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Research Article

16s rDNA BASED IDENTIFICATION OF BACTERIA IN THE ORGANIC MATTER OF PACHAMALAI FORESTED STREAMS

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Abstract

Molecular tools to monitor bacterial diversity in complex microbial assemblages have developed during the last decade using 16S ribosomal DNA based polymerase chain reaction (PCR). The organic matter samples from Koraiyar and Mayiluthu forested streams of Pachamalai hills were subjected to DNA extraction and the resultant amplicons of the genomic DNA were amplified by PCR using universal primers. The resultant amplicons of PCR assays were subjected to electrophoresis and PCR fitness was identified. The molecular detection of 16S rDNA sequence of samples from both stream was done. This showed the availability of a simple method to confirm the bacterial colonization on a broad range of species and this would be useful in the evaluation of organic matter degradation in streams.

Keywords: PCR, 16S rDNA, primers, Koraiyar, Mayiluthu.

Introduction

Identification of bacteria is traditionally performed by isolation of the organisms and the study of their phenotypic characteristics, including Gram staining, morphology, culture requirements and biochemical reactions. However, these methods of bacterial identification have major drawback. Firstly, they cannot be used for nocultivable organisms. Second, we are occasionally faced with organisms exhibiting biochemical characteristics that do not fit into patterns of any known genus and species. Third, identification of slow growing organisms would be extremely slow and difficult (Woo et al., 2003). Since the discovery of PCR and DNA sequencing, comparison of the gene sequences of bacterial species showed that the 16S rDNA gene is highly conserved within a species and among species of the same genus, and hence, can be used as the new technique for identification of bacteria to the species level (Oleson and Woese, 1993).

Aquatic microbial diversity is well understood to be a key component of aquatic ecosystem functioning (Conter and Biddanda, 2002;Gessner

et al., 2010), and major advances toward linking microbial diversity with ecosystem function have been made in aquatic system (Horner- Devine *et al*., 2013). A recent survey of microbial diversity studies in aquatic habitats showed that microbial diversity in lotic environments is less commonly studied than in marine and lake ecosystems (Zinger *et al*., 2012). Stream and rivers are hotspots of microbially mediated carbon (C) and nutrient processing with landscapes (Hynes, 1975; Fisher *et al*., 1998; McClain *et al*., 2003). Bacterial diversity was particularly difficult to define, beyond differentiating gram-negative from gram-positive cells (Geesey *et al*., 1977) or conducting plate counts on selective media (Millner and Goulder, 1984), before the availability of molecular tools. The application of molecular techniques to measure stream microbial diversity produced new insights. Following the establishments of the ribosomal rRNA gene as a conserved marker of taxonomic lineage (Pace, 1997), studies on water column biota began to resolve longitudinal patterns in microbial diversity (Crump *et al*., 1999; Crump and Baross, 2000), and efforts to integrate molecular and traditional tools in studies of fungal diversity were mounted (Nikoicheva *et al.*, 2003; Nikolcheva and Barlocher, 2005).

Materials and Methods

Sampling procedure

Sampling of epilithic biofilms was performed using sand blasted glass tiles of 9.6 cm^2 of surface area. Two tiles were scraped per replicate using a sterile cell scraper and suspended in 1 ml sterile stream water.

Isolation of Bacteria

The samples were collected in a sterile plastic container and transported to laboratory for bacteriological analysis. Bacterial isolates were screened on Nutrient Agar (NA) plates by the standard pour plate method. Plates were incubated at 37°C for 24h. A total of one hundred and forty-four isolates were obtained, after incubation. Two isolate was selected from the group of isolates and used for further studies. The isolated bacteria were identified based on colony characteristics, gram staining methods and by various biochemical tests as given by Bergey's (1984) Manual of Determinative bacteriology.

Biochemical Characterization of the bacteria

The selected bacterial strain was grown in nutrient broth culture medium containing 2.5% peptone, 1.0% yeast extract, and 0.5% beef extract. Cultures (50 ml in 250-ml conical flasks) were inoculated with 5% (v/v) inoculums and incubated at 37 °C with vigorous orbital shaking at 120-150 rpm. To make a solid medium, 1.5% agar was added to the broth (Himedia, India). The shape and color of the colonies were examined under the microscope after Gram staining. Isolates were biochemically analyzed for the activities of Oxidase, Catalase and MR-VP test, Urease test, Motility, Indole production (Table- 1) and Citrate utilization (Table- 2) tests were also conducted. These tests were used to identify the isolates according to Bergey's Manual of Determinative bacteriology.

Bacterial DNA isolation

Bacterial genomic DNA was isolated as per the standard protocol (Hoffman and Winston, 1987). Nutrient broth was inoculated with single colony and cultured for overnight at 37ºC. Cells were harvested from 5 mL of the culture and to this 100µL of lysozyme was added and incubated at Room temperature for 30 min, followed by the addition of 700µL of cell lysis buffer (Guanidiumisothiocyanate, SDS, Tris- EDTA). The contents were mixed by inverting the vial for 5 min with gentle mixing till the suspension looked transparent. 700µL of isopropanol was added on to the top of the solution and were mixed gently till white strands of DNA were seen. The DNA was extracted from the aqueous layer was precipitated with ethanol. The DNA pellet was dried and dissolved in 50µL of 1X TE buffer. The purity of the DNA was analyzed by running on 0.8% agarose gel stained with ethidium

bromide (0.5 µg/µL). A single intense band with slight smearing was noted.

The extracted genomic DNA was used as template DNA for the universal bacterial primers (Forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer (5' GGTTACCTTGTTACGACTT 3') were used for the amplification of the 16S rRNA gene fragment. The reaction mixture of 50µl consisted of 10 ng of genomic DNA, 2.5 Units of Taq DNA polymerase, 5µl of 10X PCR amplification buffer (100 mMTris- HCl, 500 mMKCl pH-8.3), 200µM dNTP, 10 p moles each of the two universal primers and 1.5mM $MgCl_2$. Amplification was done by initial denaturation at 94°C for 3min, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing temperature of primers was 55°C for 30 second and extension at 72°C for 1 minute. The final extension was conducted at 72°C for 10 minutes.

Agarose Gel Electrophoresis

10 µL of the reaction mixture was then analyzed by submarine gel electrophoresis using 1.0 % agarose with ethidium bromide (0.5 µg/µL) as per the standard protocols (Sambrook*et al.*, 2001) at 80V/cm and the reaction product was visualized under Gel Documentation System (Alpha Innotech).

Purification of PCR Product by Exosap-IT

The PCR product was subjected to purification by using Exosap-IT. It is a mixture of Exonuclease I and Shrimp Alkaline Phosphatase that removes left over primers and free nucleotides from the PCR reaction. To 5 µL of PCR product add 2 µL of Exosap. Further the samples were incubated at 37ºC for 15 minutes for the degradation of primers and free nucleotides. Then the Tube was transferred to water bath at 80ºC and incubated for 15 minutes to inactivate the Exosap-IT enzyme. The sample was then ready for sequencing reaction.

DNA sequencing of 16S rRNA gene fragment

The 16S rRNA purified PCR product (100ng concentration) was subjected for the amplification of the 16S rRNA gene.

PCR amplification of 16S rRNA gene:

PCR reaction was performed in a gradient thermal cycler (Eppendorf, Germany). The sequencing using ABI DNA 3730 XL sequencer (Applied BiosystemInc). Sequencing of the 16S rRNA gene of the bacterial isolate was done from both the directions, the sequence obtained was subjected to BLAST search and the bacterial species were determined. The percentages of sequence matching were also analyzed.

Computational analysis (BLAST) and Identification of Bacterial species

BLAST (Basic Local Alignment Search Tool) is a web-based program that is able to align the search sequence to thousands of different sequences in a database and show the list of top matches. This program can search through a database of thousands of entries in a minute. BLAST (Altschul*et.al.*,1990) performs its alignment by matching up each position of search sequence to each position of the sequences in the database. For each position BLAST gives a positive score if the nucleotides match, it can also insert gaps when performing the alignment. Each gap inserted has a negative effect on the alignment score, but if enough nucleotides align as a result of the gap, this negative effect is overcome and the gap is accepted in the alignment. These scores are then used to calculate the alignment score, in "bits" which is converted to the statistical E-value. The lower the E-value, the more similar the sequence found in the database is to query sequence. The most similar sequence is the first result listed.

Results and Discussion

Bacterial population analysis in biofilms

PCR amplification of DNA extracted from epilithic biofilm was performed on samples of Pachamalai forested stream. The traditional identification of bacteria on the basis of phenotypic characteristics is generally not as accurate as identification based on genotypic methods. Comparison of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique. The sequence of the 16S rRNA gene has been widely used as a molecular clock to estimate relationships among bacteria (phylogeny), but more recently it has also become important as a means to identify an unknown bacterium to the genus or species level. The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Patel, J. B. 2001).The rRNA based analysis is a central method in microbiology used not only to explore microbial diversity but also to identify new strains.

Total of one hundred and forty-four isolates obtained, from the Pachamalai forested stream two isolate were used for further analysis. Isolated colony from mixed populations, on nutrient agar plates were characterized and sub cultured to obtain pure cultures, and the isolated bacteria were identified based on colony characteristics, and were biochemically analyzed for the activities of Oxidase, Catalase, MR-VP test, Urease test, Motility, Indole production (Table- 1), Sugar utilization test (Table -2) and Gram staining (Figure: 1) tests. From the tests the isolate was found to be *Bacillus spp..*, further confirmation was done using molecular approach. Bacterial genomic DNA was isolated as per the standard protocol (Hoffman and Winston, 1987). The presence of bacterial genomic DNA isolated was confirmed on 0.8% agarose gel stained with ethidium bromide. An intense single band was seen along with the DNA marker. (Fig-2) The extracted DNA was used as template for amplification of 16S rRNA gene. The universal primers 27F and 1429R were used for the amplification and sequencing of the 16S rRNA gene fragment. The optimum annealing temperature was found to be 55ºC. An intense single band was visible on 1% agarose gel stained with ethidium bromide (Figure: 2). The PCR product was subjected to sequencing using BDT V3.1 cycle sequencing kit on ABI 3730 XL genetic analyzer from both forward and reverse directions. The two sequences (Figure: 3) obtained were compared with the NCBI gene bank database using BLAST search program (http;//www.ncbi.nlm.nih.gov) (Marchler– Bauer *et al.*, 2000; Pruitt *et al.*, 2005). The percentages of sequence matching were also analyzed. The homology search made using BLAST sequence 1 showed 99 – 100% maximum identity with that of *Bacillus subtilis strain B5501A*,NCBI Gene Bank Accession No: FJ 55787.1 and E-value equal to 0 for all closely related taxa. Other close homologs of the isolate showing 99% similarity with, *Bacillus tequilensis*BK206 99%, 99% similarity with *Bacillus vallismortis*, and 95% *Paeni Bacillus polymaxa*,*Bacillus velezenesis P42*, *Bacillus amyloguefaciens - L51*, *Bacillus sigmensis* IHBB16121, *Bacillus licheniform*, and the sequence 2 showed maximum identity with that of *Leptotrichia species* EB007,*Leptolyngbya Species* –L21- BG2 and with Cyanobacterium *phormidium*. Sequences of the bacterial isolates were used for the construction of the phylogenetic dendrogram to know the genetic relatedness between the bacterial isolates. All the closely related homologs of identified bacteria were used for the construction of the phylogenetic dendrogram to know their evolutionary origin. The dendrogram showing the relation between *Bacillus subtilis* strain B5501A *and Leptolyngbya 12077* and their close homologs is shown in (Figure:4a, b).

Table -1: Physio–biochemical characteristics of the isolate *Bacillus subtilis* B5501A

Test	Observation
Gram's stain	Gram Positive
Spore staining	Central, oval, bulging
Cell shape	Rods
Cell size	>3µm
Colony character	White, raised irregular
Motility	+
Catalase	+
Oxidase	-
Indole	-
Methyl red	-
Voges-Proskauer	-
Citrate utilization	+
Casein utilization	+
Starch hydrolysis	+
Urea hydrolysis	-
Growth at 50ºC	+
Growth in 10% NaCl	-
Anaerobic growth	+
TSI (Triple Sugar Iron)	Acid slant/ alkaline butt, gas, no H_2S produced

+,Positive , -Negative

Table-2: Sugar utilization test

Sugar utilization	Result (acid/ gas)
Glucose	+/-
Galactose	+/-
Arabinose	+/-
Mannitol	-/-
Maltose	+/-
Mannose	+/-
Raffinose	-/-
Rhamnose	-/-
Sucrose	+/-
Lactose	+/-
Fructose	+/-
Xylose	+/-

+/Positive -/ Negative

Figure –1 – Bacillus strain (Gram Staining)

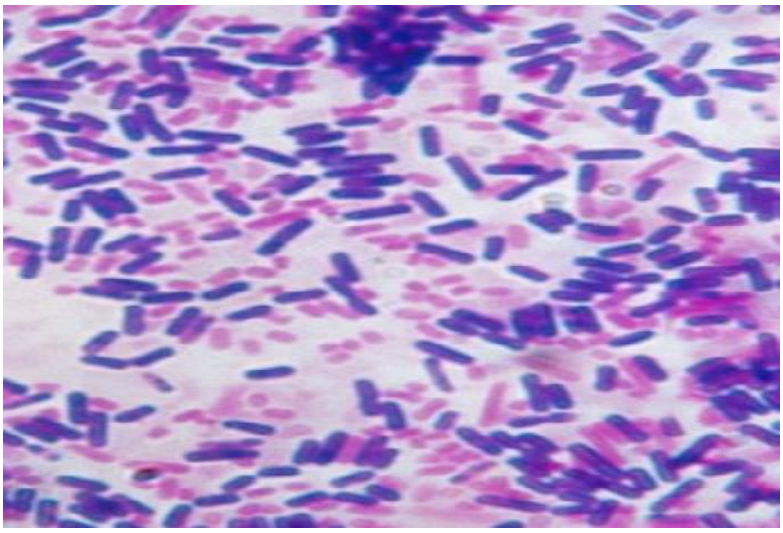
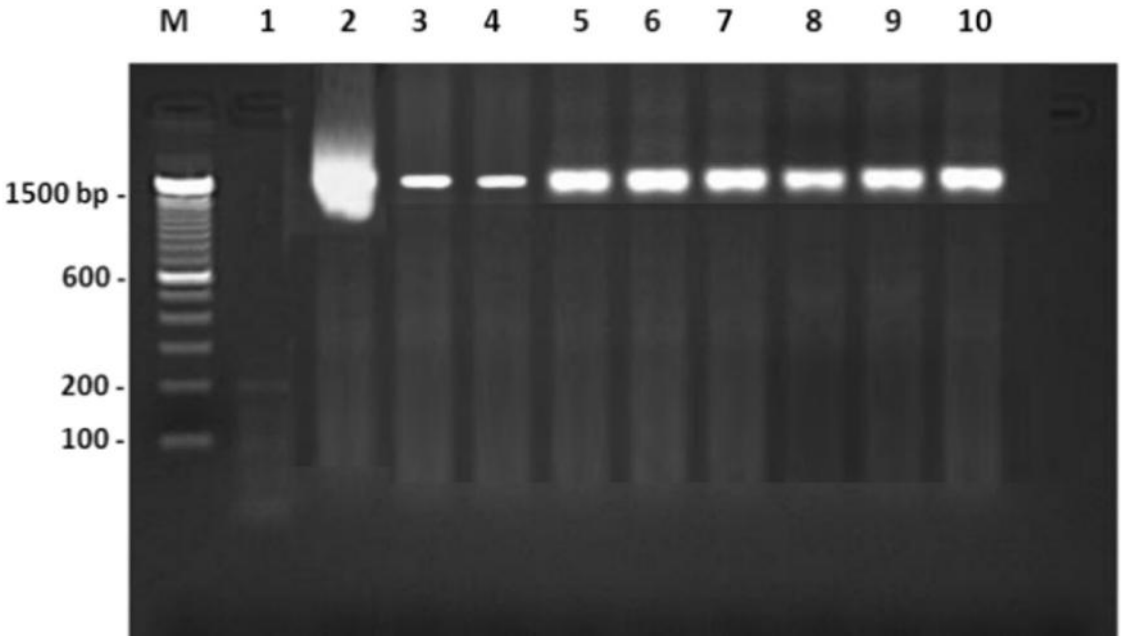


Figure- 2- Gel image of 16S rRNA amplicon



Lane – M - DNA marker,
Lane- 1 -Negative control
Lane – 2 -Positive control (genomic DNA)
Lane-3 to 10 -16S rRNA amplicon band

Figure- 3 - Partial sequence of PCR product of 16S rRNA gene

a. sequence-1 b. sequence-2

(a)

ACGCGATAAGTATCCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAGGAATTGACGGGGGCCCGACA
AGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAGGGCTTGACATCCCTCGAATCCT
CTTGAAAGAGAGGAGTGCCTTCGGGAGCGAGGAGACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAG
ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCACGTCCTTAGTTGCCAGCATTGAGTTGGGCACTCTGGGG
AGACTGCCGGGGACAACTCGGAGGAAGGTGTGGATGACGTCAAGTCATCATGCCCCTTACGTTCTGGGCTAC
ACACGTACTACAATGCTTCGGACAAAGGGCAGCCAACTAGCGATAGTGAGCTAATCCCATAAACCGAGGCAC
AGTTCAGATTGCAGGCTGCAACTCGCCTGCATGAAGGAGGAATCGCTAGTAATCGCCGGTCAGCATACGGCG
GTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGCATACGTCCTACGGGAGAAAGCA
GGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACC
AAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTA
CGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCA
GCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTTGCCTA
ATACGTAACTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTAACGCTGGCGGCAGGCCTACGGA
CGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGCTCGGAAACGGACGCTAATACCGCATA
CGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTT
GGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGGATGATCAGTCACACTGGAACTG
AGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCC
ATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGTTAAGCACTCCGCC
TGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGT
TTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTTCC
CCTTTCGGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC
CCGCACGAGCGCAACCCTTTGATCTTAGTGCAGCATCAGTGGCACCTCTA
AGGTGACTGCGTGACAACGAGAGGTGGGGATGACGTCAATCATCATGCCCCTTATGACCTGGGCTACAAGAG
AGTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCT
TGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCG
GGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCAGCAGTAG
GGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAA
GCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACACATGCAAGT
CGAGCGGCAGCACGGGTACTTGTACCTGGTGGCGAGCGG

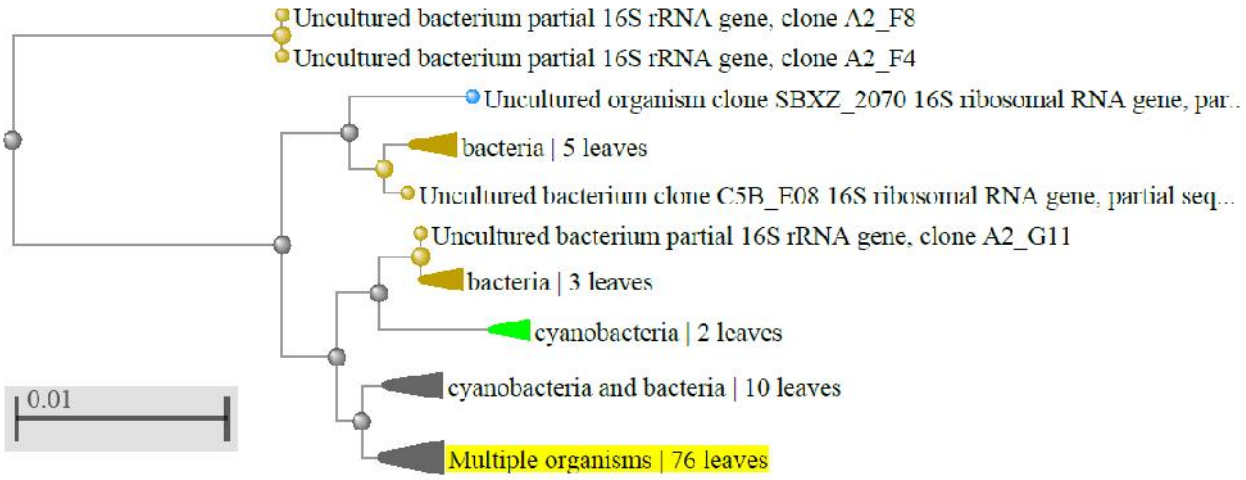
(B)

AACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAGCACGGGTACTTGTACCTGGTGGCGAGCGGCG
GACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGCTCGGAAACGGACGCTAATACCGCA
TACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAG
TTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGGATGATCAGTCACACTGGAAC
TGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGGGCGGTGGCG
GGTGCTATAATGCAGTCGAGCGAATCGATGGGAGCTTGCTCCCTGAGATTAGCGGCGGACGGGTGAGTAACA
CGTGGGCAACCTGCCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATACGTTCTTTTCTC
GCATGAGAGAAGATGGAAAGACGGTTTACGCTGTCACTTATAGATGGGCCCGCGGCGCATTAGCTAGTTGGT
GAGGTAATGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGC
CGCGTGAACGAAGAAGGCCTTCGGGTCGTAAAGTTCTGTTGTTAGGGAAGTTACCAGGTCTTGACATCCTCT
GACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCAC
TCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCT

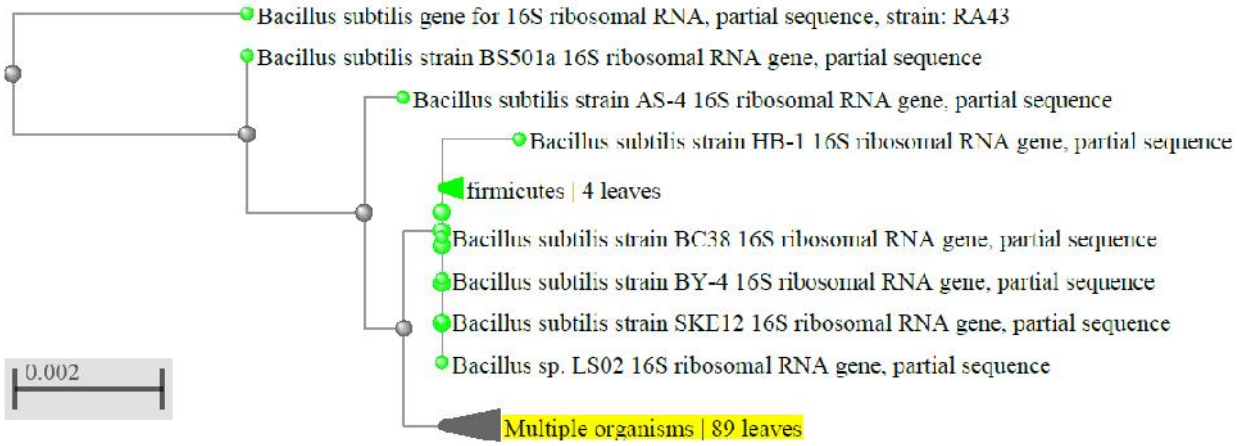
GGGCTACACACGTGCTACAATGGACAGAACAAAGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCT
GTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCAT
GCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTCGTAACACCCGAAGC
CGGTGAGGTAACCTTTTAGGAGCCAGCCGCCGAAGG
TGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAACC

Figure- 4- Phylogenetic tree representing close homologs of a. uncultured bacterial clone. b. *Bacillus subtilis*

(a)



(b)



Nikolcheva, L. G and F. Barlocher, 2004. Using taxon-specific primers study on demonstrated the presence of these major fungal taxa and found ascomycetes to be the main group of fungi colonizing decaying leaves in streams. In contrast rDNA approaches confirmed higher bacterial species richness in the Pachamalai forested stream.

Conclusion

Patterns of biofilm formation were investigated in Mayiluthu and Koraiyar streams of Pachamalai forest. From the results it was understood that a higher amount of particulate organic matter standing stock was found in the Mayiluthu stream channelFrom the tests conducted the isolate was found to be *Bacillus species* and the obtained dendrogram showed that there is a relation between *Bacillus subtilis*strain *B5501A.*

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