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Molecular Identification of Isolated Bacteria from Soils in Likouala Peat Bog Area, Republic of Congo

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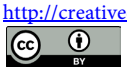

Abstract

This study aimed to the molecular identification of isolated bacteria from the soils of Likouala (Congo-Brazzaville) peat bog area. Counting and isolation were carried out on Mossel and TSB media enriched with petroleum and vegetable oil; the amplification and sequencing of 16S RNA genes by PCR and Artic Oxford Nanopore Technology. The results showed bacterial loads of $(5.81 \pm 1.08) \times 10^4$; $(6.64 \pm 1.94) \times 10^4$ et $(8.56 \pm 1.19) \times 10^3$ CFU/g on Mossel respectively for samples 1, 2 and 3 against $(2.12 \pm 4.1) \times 10^8$ et $(8.15 \pm 10.1) \times 10^7$ CFU/g respectively on TSB enriched with petroleum and vegetable oil exclusively with sample 2. The analysis of the 16S rRNA of the isolates gene made it possible, after PCR, agarose gel electrophoresis, sequencing and bioinformatics analysis, to identify eight (08) strains with similarities of 99 to 100% whose sequences genes placed in GenBank have made it possible to obtain accession numbers corresponding to: *Bacillus thuringiensis strain* (ON303633); *Bacillus cereus strain* (ON350770); *Bacillus thuringiensis strain* (ON350771); *Bacillus thuringiensis strain* (ON738723); *Priestia megaterium strain* (ON738719); *Bacillus anthracis strain* (ON738720); *Bacillus subtilis* (ON738721); *Enterobacter sp* (ON738722). The phylogenetic classification of strains was done and revealed two genuses which are Bacillus and Enterobacteriaceae.

Keywords

Bacteria, Peat Bog Area, PCR, Sequencing, 16S rRNA

1. Introduction

The research of microorganisms in peatlands has been of increasing interest in the last five years, with the aim of understanding their influence in the functioning and stability of peatlands and the interaction between microbial activities and the effects of climate change. Several studies carried out on the role of peatlands in the global carbon cycle in the face of global warming show a rise in temperatures leading to a strong degradation of organic matter following an increase in microbial activity, resulting in a significant release of CO_2 and carbon fixation by photosynthesis [1]. Microflora studies have revealed the presence of high amounts of molds in the peat with concentrations of molds close to 107 per gram of dry peat; up to 23 species of yeasts belonging to the genera *Trichosporon*, *Candida*, *Rhodotorula* and others [2], and other species of pathogenic mycobacteria such as *Mycobacterium fortuitum* [3]. Although the study of the diversity of microorganisms in peatlands is the subject of growing interest, the processes maintaining this diversity and its role in the functioning and stability of peatlands remain to date very little explored. This lack of knowledge is even greater if we consider the interaction between the effect of diversity and the effects of climate change [4]. Indeed, despite advances in knowledge of current carbon storage, there are still considerable knowledge gaps on the microbiology of these ecosystems [5]. The best understanding lies mainly in the ability to better characterize communities of microorganisms both at the taxonomic and functional level. To date, in the Republic of Congo, no identification of microbial strains has been carried out on the microorganisms of the peat soils of Likouala. In order to assess the evolution of its quality and its impact on the environment, we proposed to identify after having characterized the bacteria contained in the soils of the Likouala peat bog area.

2. Materials and Methods

2.1. Materials

The biological materials used in this study were three (3) composite soil samples collected in the Likouala peat bog area. A total of 9 soil samples collected. We used the samples collected according to [6]. After collection of soils samples, all the samples were transferred to a molecular microbiology Laboratory for analyses.

2.2. Methods

2.2.1. Enumeration and Isolation

The samples were cultured on Mossel media and TSB enriched with petroleum and vegetable oil [7] [8]. After culture, the colonies were purified on Mossel and TSB enriched. A colony is removed using a loop or Pasteur pipette using the striation technique to obtain isolated colonies. The dishes were incubated in an oven at 37˚C, and then observed after 24 h. The isolates were considered pure and then stored in sterile Eppendorf tubes containing 900 µl of liquid TBS and

100 µl of glycerol, then kept cool at −20˚C [9].

2.2.2. Phenotypic Characterization of Isolates

The phenotypic characterization of the strains was carried out by applying classic microbiology techniques based on the search for phenotypic characters: cultural characters, morphological characters (colony and cell morphology), Gram type and catalase production [10] [11].

2.2.3. Molecular Identification

1) DNA extraction

The extraction was done using the Qiagen kit whose execution was carried out as indicated by the manufacturer.

2) Evaluation of DNA concentration

DNA concentration and purity were measured using a Biorad spectrophotometer, using the A 260/A280 ratio to assess protein contamination in the DNA solution DNA electrophoresis was performed on 1% agarose gel [12].

3) PCR amplification of the 16S rRNA gene

a) Primers design

Universal primers were used to amplify genes encoding 1500 bp ribosomal RNA [12]. See in **Table 1**.

b) Mix and PCR conditions

The PCR reaction was carried out in a final volume of 50 µL according to the conditions in **Table 2**. 1 µL for each primer and 5 µLfor the buffer, all concentrations of all PCR mix are given in **Table 2**.

The PCR amplification was carried out in a Thermocycler as follows: first

Table 1. Universal primers for amplification of 16S RNA.

Primer	Nucleotidic sequence	Primer Size
fD 1	5'-AGAGTTTGATCCTGGCTCAG-3'	(1500 pb)
rP 2	5'-ACGGCTACCTTGTTACGACTT-3'	

Table 2. PCR reaction mix.

Components	Initial concentration	Final concentration	Reaction volume (µL)
Buffer taq	10×	0.25×	5
Primer F	20 Mm	0.50 µM	1
Primer R	20 Mm	0.50 µM	1
d NTP	10 Mm	0.25×	1
H_2O	-	-	39.5
Taq polymerase	5 U/Ml	0.13	0.5
ADN	-	-	2
Final volume of reaction		50 µL	

initial denaturation at 95˚C for 5 min; 30 cycles for each of the following steps; denaturation at 95˚C for 30 seconds; Hybridization was carried out at 55˚C for 30 seconds; elongation at 72˚C for 1 min 30 s, and a final elongation at 72˚C for 5 min, followed by storage of the PCR fragments (amplicons) at 4˚C.

c) DNA Electrophoresis and Sequencing

DNA electrophoresis was performed on 1% agarose gel using TBE buffer DNA was revealed with Syber green then visualized using a computer combined with a band visualization device of the Gel Doc EZ imager type (UV device). The size of the DNA amplicon is estimated using a molecular weight marker. For sequencing, the sequence library was prepared according to the Oxford Nanopore Technology protocol. Sequences were purified with the AMpurex kit, then quantified using the spectrophotometer, normalized, coded, and sequenced using the GRiDION Oxford Nanopore tool.

2.2.4. Results Analysis

The results were represented, illustrated and statistically analyzed by Microsoft Excel 2016 software. For the Bioinformatics analysis, all the sequences were the subject of an in-silico study in BLASTn which revealed allowed us to identify the eight sequences and two homologues from the bank. The comparison of the partial nucleotide sequences of the gene encoding 16S rRNA using the GenBank portal by alignment allowed us to obtain identification rates that vary from 96.75 to 100%. Using percentage of similarity, E value, ten sequences were aligned with ClustalW and we the inference phylogenetic tree was performed with PhyML [8]. The eight new sequences were definitely submitted to GenBank.

3. Results

3.1. Enumeration

Table 3 shows the bacterial loads of samples 1, 2 and 3 on Mossel medium. The total load of bacteria of the *Bacillus* genus depends on the sample. Samples 1 and 2 present loads of $(5.81 \pm 1.08) \times 10^4$ and $(6.64 \pm 1.94) \times 10^4$ CFU/g respectively against the load of sample 3 where it is $(8.56 \pm 1.19) \times 10^3$ CFU/g.

Table 4 presents the bacterial loads on TSB medium enriched with petroleum

Table 3. Loads (CFU/g) of bacteria of the *Bacillus* genus in the samples.

sample	Mossel medium
1	$(5.81 \pm 1.08) \times 10^4$ CFU/g
2	$(6.64 \pm 1.94) \times 10^4$ CFU/g
3	$(8.56 \pm 1.19) \times 10^3$ CFU/g

Table 4. Loads (CFU/g) of hydrocarbonoclasts bacteria of sample 2.

Sample	TSB + petrol	TSB + vegetable oil
2	$(2.12 \pm 4.1) \times 10^8$ CFU/g	$(8.15 \pm 10.1) \times 10^7$ CFU/g

and vegetable oil mainly with the sample 2 because of its load on Mossel medium. The bacterial load is higher with the TSB medium enriched with petroleum with $(2.12 \pm 4.1) \times 10^8$ CFU/g against $(8.15 \pm 10.1) \times 10^7$ CFU/g with the TSB medium enriched with vegetable oil.

3.2. Isolation and Characterization

Table 5 shows the phenotypic characteristics of the sequenced strains. Microscopic examination reveals the rod-shaped bacteria, the majority of which were Gram positive (Gram+) and catalase positive. These 10 isolates were selected and identified by molecular biology.

3.3. Molecular Identification by 16S rDNA Analysis

3.3.1. Extracted DNA Concentrations

After extraction of the genomic DNA, the concentration and purity of the extracts were evaluated using the spectrophotometer. DNA purity is acceptable when the A260nm/A280nm ratio is between 1.8 and 2.0 [13]. If R is less than 1.8, contaminating proteins are probably available in the solution. A value greater than 2 indicates probable RNA contamination. The A 260nm/280nm ratio varies from 1.86 to 2.09 and varies from one DNA extract to another, because it depends on the sequence composition. The resulting results are in Table 6.

3.3.2. Extracted DNA Electrophoresis

After extraction and quantification of DNA extracts, electrophoresis on 1% agarose gel. was made. Of the 10 isolates, 9 bands were observed and one band was not observed. These results are shown in Figure 1.

3.3.3. Electrophoresis of PCR Fragments

The identification of the isolates, by amplification of the purified 16S gene revealed by DNA bands of approximately 1500 bp as indicated in Figure 2.

Table 5. Phenotypic characteristics of the isolates.

Isolates	Appearance, Color and Consistency of Colonies	shape	Arrangement	Mobility	Gram	Catalase
RE 20	Circular, flat Pink, dry	Stick	Isolated	+	+	+
RE 21	Circular, flat Pink, dry	Stick	Isolated	−	+	+
RE 24	Circular, flat Pink, dry	Stick	Isolated	−	+	+
Bv1	Circular, white, dry	Stick	Isolated	+	+	+
Bv 2	Circular, yellow, creamy	Stick	Isolated	−	+	+
Bv 3	Circular, yellow, dry	Stick	Isolated	+	+	+
Bv 4	Circular, greyish, creamy	Stick	Chain	+	+	+
Bv 6	Circular, yellow, creamy	Stick	Isolated	+	+	+
Bv 7	Circular, white, creamy	Coccobacillus	Isolated	−	+	+
Bv 9	Circular, yellow, dry	Stick	Chain	+	+	+

Table 6. DNA concentration and purity after extraction.

Sample	DNA concentration(μg/mL)	DNA purity (A260/A280)
RE 20	49.5	1.91
RE 21	60.0	1.93
RE 24	101.7	1.96
Bv 3	41.0	1.95
Bv 4	127.3	2.08
Bv 1	183.6	2.09
Bv 2	2.6	2.03
Bv 6	47.2	1.87
Bv 7	46.6	1.86
Bv 9	30.5	1.98

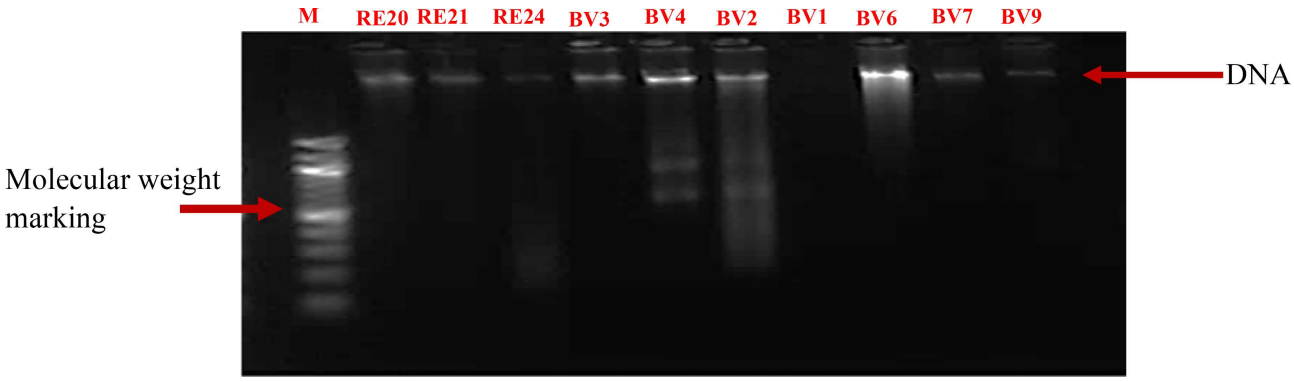

Figure 1. 1% agarose gel electrophoresis of genomic DNA extracts from isolates.

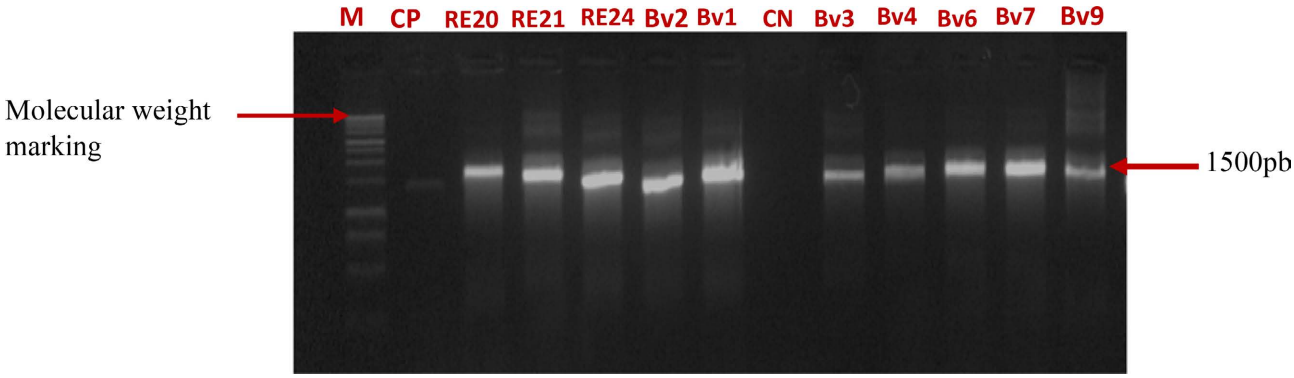

Figure 2. Electrophoresis on a 1% agarose gel of the PCR fragments.

3.3.4. Bioinformatics Analysis of 16S rRNA Sequences

The identification of the different isolates by 16S rDNA sequencing made it possible to differentiate the genus and the species of the isolates. A total of 10 sequences corresponding to the ten isolates were identified, eight of which could be submitted to GenBank. Table 7 indicates the various sequences and corresponding isolates.

Table 7. Correspondence of isolate codes and strains identified by 16S rRNA.

N°	Codes	Identified strains	new accessions numbers
1	RE 20	*Bacillus thuringiensis* strain GEB22	ON303633
2	RE 21	*Bacillus cereus* strain GE2B22	ON350770
3	RE 24	*Bacillus thuringiensis* strain GE3B22	ON350771
4	Bv 3	*Bacillus thuringiensis* strain GE4B22	ON738723
5	Bv 4	*Bacillus sp* GE5B22	-
6	Bv 1	*Bacillus sp* GE6B22	-
7	Bv 2	*Priestia megaterium* strain GE7B22	ON738723
8	Bv 6	*Bacillus anthracis* strain GE8B22	ON738720
9	Bv 7	*Bacillus subtilis* strain GE9B22	ON738721
10	Bv 9	*Enterobacter sp* GE10B22	ON738722

3.3.5. Multiple Sequence Alignment of Identified Strains with Some GenBank Homologs

The sequences of the identified strains were aligned in order to analyze the distance and the rate of similarity between strains and to understand the events that occurred during evolution. Similarly, these analyzes make it possible to test the efficiency of the 16S rRNA gene to discriminate between strains sharing a similar polymorphism. Figure 3 illustrates the multiple alignment of the studied sequences and their homologs. A strong sequence similarity was observed between the three strains *of B. thuringiensis* (GEB22, GE3B22 and GE4B22) and the homologous strain *B. thuringiensis (*B.t10*).* This observation is the same between the two strains of *P. megaterium* (GE7B22 and zs-3); the two strains of *B. subtilis (*GE9B22 and MK736123); the two strains of *Entobacter* and between the strains of *B. cereus; B. anthracis.*

3.3.6. Phylogenetic Inference

Figure 4 shows the dendrogram of the sequences of the identified strains and their homologs obtained from GenBank. The resulting phylogenetic tree is divided into two clusters; Enterobacteriaceae species on the one hand and *Bacillus* species on the other. The species composing each cluster have a strong similarity between them.

4. Discussion

This work aimed to the molecular identification of bacteria isolated from samples soil of the Likouala peat bog area (Congo-Brazzaville). Counting the bacteria on Mossel medium reveals bacterial loads of $(5.81 \pm 1.08) \times 10^4$ and $(6.64 \pm 1.94) \times 10^4$ CFU/g for samples 1 and 2 respectively. Whereas, sample 3 presented a load of $(8.56 \pm 1.19) \times 10^3$ CFU/g (Table 3). The count of sample 2 on the TSB medium, enriched with petroleum and vegetable oil, shows higher bacterial loads than on the Mossel medium. Indeed, on the TSB medium enriched with

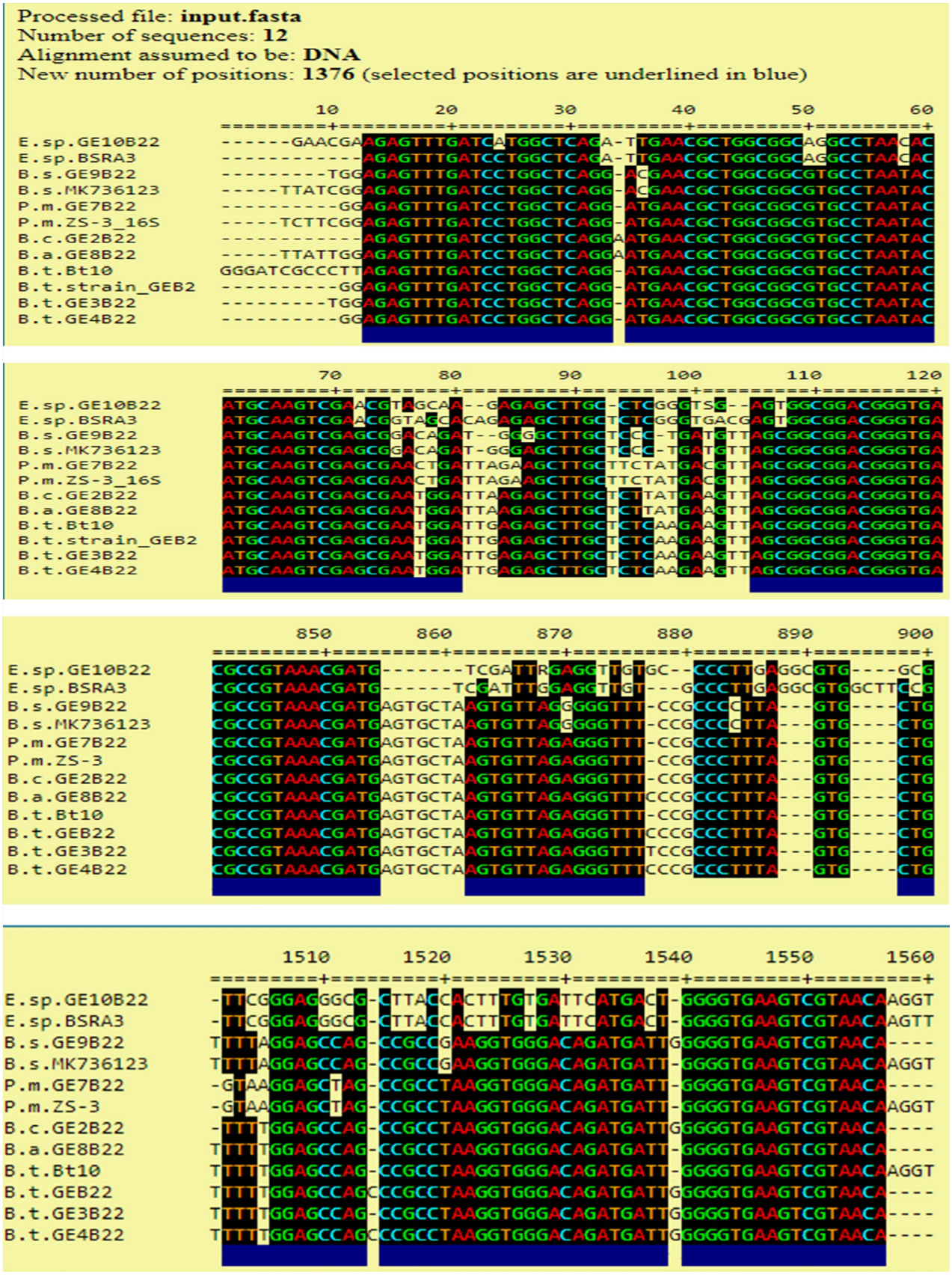

Figure 3. Part of Multiple sequence alignment of identified isolates with their homologs.

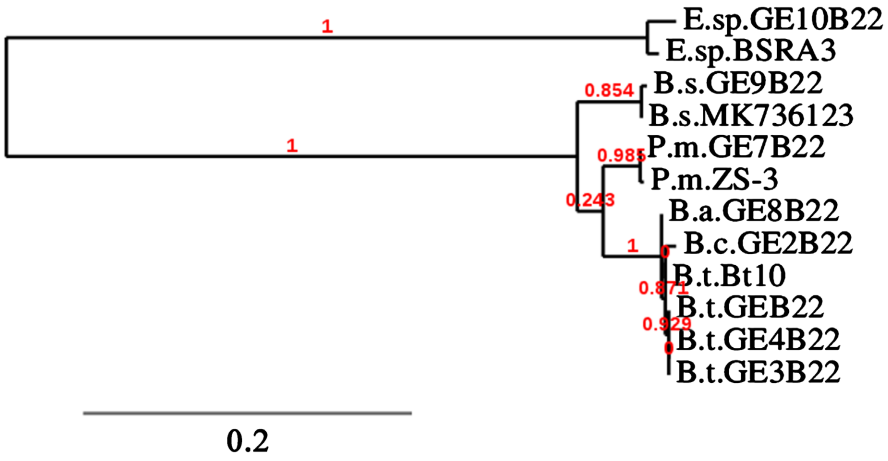

Figure 4. Phylogenetic tree of sequences in this study and their homologs.

petroleum, the bacterial load is (2.12 ± 4.1) × 10^8 CFU/g and (8.15 ± 10.1) × 10^7 CFU/g on the same medium enriched with vegetable oil. These results suggest that the total load of bacteria of the genus *Bacillus* and hydrocarbonoclasts is a function of the sample, the medium and in varying concentrations of the used source of carbon. These results are close to those obtained by [14] with samples soil of Brazzaville where the bacterial load after counting was around 9.3 × 10^4 CFU/g. The microscopic and macroscopic examinations made it possible to distinguish variable characteristics. Two forms of bacteria were characterized: cocci and rods which were more abundant, mostly gram positive (Gram+) and catalase positive thus suggesting bacteria of the genus *Bacillus.* These results corroborate those of several studies which have already shown that bacteria of the *Bacillus* genus can be isolated from food and soil [15] [16]. In order to carry out the molecular identification of bacteria isolated from peat bog soils; we ensured the efficiency of DNA amplification by different PCR reactions allowing to amplify different fragments up to 1500 bp. The 260/280 ratio was determined, which made it possible to detect contamination of the nucleic acids by proteins. Its value varied between 1.8 and 2.0 for DNA and between 2.0 and 2.2 for RNA. The A260nm/A280nm ratio varied from one fragment to another, respectively from 1.74 to 1.88 because it depends on the sequence composition, these results are in accordance with the statement of Jay A. [13]. Most sequences showed 99% similarity to the closest sequences already presented in NCBI databases. The more significant the alignment score, the results are expressed as a percentage of similarity of the strains identified for our study with the closest species with regard to the intervals. In **Figure 3** the bloc from 841 to 854 is showing the conserved region of ADNr16S, all identified sequences and homologs present a highly this conserved region. This evidence corroborates the postulate in which all procaryotes specially bacteria can be identified by the sequencing of the conserved region of ADNr16S. Authors [8] [17] have already found the same results. When watching the bloc [855 - 861] of **Figure 3** the two sequences of *Enterobacter sp* are different from those of all *Bacillus strains*. Phylogenetic Inference

has confirmed the phylogeny of reference which is well established between the Bacillus strains and the Enterobacter sp strains. The phylogenetic tree dispays two monophyletic groups, these results have been already found by others authors [17] [18] [19].

PCR amplification and then sequencing of the gene encoding rRNA 16S identified eight (08) species of bacteria including three (3) species of *Bacillus thuringiensis, Bacillus cereus, Priestia megaterium, Bacillus antracis, and Bacillus subtilis and one species Enterobacter sp.,* isolate whose species could not be determined because of the low rate of similarity, this observation has already been made by other auhors [8]. The association of other more discriminating molecular markers in addition to the gene encoding 16S rRNA is possible in the future in order to assess the microbial biodiversity colonizing peatland ecosystems.

5. Conclusion

This study contributes to the molecular identification of bacteria isolated in soil samples constituting the surface part of the peat bog in the Likouala area in the Republic of Congo. The results of the microbiological analyses of this present study showed that the soils of the peat bogs of the Likouala zone are rich in bacteria of the Bacillus genus. PCR amplification, followed by sequencing of the 16S rRNA gene allowed the identification of eight (8) species of bacteria including six (6) of the *Bacillus* genus: *Bacillus thuringiensis, Bacillus cereus Bacillus thuringiensis; Bacillus thuringiensis; Bacillus anthracis; Bacillus subtilis; a strain of Enterobacter sp* and a strain of *Priestia megaterium*. This present work constitutes a scientific support in the study, the understanding and the interaction of the bacterial diversity colonizing the soils of the peat bogs in the Republic of Congo.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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