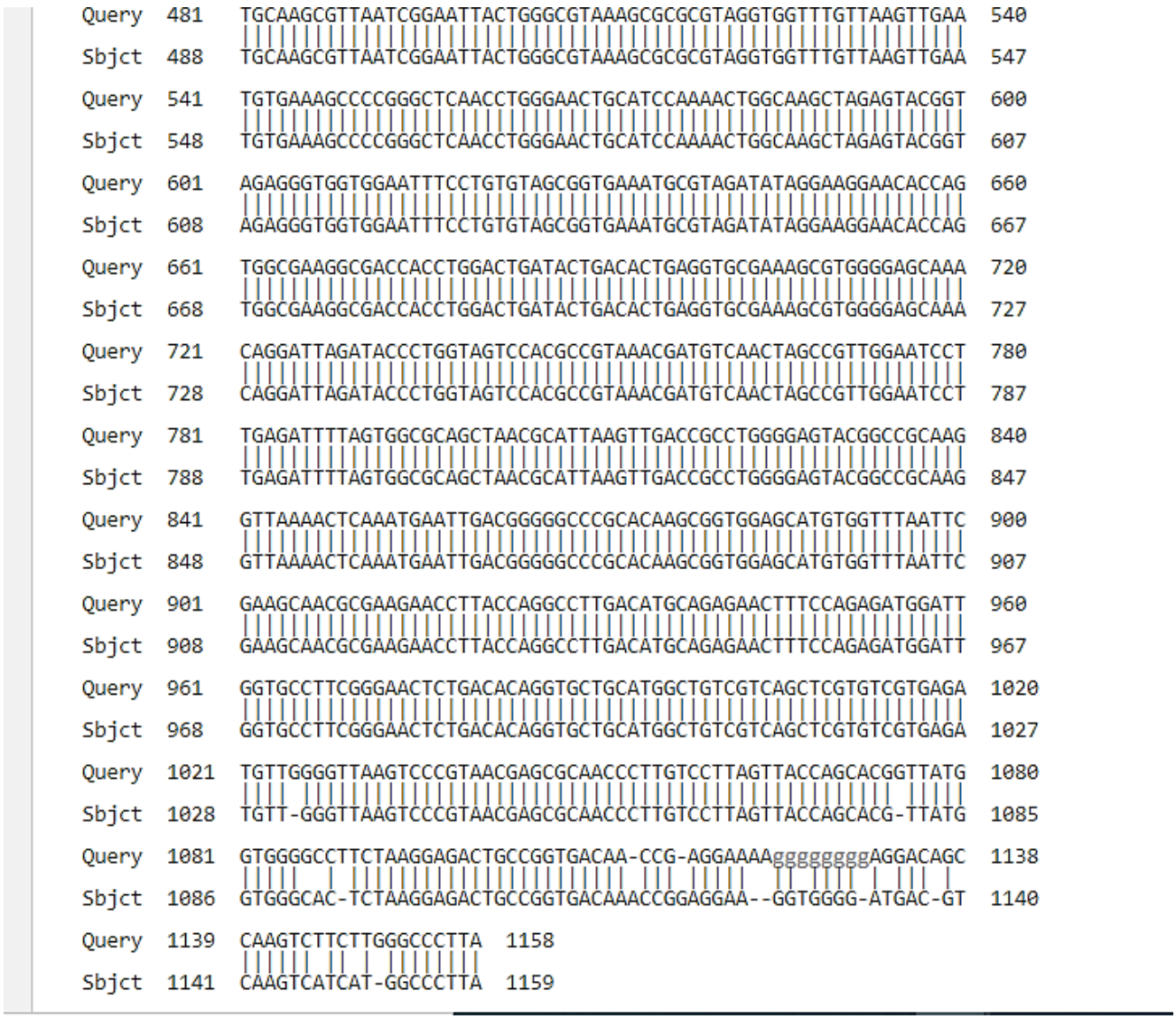

Fig. 2. Compatibility ratio and genome fragment loci of the Iraqi isolate Query from the whole genome of the bacteria isolate under study.

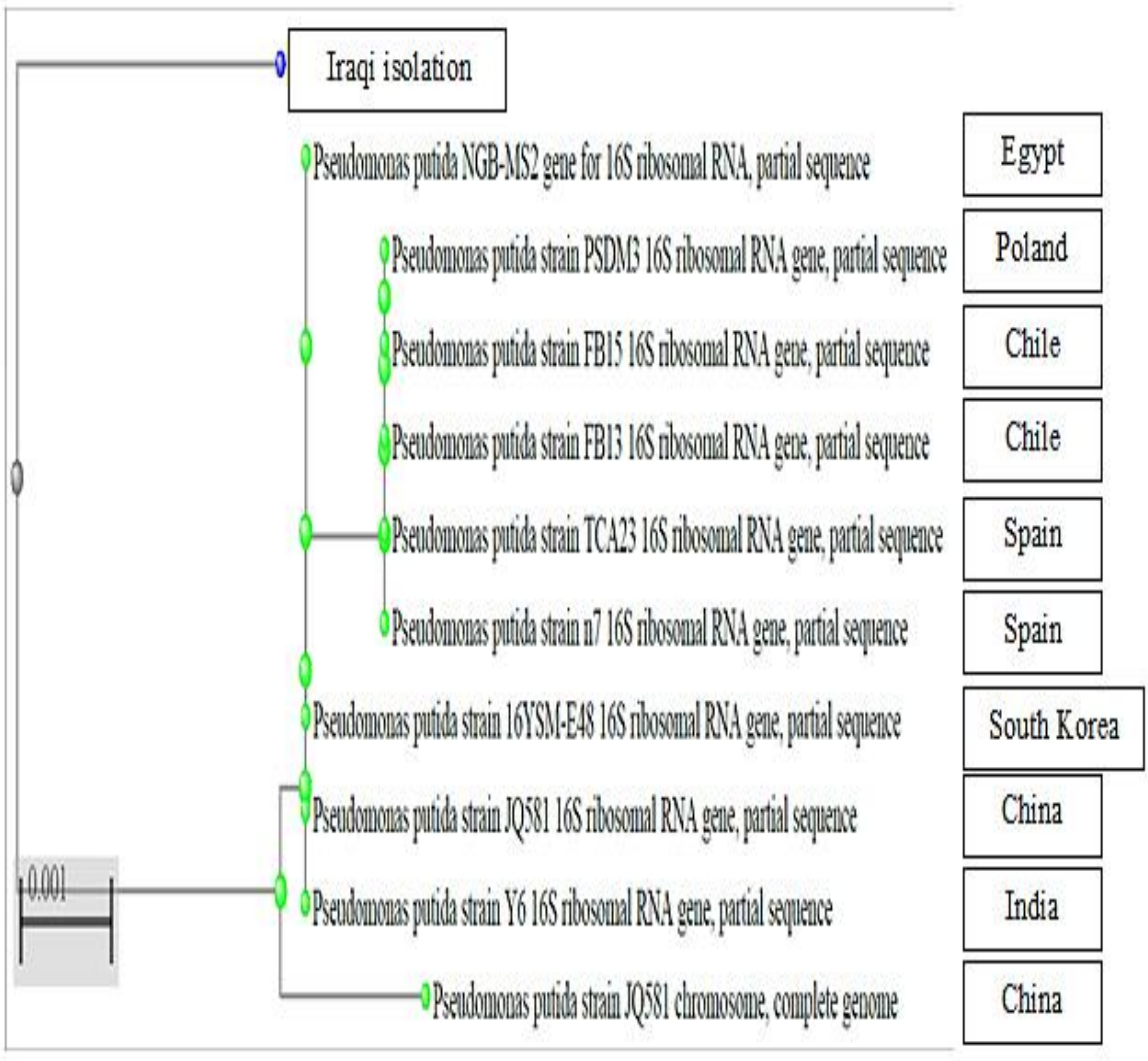

Fig. 3. Genetic tree showing the relationship between the Iraqi isolate under study and global isolates of *Pseudomonas putida*.

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