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Global Registration of Environmental Bacteria Recorded in NCBI, ENA and DDBJ for New Iraqi Isolates

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Abstract: The current study included the identification and new global registration of bacteria isolated from two environmental sources (soil and water). The Goal of this research is screening the microbiome community with a highly adaptability of new bacterial strains grown in environment. The molecular identification of 28 isolates was successfully performed using the amplification and sequencing of the 16SrRNA gene. Additionally, bacterial identification was confirmed through Gram staining. Fifteen samples isolated from soil and Thirteen from water were recorded as new strains in the National Center for Biotechnology Information (NCBI) and the Gene bank Data. Results showed, that the bacterial species involved: (6) *Bacillus subtiles, (1) Psychrobacter nivimaris, (2) Terribacillus aidingensis, (1) Zobellella denitrificans, (1) Bacillus cabrialesii, (2) Staphylococcus gallinarum , (1) Photobacterium halotolerans, (1) Bacillus paramycoides, (1) Pseudomonas putida, (4) Staphylococcus epidermidis, (3) Staphylococcus haemolyticus, (1) Rheinheimera aquimaris, (1)Planomicrobium okeanokoites,, (1)Micrococcus endophyticus, (1) Rheinheimera baltica, and (1) Staphylococcus saprophyticus*. Gene investigations were performed on all isolates and the phylogenetic tree of the isolates was constructed using MEGA 11 software. These strains identified based on phenotypic properties and molecular techniques. The new bacterial strain exhibited a significant performance in environment.

Key words: Bacteria , soil, water, global registration, gene bank , Iraq.

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Introduction

Microbes are omnipresent, extremely diverse and perform specialized roles in the environment. Taxonomic information of an unknown microorganisms are highly essential to establish its biodiversity, relationship among other microbes in the environment and its functional aspects (1). Microbial biologists simply lacked most of the classical tools, concepts, and theory obtainable to systematics, ecologists, and evolutionary biologists. Advances in molecular biology, macromolecular sequencing techniques, and genomic technologies, however, are changing the playing field dramatically for microbial biologists (2). Environment is not only spatial place for human but also landfill for their waste which generated from their activity. It has been the ability to revive the situation and neutralize its own condition and restore their initial state, if the waste is below the threshold of

environment carrying capacity (3).This leads to the changes on environmental quality of water, soil and air that also effect on the flora, fauna and microorganisms life (4). In the environment the number and type of microorganisms is influenced by ecological characteristic also that flow into the environment. It either inhibits or stimulates the growth of microorganisms. Now, serious inroads are being made in microbial ecology and evolution, paved largely by the application in molecular methods. Water is very essential to society for different uses, including drinking, agriculture, industry, and more, from the beginning of human civilization (5). Water is one of the most widespread substances on planet Earth, since almost 70% of the total surface of the globe is covered by water, forming one of its shells ,Several sources from water in developing nations are dangerous due to the presence of contaminants and dangerous physical, and chemical and biological (6). Soil is one of the most valuable resources on earth, essential for food security on a global level, On the other hand, the diversity of soil bacterial communities is enormous a single gram of soil may contain $1x10^3$ to $1x10^6$ unique 'species' of bacteria (7-9). While the vast majority of this diversity remains uncharacterized, recent molecular advances (2) allow us to survey the full extent of soil bacterial diversity at an ever-increasing pace. Although such molecular surveys provide essential information on the composition of soil bacterial communities, they are only the first step towards understanding the ecology of such communities. At present, we are mostly incapable of to interpret the taxonomic survey data in an ecologically significant way, nor do we understand why certain taxa are more abundant in some soils compared to others. A select number of bacterial taxa have been well studied and their ecological characteristics are reasonably well defined. However, these taxa are the exceptions: the majority of soil bacterial taxa, even those that are numerically dominant, have not been extensively studied and their ecological characteristics remain largely unknown (10).Generally, microbial biology has been developed along research lines largely independent of other biological disciplines, mainly for technical reasons. Bacteria are widely used in aquaculture systems as the considered as the main food sources of the marine finfish (11). Because of their high economical value, extensive research had been carried out on the ecophysiology of Bacterial strains or species (12). These studies focused on the understanding of dynamics bacteria in laboratory (13).

The term strain refers to an isolate or a group of isolates that can be distinguished from other isolates of the same bacterial species, in addition to the definition Isolate is pure culture of bacteria presumably derived from a single organism. The process of differentiating bacterial isolates beyond the species level is referred to as "strain typing" or "subtyping" and is based on genotypic (the genetic information dictating a particular trait) or phenotypic (visible, expressed traits influenced both by the genotype and environmental factors) characteristics (14). Identification of organisms by 16S rRNA gene sequencing has been described in reports focusing on a particular bacterial phylum or genus, such as Mycobacterium (15,16). and we

previously reported a series including environmental bacteria (17). Although 16S rRNA gene sequence analysis has become a common method for the accurate identification of bacterial isolates (18). and its definition of bacterial species has been precise (19).The ability to obtain DNA sequence information from an environmental sample (by PCR amplification followed by cloning or direct sequencing) allowed characterization of phylogenetically relevant markers, such as 16S rRNA gene sequences, regardless of the viability of the organism that harbored the DNA (20).

However, rRNA, and other core genes involved in information transfer, and therefore appear not to have been as extensively laterally exchanged, provide the most coherent frame work for understanding and interrelating the main evolutionary branches on the extant collection tree of life (21).

The phylogenetic tree visually presents the evolutionary history and phylogenetic relationships between different taxonomic units, helping people's understanding of the causes of species morphological diversity and evolutionary patterns (22). On the one hand, a phylogenetic tree can drive the development of phylogenetic systematics (23). On the other hand, it can help reveal patterns such as genetic structure, gene flow, and genetic drift among populations, providing important clues for population genetics research (24,25). Mainly, for phylogenetic purposes these sequences can be used for the genetic identification of the various bacteria species (26).

Materials and Methods

Samples collection

A total of fifty bacterial samples were collected from various locations in Al-Basrah city, specifically from soil and water. These samples were meticulously handled and kept in transport box. Each sample, consisting of 100 ml, was collected using sterile falcon tubes and subsequently transferred to the laboratory for bacteriological investigation, conducted from December of 2023 to March of 2024.

Isolation of bacteria

After being re-suspended, the primary cultured on Nutrient agar and incubated at 37°C for 24 hours. then subcultured. To obtain pure cultures, a single colony was streaked onto Nutrient agar. The pure culture was stained by Gram using the technique outlined by (27). The positive samples for bacterial growth then cultured again on nutrient agar tubes with a slant to maintain the bacteria for long-term storage the maintenance protocol was activation of bacteria in 5 ml of nutrient broth at 37°C for 24 hours.

Molecular methods

DNA Extraction

DNA extraction begin after a single colony of bacterial isolate activated in 5 ml of sterilized nutrient broth and incubated at 37°C for 24 h according to the procedure of Presto™ Mini g DNA bacteria kit Serial No. L126505 by the Geneaid company**.**

Detection of Genomic DNA by Agarose Gel Electrophoresis

Electrophoresis was performed using TBE buffer, Bromophenol blue, Ethidium bromide and Agarose (28,29). A 0.8% agarose gel was prepared by dissolving 0.2 g of agarose in 25 ml of 1× TBE buffer, followed by the addition of 0.2 µl of Ethidium bromide. The gel was cast, allowed to solidify,and then loaded with a mixture of 4 µl of DNA and 2 µl of Bromophenol blue. The gel was run at 60

V until the dye migrated and DNA bands were visualized under a UV transilluminator.

Identification of bacteria by 16S rDNA gene

Bacterial isolate was identified by sequencing 16S rDNA gene using universal primer Forward 27 F 5'-AGAGTTTGATCCTGGCTCAG-3'and Reverse 1492 R 5'-GGTTACCTTGTTACGACTT-3'.The first step to bacterial identification is amplify the 16S r DNA gene by PCR technique (30).

The Reagent (50 µl) of PCR for amplifying 16S rDNA gene contained : Taq green master mix (25µl), DNA template (2µl) ,Primer forward (2µl) ,Primer reverse (25µl),Nuclease free water (19µl) and total volume (50µl), for the PCR amplification program see Table (1).

Table (1): PCR program of 16S rDNA amplification.

Stage	Temperature	Time	Cycles	Size of product
Initial denaturation	95 ° C	5 min	1	1500 bp
Denaturation	95° C	30 sec	35	
Annealing	55° C	30 sec		
Extension	72°C	1 min		
Final extension	72° C	5 min	1	

Sample preparation for sequencing

The samples of product was sent to Macrogen company for sequencing PCR product of each sample was labeled with a number identical to the number of excel sheet which is given by Macrogen company, the products purification were accomplished by the company for sequencing (31).

Bacterial species

Bacterial species were identified by Basic Local Alignment search tool (BLAST) followed by National Center for Biotechnology Information (NCBI) as (32) .Then the program was identified the bacterial species with others by comparing their sequences together.

Analysis of the sequence data

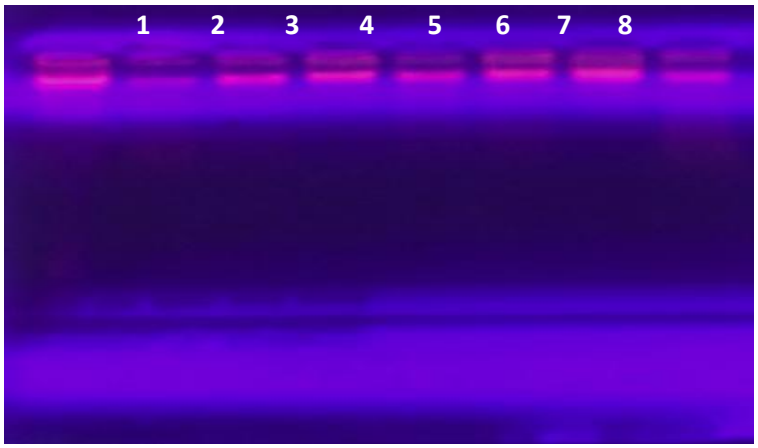
The phylogenetic is a tree of life, or evolutionary tree (33-35). For the construction of the Neighbor Joining Tree, all 16S rDNA sequences obtained were trimmed according to the quality of the sequencing Multiple cluster alignment and phylogenetic analysis were performed on MEGA 11 software (Molecular Evolutionary Genetics Analysis) Version 11 based on the neighbor binding method using a 1000 repetition bootstrap to evaluate statistical support (36).

Results and Discussion

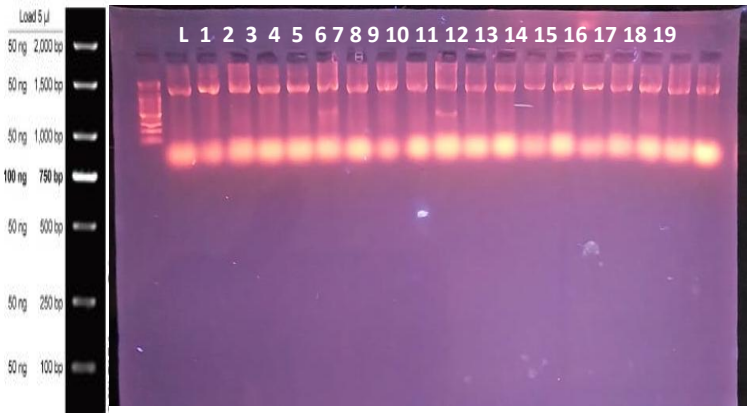
Identification and Detection of New Global Bacterial Strains.

From 50 samples collected from water and soil during the current study, 28 new global bacterial isolates were obtained: 18 were identified as Gram positive (64.2%) and 10 as Gram negative bacteria(35.7%) , There were other samples from their no registered the new strain in gene bank .Figure (1) showed the extracted DNA for all samples were subjected after that amplify the 16S rDNA gene using PCR. The amplified bands of all bacterial isolates were visualized under UV trans illuminated which showed a single band for each isolate with size roughly 1500bp when compared to a typical ladder figure(2). The 16S rDNA gene of 28 isolates was successfully sequenced, and the bacterial species were identified after trimming and treating using (BLAST).The new isolates

data bases were recorded in DDBJ, published on the NCBI and the GenBank as bellow : Bacillus included 3 species *B.subtilis* was the highly frequented (6 isolates) 21.4% , whereas all the other Bacillus species frequency was (1 isolate) for each 3.5% *B. cabrialesii and B. paramycoides* , in addition to Staphylococcus included 4 species : *S. epidermidis* (4 isolates) 14.2% , *S. haemolyticus (* 3 isolates) 10.7% *S. gallinarum* (2 isolates) 7.1% *and S. saprophyticus (* 1 isolate) 3.5% on the other hand , Rheinheimera included 2 species frequency was 1 isolate for each *R. aquimaris* and *R. baltica* 3.5% **,** *Terribacillus aidingensis*(2 isolates) 7.1% whereas all the other species frequency was 1 isolate for each 3.5% *Psychrobacter nivimaris* (1), *Pseudomonas putida* (1) , *Zobellella denitrificans (*1), *Photobacterium halotolerans* (1) ,*Micrococcus endophyticus* (1) and *Planomicrobium okeanokoites* (1) . We observed in our results the mutation divided in two types Transition and Transversion , Mutation locus ,Mutant nucleotide and what the type of amino acid transferred was explained in table (2) for all 28 isolates.



Figure(1):Agarose gel electrophoresis (0.2 gm) showed of whole Genomic DNA , Lane 1- 8: for bacterial isolates bands.



Figure(2):PCR amplification results show 1-19 Gene 16S rDNA (1500bp) bands for bacterial isolates and Lane L: 100bp-2Kbp Ladder.

Table(2): NCBI registered bacterial isolates with the mutation types and nucleotides locus.

No	Bacteria Name	Sample type	Gram stain	Mutation locus	Mutant nucleotide	Mutation type	Type of amino acid
1.	*Bacillus subtiles*	Soil	+ve	151	A=G	Transition	Ser=Gly
2.	*Staphylococcus gallinarum*	Soil	+ve	682	A=G	Transition	Ser=Gly
3.	*Psychrobacter nivimaris*	Water	-ve	974	G=T	Transversion	Gly=Arg
4.	*Terribacillus aidingensis*	Soil	+ve	894, 906	G=A A=G	Transversion Transition	Asp=Asn Thr=Ala
5.	*Zobellella denitrificans*	Soil	+ve	98 296 475	G=C C=G A=G	Transversion Transversion Transition	Ala=Pro Pro=Arg Lys=Glu
6.	*Bacillus cabrialesii*	Soil	+ve	863	G=C	Transversion	Arg=Pro
7.	*Staphylococcus gallinarum*	Water	-ve	629 647 650 919	T=G A=G G=A G=T	Transversion Transition Transition Transversion	Ile= Ser Glu=Arg Arg=Lys Cys=Phe
8.	*Photobacterium halotolerans*	Water	-ve	233 275 325 359, 360 373 379	G=C G=A G=A T=G, G=T A=G A=G	Transversion Transition Transition Transversion, Transversion Transition Transition	Try =Ser Cys=Tyr Val =Ile Met=Ser Arg=Gly Arg=Gly
9.	*Bacillus subtilis*	Soil	+ve	845	A=C	Transversion	His=Pro
10.	*Bacillus paramycoides*	Water	+ve	366	A=G	Transition	Phe=Leu
11.	*Pseudomonas putida*	Soil	-ve	746	A=T	Transversion	Glut=Val
12.	*Bacillus subtilis*	Soil	+ve	1108	A=C	Transversion	Asp=Thr
13.	*Staphylococcus epidermidis*	Water	+ve	228 749	A=G G=A	Transition Transition	Tyr=Cys Glu=Lys
14.	*Staphylococcus haemolyticus*	Soil	-ve	643 748 873	G=A G =A C=G	Transition Transition Transversion	Asp=Lys Glu=Lys Cys=Try
15.	*Bacillus subtilis*	Soil	+ve	859	G=C	Transversion	Ala=Pro
16.	*Rheinheimera aquimaris*	Water	-ve	726	G=C	Transversion	Glu=Gln
17.	*Planomicrobium okeanokoites*	Water	-ve	1082	G=C	Transversion	Glu=His
18.	*Bacillus subtilis*	Soil	+ve	862	G=C	Transversion	Ala=Pro
19.	*Micrococcus endophyticus*	Soil	+ve	866 875	T=G G= C	Transversion Transversion	Val= Gly Arg=Pro
20.	*Staphylococcus epidermidis*	Water	+ve	353 715 734 736 764	G=A T=C T=G A=G A=G	Transition Transition Transversion Transition Transition	Ser=Asp Ser=Pro Ile=Ser Ile=Val His=Arg

No	Bacteria Name	Sample type	Gram stain	Mutation locus	Mutant nucleotide	Mutation type	Type of amino acid
21.	*Terribacillus aidingensis*	Water	+ve	643 839	G=A T=C	Transition Transition	Arg=Lys Leu=Pro
22.	*Rheinheimera baltica*	Water	-ve	352 748	C=T A=G	Transition Transition	Arg=Cys Arg=Gly
23.	*Bacillus subtilis*	Soil	+ve	862	G=C	Transversion	Ala=Pro
24.	*Staphylococcus saprophyticus*	Water	+ve	375 884 956	G=C G=T A=G	Transversion Transversion Transition	Asn=Lys Cys=Phe His=Arg
25.	*Staphylococcus epidermidis*	Water	+ve	605 752 763	A=G G=A A=G	Transition Transition Transition	Lys=Arg Arg=Lys Thr=Ala
26.	*Staphylococcus haemolyticus*	Soil	-ve	581 683 736 755 765	A=G A=G T=G G=A A=G	Transition Transition Transversion Transition Transition	Asn =Ser Glu= Ala Ser=Ala Arg=Lys Thr=Ala
27.	*Staphylococcus epidermidis*	Water	+ve	641 752	A=G A=G	Transition Transition	Glu=Gly Arg=Lys
28.	*Staphylococcus haemolyticus*	Soil	-ve	739	G=C	Transversion	Asp=His

Registration of global bacterial isolates

The present study showed recording of 28 new bacterial isolate and recorded in NCBI with Accession number, Percentage identify and Name of the new strain in Gene Bank as table (3).The similarities between the recorded strains and their type strains in gene bank were 99% that is excellent in classification see the Accession number for all isolates in table (3). The new strains were resulted from mutation resulted from mistake or error in the DNA or RNA strand leading to a change in the nucleotide sequence (37). The new strains in this study were isolated from environmental (water and soil) sources.

Table (3): The total numbers of Bacterial isolates mutations (New), Accession number, Percentage identify and Name of the new strain in Gene Bank

No. of isolates	Strain name (NEW)	Accession number	Percentage identify	Name of the new strain in Gene Bank
1	*Bacillus subtilis*	PQ203227	99%	HAB1
2	*Staphylococcus gallinarum*	PQ203250	99%	HAB2
4	*Psychrobacter nivimaris*	PQ215580	99%	HAB4
5	*Terribacillus aidingensis*	PQ215664	99%	HAB5
6	*Zobellella denitrificans*	PQ215786	99%	HAB6
7	*Bacillus cabrialesii*	PQ215811	99%	HAB7
9	*Staphylococcus gallinarum*	PQ222364	99%	HAB9
10	*Photobacterium halotolerans*	PQ215861	99%	HAB10

No. of isolates	Strain name (NEW)	Accession number	Percentage identify	Name of the new strain in Gene Bank
11	*Bacillus subtilis*	PQ222381	99%	HAB11
12	*Bacillus paramycoides*	PQ219062	99%	HAB12
13	*Pseudomonas putida*	PQ222366	99%	HAB13
14	*Bacillus subtilis*	PQ222382	99%	HAB14
15	*Staphylococcus epidermidis*	PQ219239	99%	HAB15
17	*Staphylococcus haemolyticus*	PQ236499	99%	HAB17
20	*Bacillus subtilis*	PQ236500	99%	HAB20
21	*Rheinheimera aquimaris*	PQ236501	99%	HAB21
22	*Planomicrobium okeanokoites*	PQ236502	99%	HAB22
26	*Bacillus subtilis*	PQ222383	99%	HAB26
29	*Micrococcus endophyticus*	PQ236503	99%	HAB29
31	*Staphylococcus epidermidis*	PQ219240	99%	HAB31
34	*Terribacillus aidingensis*	PQ236504	99%	HAB34
37	*Rheinheimera baltica*	PQ236505	99%	HAB37
39	*Bacillus subtilis*	PQ222384	99%	HAB39
45	*Staphylococcus saprophyticus*	PQ236506	99%	HAB45
47	*Staphylococcus epidermidis*	PQ219241	99%	HAB47
48	*Staphylococcus haemolyticus*	PQ236507	99%	HAB48
49	*Staphylococcus epidermidis*	PQ219242	99%	HAB49
50	*Staphylococcus haemolyticus*	PQ236508	99%	HAB50

16S rDNA gene amplification and phylogenetic tree

Bacterial species were identified by 16S rDNA figure (2): instead of biochemical tests of time-consuming and may give false negative results, while molecular method including 16S rDNA sequencing of a housekeeping gene is an excellent tool for classifying and phylogenetic relationships. It is found in all bacterial species and enough for bioinformatics. The function of 16S rDNA as a protein synthesis from a conserved gene and does not changed across the time makes it as an internal control in Real time PCR (38). The phylogenetic relationship among twenty-eight bacterial species showed in figure (3).In addition, each species group was represented by only one isolate to avoid the overlapping in the tree.

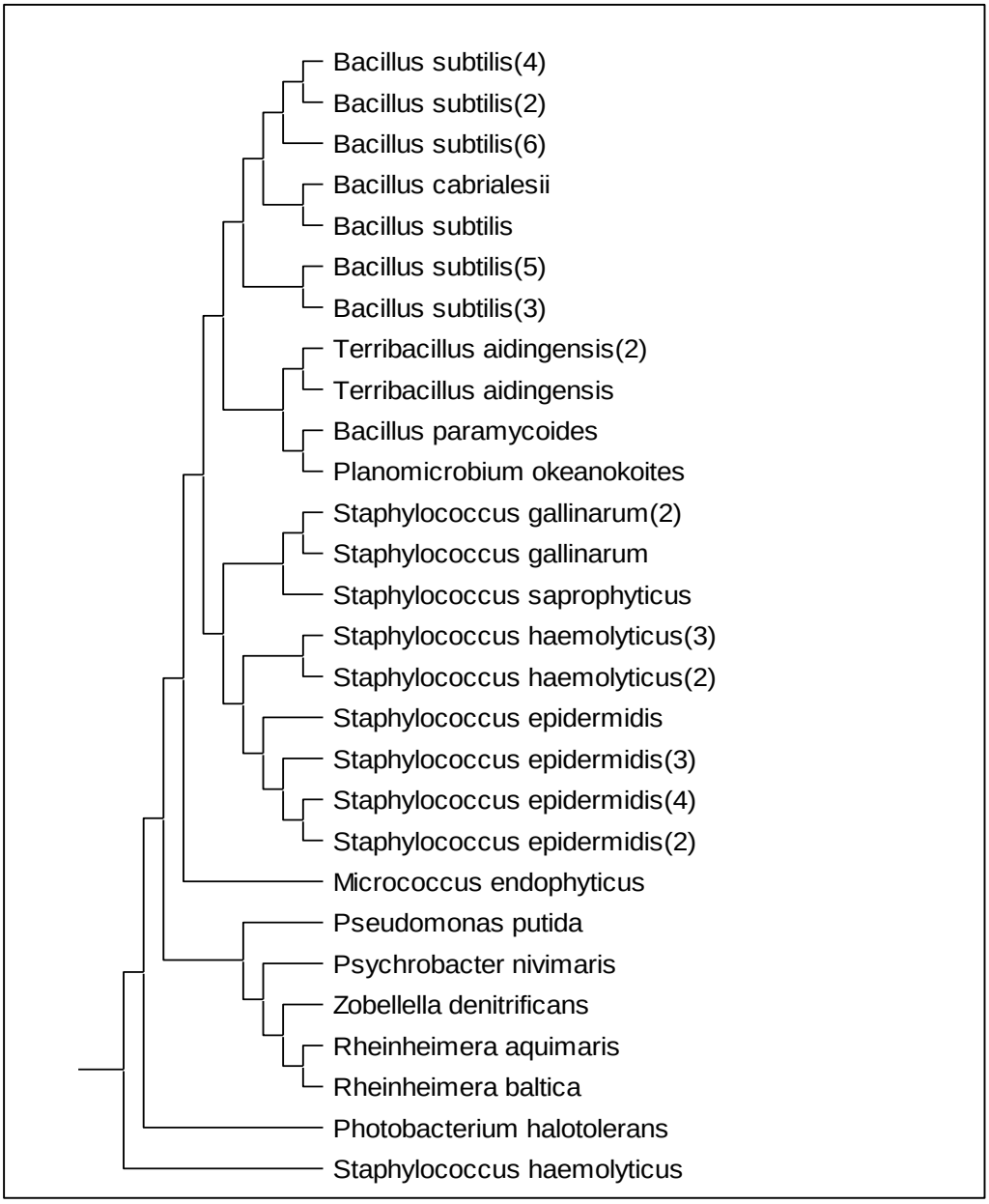


Figure (3): Rooted Neighbor Joining phylogenetic tree showing phylogenetic relationship of different 28 bacterial isolates that is constructed from MEGA11 software algorithm.(36).

Conclusion

Regarding to our results we concluded that, there are 28 new isolates were registered as a global strains which are *Bacillus subtiles Psychrobacter nivimaris, Terribacillus aidingensis, Zobellella denitrificans, Bacillus cabrialesii, Staphylococcus gallinarum , Photobacterium halotolerans, Bacillus paramycoides, Pseudomonas putida, Staphylococcus epidermidis, Staphylococcus haemolyticus, Rheinheimera aquimaris, Planomicrobium okeanokoites, Micrococcus endophyticus , Rheinheimera baltica, and*

Staphylococcus saprophyticus . The new strain exhibit a critical role in biodiversity and indicated that the different proportions according to their site of origin .

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