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Distribution, occurrence and molecular characterization of *Bacillus* related species isolated from different soil in Basrah Province, Iraq

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Abstract. *Alyousif NA. 2021. Distribution, occurrence and molecular characterization of* Bacillus *related species isolated from different soil in Basrah Province, Iraq. Biodiversitas 23: 679-686. Bacillus* related species are widely predominant in various environments such as soil with diverse environmental conditions. These bacteria are one of the most explored groups of bacteria in industrial biotechnology due to their enzymes. The present study was aimed to isolate, screen and determine the distribution of *Bacillus* related species from soil samples. The total *Bacillus* related species counts in one gram of each soil sample ranged between 3.2 X 10^3 cfu/g and 4 X 10^3 cfu/g. A total of 43 isolates with different morphologies were isolated from the soil samples out of ten different habitats and the number of morphotypes recovered from each habitat varied from 2 to 7. The molecular identification of isolates by PCR technique and sequencing of 16S rDNA gene indicated that 43 isolates belonged to the genera *Bacillus*, *Cytobacillus*, *Priestia* and *Peribacillus.* The species *Bacillus licheniformis* and *Bacillus subtilis* were the most distributed species in the habitats. The bacterial isolates were showed 100% similarity, except 8 isolates were showed 99% similarity were identified as new strains and their sequences were deposited at the National Center for Biotechnical Information (NCBI). The phylogenetic tree was constructed by MEGA X software based on 16S rDNA gene sequences of *Bacillus* related species to evaluate their close relationship and evolution. The study revealed the higher diversity and distribution of *Bacillus* related species in the soil samples and the molecular technique was the best method for identifying *Bacillus* related species.

Keywords: 16S rDNA gene, *Bacillus* species, genetic diversity, sequence, soil bacteria

INTRODUCTION

Bacillus related species are Gram-positive, rod shaped, spore-forming, aerobic or facultatively anaerobic, motile bacteria with peritrichous flagella (Foysal and Lisa 2018). *Bacillus* related species are widely predominant in various environments such as soil with diverse environmental conditions, fresh and saltwater, plants and animals (Pignatelli et al. 2009; Liu et al. 2017). These bacteria are one of the most important components of the soil microbial populations, and they are frequently found in high abundance in harsh environments including desert soils, acidic soils and saline-alkali soils, indicating that bacteria play a vital role in these soils (Cihan et al. 2012; Amin et al. 2015). The survival of *Bacillus* related species for long time in different harsh environments is attributed to the formation of resistant endospores that resist harsh environmental conditions (Mandic-Mulec et al. 2016).

Few *Bacillus* genus is pathogens for animals and humans and considered medically important. *Bacillus anthracis* causes anthrax, a potentially lethal disease and *Bacillus cereus* is known for causing food poisoning, can also cause local and systemic infections (Jeßberger et al. 2015; Celandroni et al. 2016). *Bacillus* related species is one of the most explored groups of bacteria in industrial biotechnology due to their enzymes, which are tolerant to a wide range of pH and high temperature values, particularly in harsh industrial processes (Cihan et al. 2012). Several species of *Bacillus* genus have been produced numerous valuable antibiotics, some of these antibiotics exhibited a narrow spectrum of activity, while others exhibited a broad spectrum of activity (Yahya et al. 2021). *Bacillus subtilis* is a major species that produces effective antibiotics against multidrug-resistant bacteria like methicillin-resistant *Staphylococcus aureus* (Chalasani et al. 2015). Several species of genus *Bacillus* are known for their ability to produce a variety of metabolites which are exploited for biological control of plant pathogens (Saxena et al. 2019). Many members of the genus *Bacillus* are known for using Plant growth promoting rhizobacteria (PGPR) in many fields and horticultural crops due to their ability to produce phytohormones (Saxena et al. 2017). PGPR is a bacterium that can produce growth hormones to help plant growth, provide nutrients and become biological agents capable of suppressing tungro disease (Salamiah and Wahdah 2015; Widawati 2015)

It is difficult to find an appropriate method to classify and generalize the *Bacillus* related species because these bacteria have a wide range of characteristics that allow these species to colonize almost all-natural habitats, including soil, air, water, and harsh conditions environments (Gopal et al. 2015). In addition, the molecular methods are more suitable than the traditional culture-dependent methods for discovering bacterial diversity and novel bacterial species (Li et al. 2014). A variety of molecular methods, such as polymerase chain reaction (PCR) and 16S rDNA sequencing technique, are currently being successfully utilized to identify *Bacillus*

related species isolated from various environmental sources (Srinivasan et al. 2015; Foysal and Lisa 2018). The present study aims to isolate and screen *Bacillus* related species from soil of different environmental habitats of Basrah province, characterize these bacteria by 16S rDNA sequencing technique, determine their distribution, and construct a phylogenetic tree of isolated *Bacillus* related species.

MATERIALS AND METHODS

Collection of soil samples

A total of 10 soil samples were collected from different environmental habitats of Basrah province (30°22′N 47°22′E) as shown in Figure 1 include 10 sites (Al-Dair, Al-Nashwa, Karmat Ali, Al-Hartha, Um Qasr, Abu Al-Khaseeb, Al-Maqil, Shat Al-arab, Al-Zubair and Al-Qurna) during the period August 2020 till October 2020. Approximately 20 g of each sample were collected from at least 5 cm of depth in dry and sterile plastic bags with a sterile spoon. Then the soil samples were transported to the laboratory under sterile conditions for processing.

Isolation of *Bacillus* related species

Four grams of each soil sample was suspended in 250 ml Erlenmeyer flask containing 96 ml of sterile distilled water and shaken vigorously for 2 min. The samples were heated at 60°C for 60 min in a water bath (Chilcott and Wigley 1993). Then the soil suspensions were sequentially diluted in sterile distilled water with different aqueous dilutions from 10^{-1} to 10^{-4}. A volume 100 µl from each dilution of soil samples was plated on a nutrient agar medium using spreading method. The plates were incubated at 35°C for 24 h. The colonies with different morphological appearances were isolated and sub-cultured onto fresh nutrient agar medium for obtaining pure culture.

Genomic DNA extraction

According to the manufacturer's instructions, the genomic DNA of 43 bacterial isolates was extracted from the cultures growing on nutrient agar for 18 h using Presto™ Mini g DNA bacteria kit (Geneaid, Taiwan). The extracted genomic DNA was detected by gel electrophoresis and then stored at -20°C for further use.

PCR preparation for bacterial identification by 16S rDNA

The gene encoding 16S rRNA was amplified by PCR using universal primers 27F (5-AGAGTTTGATCCTGGC TCAG-3) and 1492R (5-GGTTACCTTGTTA CGACTT-3). The PCR reaction mixture was made in a total volume of 50 µL containing 25 µL of master mix (Promega, USA), 2 µL of template DNA, 2 µL each of forward and reverse primers, and 19 µL of nuclease-free water. The PCR amplification conditions were as follows: an initial denaturation step of 96°C for 3 min followed by 27 cycles of 96°C for 30 s, annealing of 56°C for 25 s and elongation at 72°C for 15 s and final extension step at 72°C for 10 min (Miyoshi et al. 2005). The PCR products were measured by gel electrophoresis along with DNA ladder (Intronbio, South Korea) as the marker and visualized on a UV transilluminator and photographed.

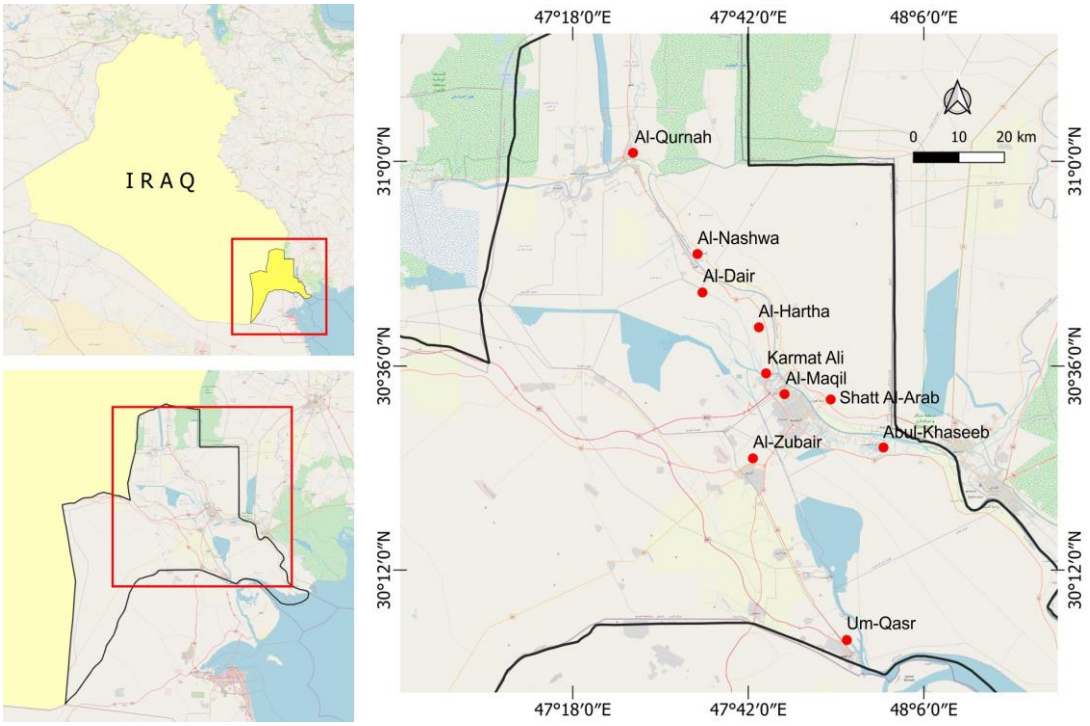

Figure 1. Map showing study area and sampling habitats in Basrah Province, Iraq

Sequencing the PCR products of 16S rDNA

The amplified PCR products of 16S rDNA were sent to Macrogen company (South Korea) for purifying and sequencing. The obtained 16S rDNA gene sequences of 43 bacterial isolates received in raw format were edited using MEGA-X and aligned with nucleotide sequences databases of NCBI using BLAST tools "http://www.ncbi.nlm.nih.gov to estimate the sequence homology and identification of bacterial isolates. The phylogenetic tree was constructed using MEGA X (Kumar et al. 2018).

RESULTS AND DISCUSSION

Numeration of viable *Bacillus* related species

Bacillus related species are abundant bacteria of the microbial community in soils. The ten soil samples from different environmental habitats of Basrah province were used to isolate Bacillus-related species in the present study. The total *Bacillus* related species counts in one gram of each soil sample obtained after 24 h of incubation are varied between 3.2 X 10^3 cfu/g (Soil sample from Al-Zubair district) and 4 X 10^3 cfu/g (Soil sample from Al-Nashwa district) as shown in Figure 2 and Table 1.

A total of 43 isolates with different morphologies were isolated from the soil samples out of ten different areas by diluted and heat-shocked methods cultured on nutrient agar media. The number of morphotypes recovered from each habitat varied from 2 to 7.

The colonies that grew on nutrient agar were successfully purified to single colonies by streaking method on nutrient agar. Morphological characterization of purified isolates was recorded according to microscopic investigation and macroscopic measurement of pure colonies.

Bacterial identification by 16S rDNA

The extracted genomic DNA from purified isolates was used for identifying all isolates by PCR amplification and sequencing of the 16S rDNA gene. The bands of PCR products were observed on agarose gel electrophoresis under UV transilluminator at the position nearly 1500 bp in comparison with the DNA ladder, as shown in Figure 3. The sequences of the 16S rDNA gene for bacterial isolates were edited using chromas (version 2.6.6) to remove the overlapping sequences and analyzed by using BLAST search tool at NCBI to identify the bacterial species by comparing the sequences with sequences of NCBI database.

The molecular identification of isolates was performed by PCR amplification and sequencing of 16S rDNA gene, as shown in Table 2. *Bacillus subtilis* and *Bacillus licheniformis* were the most distributed species in the habitats.

The comparison of the generated sequences with sequences of the GenBank database indicated that 43 isolates from the soil samples of Basrah province belonged to the genera *Bacillus*, *Cytobacillus*, *Priestia* and *Peribacillus*. The species of *Bacillus* genus were more frequent in soil samples include *B. subtilis* (11 strains), *B. licheniformis* (10 strains), *B. tequilensis* (1 strain), *B. safensis* (2 strains), *B. pumilus* (1 strain), *B. cereus* (2 strains), *B. infantis* (1 strain), *B. vallismortis* (1 strain), *B. albus* (1 strain), *B. sonorensis* (1 strain) and *B. velezensis* (1 strain). The two species were recorded in the soil samples for each genus of *Cytobacillus* and *Priestia* include *C. firmus* (4 strains), *C. oceanisediminis* (3 strains), *P. flexa* (1 strain) and *P. megaterium* (2 strains), while one species was recorded in the soil samples for genus of *Peribacillus* include *Peribacillus simplex*.

Table 1. *Bacillus* related species count of soils samples from different environmental habitats of Basrah province

Habitats	Habitats ID	No. of bacteria cfu/g	Temp. of soil
Al-Dair	A	3.6 X 10^3	28
Al-Nashwa	B	4 X 10^4	30
Karmat Ali	C	3.5 X 10^4	33
Al-Hartha	D	2.7 X 10^4	33
Um Qasr	E	7.9 X 10^3	28
Abu Al-Khaseeb	F	7.4 X 10^3	32
Al-Maqil	G	1.5 X 10^4	33
Shat Al-arab	H	1.9 X 10^4	27
Al-Zubair	I	3.2 X 10^3	26
Al-Qurna	J	3.3 X 10^3	24

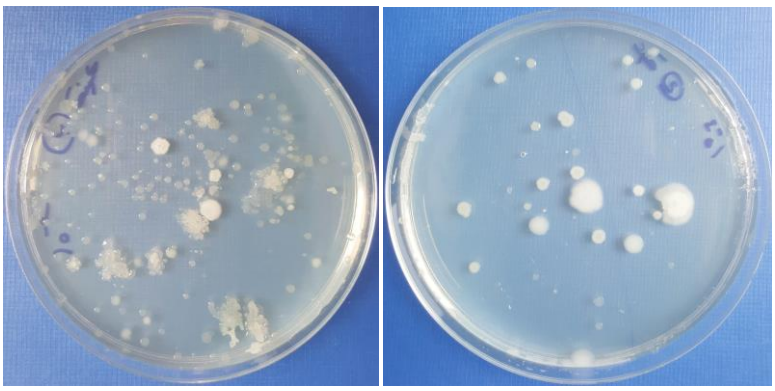

Figure 2. *Bacillus* related species isolated on nutrient agar by heat-shock method

The GenBank accession numbers of new strains

According to 16S rDNA sequence, the bacterial isolates were showed 100% similarity, except 8 isolates were showed 99% similarity, including isolates A5, A6, B2, D1, G4, H2, H3, and I3 were identified as new strains and their sequences were deposited at the National Center for Biotechnical Information (NCBI) under the Genbank accession numbers as shown in Table 3.

Distribution, frequency and phylogenetic tree of *Bacillus* related species

The distribution and frequency of *Bacillus* related species among the studied habitats were demonstrated in Table 2 and Figure 4. *B. licheniformis* and *B. subtilis* were the most dominant species in the study habitats. The strains of *B. licheniformis* bacteria are distributed in 8 habitats out of ten habitats, while the strains of *B. subtilis* bacteria are distributed in 7 habitats out of ten habitats. The strains of *Cytobacillus firmus* are distributed and frequented in 3 habitats, the strains of *B. safensis, B. cereus, C. oceanisediminis* and *P. megaterium* are distributed and frequented in 2 habitats, whereas the other bacterial species are recorded in one habitat, these species include *B. tequilensis, B. pumilus, B. infantis, B. vallismortis, B. albus, B. sonorensis, B. velezensis, P. flexa* and *Peribacillus simplex.*

On the other hand, the result showed the bacterial strains that was recorded among the studied habitats are 7 isolates at B habitat, 6 isolates at both A and G habitats, 5 isolates at both F and H habitats, 4 isolates at C habitat, 3 isolates at E, I and J habitats whereas 2 isolates are recorded at D habitat. The phylogenetic tree was constructed by MEGA X software from partial 16S rDNA sequences of 43 *Bacillus* related species isolates obtained in present study with selected type sequences downloaded from GenBank. The Clustal W program in MEGA X was used to align the sequences. The phylogenetic tree showed the relationships among the isolated *Bacillus* related species for each cluster as shown in Figure 5 which showed 9 clusters. Cluster I contained 11 strains belonging to *B. subtilis* and 1 strain belonging to *B. tequilensis,* 2 out 12 strains group in one subcluster. Cluster II included 2 strains that belong to *B. vallismortis* and *B. velezensis.* The cluster III contained 10 strains that belong to *B. licheniformis* and 1 strain that belonged to *B. sonorensis,* whereas some strains group in 2 subclusters. Cluster IV had 3 strains that belonged to *B. safensis and B. pumilus.* Cluster V contained 1 strain that belongs to *B. infantis.* Cluster VI held 7 strains that belong to *C. oceanisediminis* and *C. firmus,* whereas 3 strains were grouped in 1 subcluster. Cluster VII contained 3 strains that belong to *P. megaterium and P. flexa.* Cluster VIII contained 1 strain which belong to *Peribacillus simplex* whereas, cluster IX had 4 strains which belong to *B. cereus* and *B. albus*

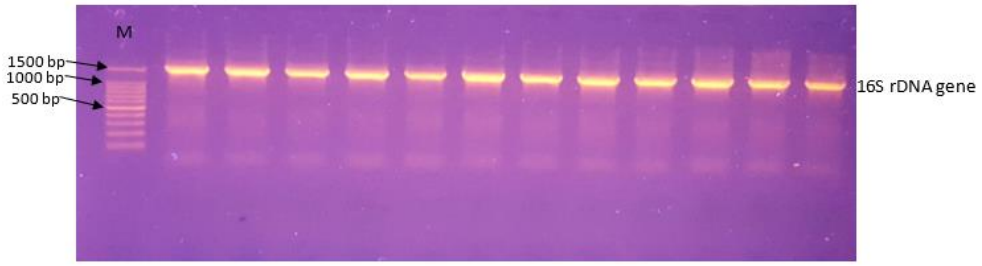

Figure 3. Agarose gel electrophoresis for PCR products of 16S rDNA (1500bp) for bacterial isolates. lane M: 100 bp DNA

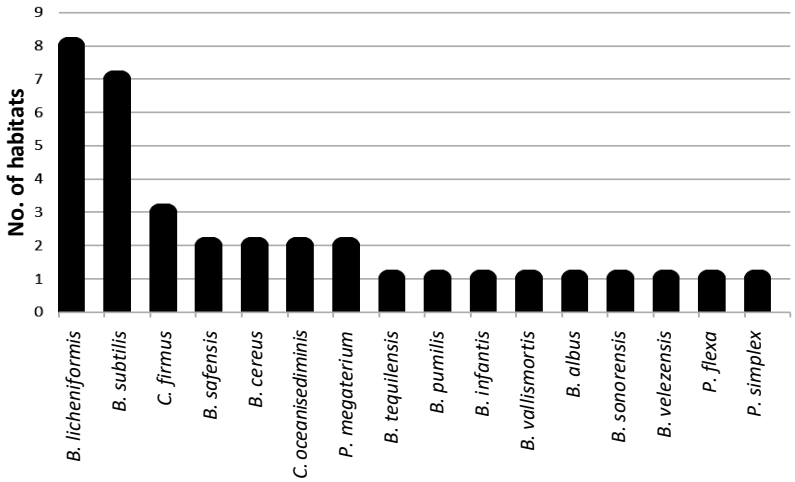

Figure 4. Distribution and frequency of *Bacillus* related species at various habitats

Table 2. Identification of bacteria by 16S rDNA gene sequencing

Habitats	Isolates code	Accession no. of closet species	species	Sequence identity%
A	A1	KX427024.1	*Bacillus subtilis* strain SXKF16-3	100
	A2	JX083969.1	*Cytobacillus firmus* strain NFB28	100
	A3	MG892780.1	*Bacillus licheniformis* strain SIITMB5	100
	A4	KC915230.1	*Bacillus licheniformis* strain ESR26	100
	A5	MW776610.1	*Bacillus subtilis* strain B4 16S	99
	A6	MK130899.1	*Bacillus subtilis* strain TPS4	99
B	B1	KT210121.1	*Priestia flexa* strain NJNPD41	100
	B2	KF876849.1	*Bacillus tequilensis* strain YJ-S4	99
	B3	MK318796.1	*Priestia megaterium* strain DK2	100
	B4	MK483703.1	*Bacillus safensis* strain kp9	100
	B5	KP192031.1	*Bacillus pumilus* strain LX11	100
	B6	HQ143565.1	*Bacillus licheniformis* strain PG5	100
	B7	MK210556.1	*Bacillus safensis* strain P9	100
C	C1	MT457444.1	*Cytobacillus oceanisediminis* strain C26	100
	C2	MT072145.1	*Bacillus licheniformis* strain QT201	100
	C3	MT372156.1	*Bacillus subtilis* strain YEBN5	100
	C4	MK578213.1	*Bacillus cereus* strain 2.3AL8	100
D	D1	MT078672.1	*Cytobacillus firmus* strain BF3-5	99
	D2	MN818696.1	*Bacillus cereus* strain AA6	100
E	E1	CP021920.1	*Bacillus sonorensis* strain SRCM101395	100
	E2	JF496512.1	*Bacillus licheniformis* strain WAS3-5	100
	E3	MT904796.1	*Bacillus subtilis* strain A5	100
F	F1	MF045813.1	*Bacillus licheniformis* strain KKR2017	100
	F2	HM753634.1	*Bacillus licheniformis* strain WSR-KSU302	100
	F3	MG892784.1	*Bacillus infantis* strain SIITMB9	100
	F4	DQ289059.1	*Bacillus cereus* isolate HKS 2-2	100
	F5	MN750767.1	*Peribacillus simplex* strain EH12	100
G	G1	MW186208.1	*Bacillus subtilis* strain ge25	100
	G2	MF077123.1	*Cytobacillus oceanisediminis* strain 224-LR35	100
	G3	MT383634.1	*Cytobacillus firmus* strain NPBR2	100
	G4	HM753621.1	*Bacillus licheniformis* strain SUM-KSU304	99
	G5	MN330424.1	*Bacillus vallismortis* strain T25-9	100
	G6	MK993461.1	*Bacillus albus* strain FA80	100
H	H1	MF045813.1	*Bacillus licheniformis* strain KKR2017	100
	H2	MW776612.1	*Bacillus subtilis* strain B29	99
	H3	MT214117.1	*Cytobacillus oceanisediminis* strain GA51	99
	H4	MW811465.1	*Bacillus subtilis* strain WZ-2	100
	H5	MK318796.1	Priestia megaterium strain DK2	100
I	I1	MG012485.1	Bacillus licheniformis isolate S8	100
	I2	CP043416.1	Bacillus velezensis strain ONU 553	100
	I3	MT111083.1	Bacillus subtilis strain MK736112.1	99
J	J1	KY753238.1	Cytobacillus firmus strain MR-39	100
	J2	KC456632.1	Bacillus subtilis strain TUST018	100
	J3	MW776612.1	Bacillus subtilis strain B29	100

Table 3. The bacterial isolates were recorded as new bacterial strains

Habitats	Isolates code	New bacterial strains	Sequence identity (%)	Accession no. of new strain
A	A5	*Bacillus subtilis* strain BSRNA1	99	MZ798373.1
	A6	*Bacillus subtilis* strain BSRNA3	99	MZ798375.1
B	B2	*Bacillus tequilensis* strain BSRNA2	99	MZ798374.1
D	D1	*Cytobacillus firmus* strain BSRNA4	99	MZ798376.1
G	G4	*Bacillus licheniformis* strain BSRNA7	99	MZ798379.1
H	H2	*Bacillus subtilis* strain BSRNA5	99	MZ798377.1
	H3	*Cytobacillus oceanisediminis* strain BSRNA6	99	MZ798378.1
I	I3	*Bacillus subtilis* strain BSRNA8	99	MZ798380.1

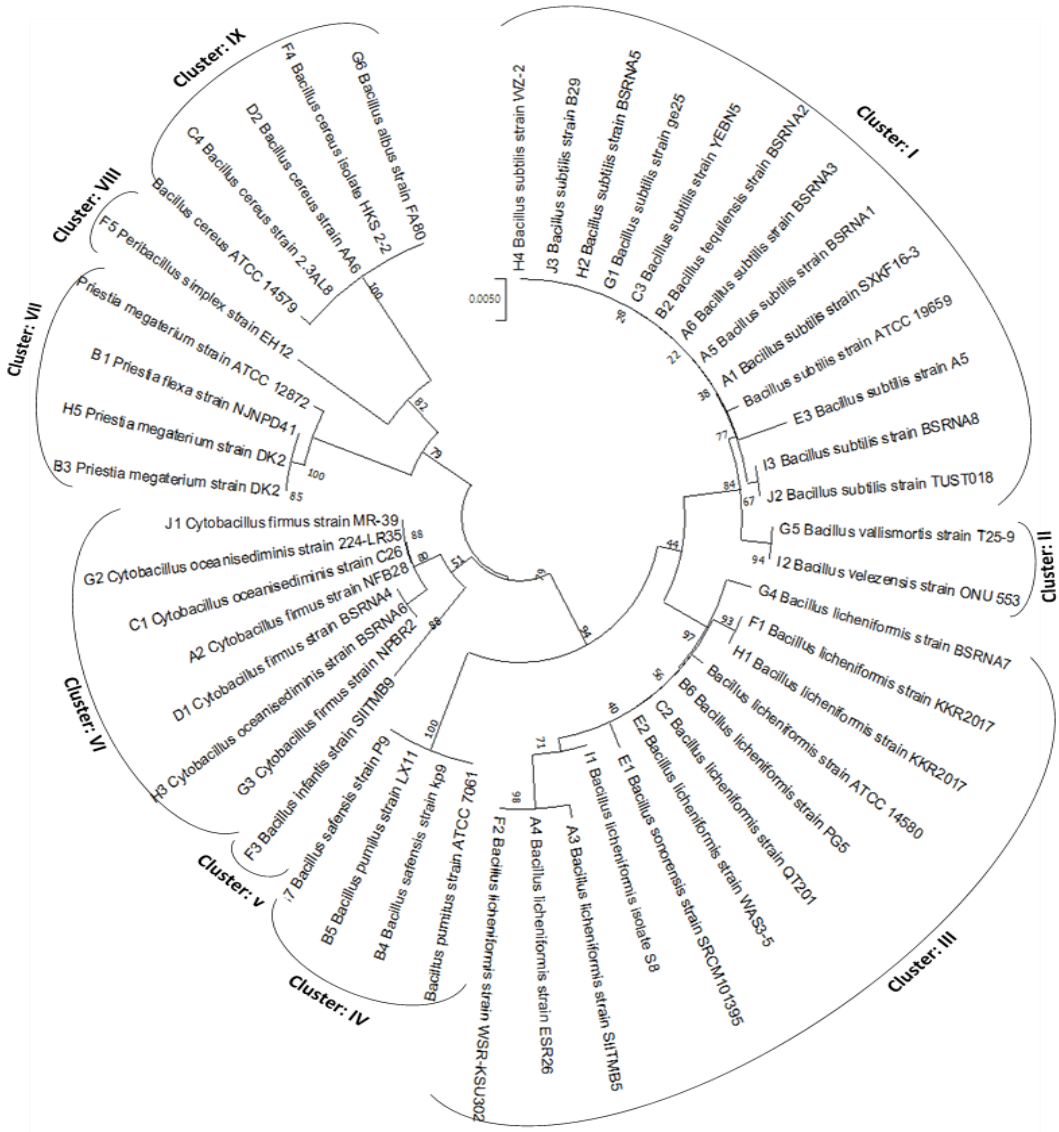

Figure 5. Neighbor-joining phylogenetic reconstruction tree showing evolutionary relationships of *Bacillus* related species with type strains available in NCBI GenBank database

Discussion

Bacillus related species are known and abundant to inhabit in soil, these bacteria are tolerant to extreme environmental conditions such as temperature, desiccation and radiation because *Bacillus* related species form resistant a thick outer wall spore (Liu et al. 2019). The heat-shock method applied in the current study proved a rapid and efficient approach in isolation and selection of spore-forming *Bacillus* like isolates and in suppression of other non-spore forming microbial isolates as reported in a previous study (McHugh et al. 2017). The heat-shock method was an efficient approach that could give important information on cultivable *Bacillus*-like communities living in soils and other habitats (Liu et al. 2016). The 16S rDNA gene is commonly used to identify the bacteria and is considered the best tool to study the bacterial phylogeny and taxonomy due to its presence in all bacteria, the function of the 16S rDNA gene has not changed over time and the length of 16S rDNA gene is suitable, in addition to accurate and reliable analysis that led to detect of new bacterial strains comparing with conventional techniques (Yadav et al. 2018; Al-Dhabaan 2019). The 16S rDNA sequence analysis allowed the identification of *Bacillus* related isolates at the species level, the sequence analysis revealed that the majority of Bacillus like isolates that were obtained in the present study belonged to the genera *Bacillus, Cytobacillus, Priestia* and *Peribacillus. B. licheniformis* and *B. subtilis* were the most dominant species in the study habitats. Both species were abundant and isolated from Basrah province's soil earlier (Alyousif et al. 2020). One strain of *B. tequilensis* and *B. vallismortis* species was isolated in the present study, these two species are most closely related to *B. subtilis. B. tequilensis* and strains of *B. subtilis* formed cluster I of the phylogenetic

tree as shown in Figure 5, whereas *B. vallismortis* formed a distinct cluster with *B. velezensis* species. *B. tequilensis* was recorded as a distinct species by Gatson et al. (2006). One strain of *B. sonorensis* species was isolated from one habitat in present study, this species showed high similarity with strains of *B. licheniformis* and all of them included in cluster III of phylogenetic tree. *B. sonorensis* was recorded as a distinct species for first time by Palmisano et al. (2001), this species was isolated in previous study from the produced water of the Al-Rafidiyah oil field in Basrah province (Hamzah et al. 2020). Two strains of *B. safensis* and one strain of *B. pumilus* were isolated from soil sample of B habitat, where both species formed one cluster in phylogenetic tree. One strain of *B. infantis* was isolated from F habitat in present study, this species formed distinct cluster in one branch. *B. infantis* was recorded as a distinct species for first time by Ko et al. (2006). Four strains of *Cytobacillus firmus*, three strains of *Cytobacillus oceanisediminis*, two strains of *Priestia megaterium*, one strain *Priestia flexa* and one strain of *Peribacillus simplex* were isolated from soil samples of different habitats, these species formed three distinct clusters in phylogenetic tree as shown in Figure 5. *Cytobacillus* and *Peribacillus* genera were proposed as new genera out *Bacillus* genus by Patel and Gupta in 2020, while genus *Priestia* was proposed as new genus by Gupta et al. (2020). Three strains of *B. cereus* species and one strain of *B. albus* were isolated in present study, these two species formed distinct cluster. *B. albus* was recorded as novel species of the *Bacillus cereus* group by Liu et al. (2017). *Bacillus cereus* strains were isolated from the soil samples of Basrah province in previous studies (Alyousif et al. 2020; Hamzah et al. 2020). In the current study eight *Bacillus* related species were identified as new strains and their sequences were deposited at the National Center for Biotechnical Information (NCBI) under the Genbank accession numbers as shown in Table 3. The DNA sequence that may change when bacteria change their original living environments, exposure to environmental factors and chemical mutagens leading to lose the ability to repair the damage in DNA and become inherited (Najafi and Pezeshki 2013). The distribution and diversity of *Bacillus* related species in the current study are different among the collected soil samples, and this is due to the nature and composition of the soil, which affects the number and species of *Bacillus* related species despite its resistance to harsh conditions due to its ability to form endospores. Ge et al. (2016) found the distribution and diversity of cultivable *Bacillus* related species in soils of Mount Wuyi, China, differed among the sites. In Taiwan, 136 species of *Bacillus* related bacteria were isolated by Liu et al. (2016) from 20 soils samples, and the results showed no correlation between *Bacillus* related bacteria distribution and sampling sites, while Liu et al. (2019) reported the distribution of *Bacillus*-like bacterial communities was positively correlated with the latitudes of sampling sites and soil total carbon content. Therefore, *Bacillus* related species are a significant part of the microbiota in many soils, and many species of these bacteria have important practical applications because they produce enzymes and other industrially useful products as well as they play important roles in recycling nutrients, nitrogen fixation and mineral enrichment of soil (Yahya et al. 2021). *B. licheniformis* is the most abundant isolate in the study habitats, this bacterium has high biotechnological application in a wide range of fields include agriculture, biomineralization, biomedicine, biofuel production, bioremediation, and anti-biofilm activity as a result of enzymes and biomolecules production by *B. licheniformis* (Muras et al. 2021). *Bacillus subtilis* is a second abundant isolate in the study habitats, this bacterium is a highly efficient producer for many industrial products including antibiotics, chemicals, vitamins, enzymes and amino acids. These products of B. subtilis play an important role in different fields such as agriculture, food processing, cosmetics and pharmaceuticals (Su et al. 2020). Both *B. licheniformis* and *B. subtilis* are licensed as probiotics for humans, veterinary and aquaculture under commercial products such as Primal Defense™, AlCare™ and BioPlus® 2B (Muras et al. 2021). The present study revealed the higher diversity and distribution of *Bacillus* related species in the soil samples collected from different habitats of Basrah province. The molecular technique was the best method for identifying *Bacillus* related species. In the current study further studies of obtained species are required to apply these bacteria in various fields.

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