

Molecular Identification and Classification of Unknown Microorganisms Based on 16s rRNA

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Traditional microbiological methods creates a indistinct thought about their classification and their taxonomic status. Several DNA-based typing methods provide information for delineating bacteria into different genera and species and have the potential to resolve differences among the strains of a species. Therefore, newly isolated strains must be classified on the basis of the polyphasic approach. also previously classified organisms, as and when required, can be reclassified on this ground in order to obtain information about their accurate position in the microbial world. The complete 16s rRNA gene sequencing and its comparative analysis of phylogenetic trees for determining taxonomic position of microbes. From the comparative study of phylogenetic tree which is a quantitative identification of the number of positions that differ between the aligned macromolecules, our sample microorganisms belongs to Volume 2, The Proteobacteria, Class III gamma (γ) proteobacteria.

Key words: EDTA: Ethylene Diamine Tetra Acetic acid, TE buffer: Tris EDTA buffer.

Classification of microorganisms on the basis of traditional microbiological methods (morphological, physiological and biochemical) creates a blurred image about their taxonomic status and thus needs further clarification. It should be based on a more pragmatic approach

of deploying a number of methods for the complete characterization of microbes. Hence, the methods now employed for bacterial systematic include, the complete 16S rRNA gene sequencing and its comparative analysis by phylogenetic trees, DNA-DNA hybridization studies with related organisms, analyses of molecular markers and signature pattern

Collectively these genotypic, chemotaxonomic and phenotypic methods for determining taxonomic position of microbes constitute what is known as the 'polyphasic approach' for bacterial systematic. This approach is currently the most popular choice for classifying bacteria and several microbes, which were

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previously placed under invalid taxa have now been resolved into new genera and species. Several critical attributes favor the use of ribosomal sequences for classification of microorganisms in the environment. The first is their universal distribution among all cellular life forms. The second is that their essential function in all organisms translates into a very slow genetic evolution and as a result the sequences coding for rRNA are highly conserved. Furthermore, the mutation rate of rRNAs corresponds to evolutionary divergence of organisms.

Study Area

The primary structure of all ribosomal sequences consists of alternating conserved and variable domains which makes them very suitable for the detection and identification of microbial species and ideal targets for specific DNA probes. The 5s rRNA is rather small giving limited information, while the 16s rRNA, consisting of about 1,500 nucleotides(nt), provides a large amount of information for phylogenetic inference and is a reasonable size for sequencing. The 23S rRNA, generally 3,000 nt, offers substantial information but requires more sequencing, so 16s rRNA has essentially become the established reference. Internal transcribed spacer (ITS) regions separately rRNA genes and also mutate so rapidly that they may only provide taxonomic information at the sub species to strain level.

16S rRNAs and 18S rRNAs are commonly used to determine evolutionary relationships between organisms. While these methods frequently yield a high accuracy, only a small fraction of fragments can be taxonomically characterized, depending on the size of the used marker gene database. To overcome this limitation, novel methods have recently been devised that analyze the presence of short oligo nucleotides or motifs to classify environmental DNA sequences into taxonomic groups^{30,31}. but to our knowledge cannot be applied to genomic sequences shorter than 1000 bp. On the other hand, simply classifying genomic fragments based on a best BLAST hit will only yield reliable results if close relatives are available for comparison³². The recently published MEGAN software addresses this problem by classifying DNA fragments based on a lowest common ancestor algorithm

MATERIAL AND METHODS

Materials

Soil sample, nutrient agar, nutrient broth. Isolation of DNA: centrifuge, TE buffer, 50mM, 250mM tris (pH-8.0), 50mM EDTA, 1Mm EDTA, RNase, chloroform.

Agarose gel electrophoresis

Agarose, 50x buffer, Electrophoretic unit, Ethidium bromide, Agarose, 50x buffer, Electrophoretic unit, Ethidium bromide. PCR amplification: master mix, forward primer, reverse primers. Elution & Purification of DNA: 10mM Tris(pH- 8.0), 1mM EDTA, tris buffered phenol, 4M NaCl, 70% ethanol.

Methodology

Soil sample was collected and grinded into fine powder one gram was weighed and serially diluted from stock solution up to 10^{-6} . From 10^{-4} , 10^{-5} , 10^{-6} 1ml culture was taken and spreaded on respective nutrient agar plates After incubation a single isolated colony was picked out from the agar plate and it was inoculated into 100ml of Nutrient broth. After incubation at 37°C for 24 hours. From the 24 hrs fresh culture

Methods for isolation of DNA

1ml culture was centrifuged at maximum rpm for 30seconds. To the pellet 400 μ L of TE buffer and 50 μ L of 10%SDS was added and vortexed and incubated at room temperature for 1hour. To that 500 μ L of phenol: chloroform (1:1) and twice 500 μ L of chloroform was added. Nucleic acids were precipitated by adding 25 μ L of 5M NaCl and 1ml of 95% ethanol. Mixture was vortexed and centrifuged for 10minutes at maximum rpm and pellet was dried and was re suspended in 100 μ L of TE buffer and incubates at room temperature for 30minutes. DNA was precipitated by adding 40 μ L of 5M ammonium acetate and 250 μ L of iso propanol and incubated for five minutes at room temperature and again centrifuge for 10minutes at maximum speed. Pellet was washed with 70% ethanol and the pellet was dried. The dried pellet was re suspended in 100 μ L of TE buffer. The DNA was checked using Agarose gel electrophoresis the bands were observed under the U.V transilluminator.

The DNA was amplified in PCR with master mix (Taq DNA polymerase, Mg⁺², DNTPs, 10x assay buffer) then add 1 μ l of forward

primer and 2 µl of reverse primer which are commercially available in market. These primers only specific 16s r RNA gene. Elevate the DNA in the pool of amplified DNA sample using 10kb, 20kb, 30kb markers in gel electrophoresis. The DNA which was code for 16s rRNA confirmed by observed the bands between 10kb and 20kb marker wells.

Elution & purification of PCR product

The slice containing DNA was cut and doubled the volume of DNA with T E buffer and melted at 65° centigrade. Then tris buffered phenol was added centrifuged at 10000 rpm for 3 minutes, aqueous layer washed out. Phenol extract was carried again. Then 0.1 Volume of 4 M NaCl was added to the sample White precipitate was formed immediately, and again centrifuged for 3 minutes at 10000 rpm. Then supernatant was discarded, leaving a transparent pellet behind. Then 2.5 volumes of cold ethanol was added to precipitate the nucleic acids. The sample was kept at – 20°C for 5 to 10 minutes centrifuge the sample at 10000 rpm for 5 minutes. Supernatant was discarded and the pellet was washed with 1 ml of 70% ethanol. The pellet was dried and resuspended in 40 µl of TE buffer.

Sequencing

The purified form of 16s rRNA gene fragment which was obtained through elution process was now subjected to sequencing in automatic sequencer.

Blast

The query sequence was compared with the sequences in the data bases(or) Library. Phylogenetic trees were developed by comparing molecule as sequences. To compare two molecules their sequences must first aligned, so that similar parts match up. Once the molecule have been aligned, a number of positions that vary in sequences can be determined. These data need to calculate a measure of the distance between the sequences called Evolutionary distance. This is simply a quantitative identification of the number of positions that differ between the aligned macromolecules.

RESULTS AND DISCUSSION

Total of ten different soil samples were collected from helini biomolecules garden,

Guntur, were studied for molecular identification and claffication based on 16s rRNA during the period of May and June 2008. Total of ten samples, 8 samples were sequenced and the query sequences were compared with the sequences in the data base (or) library. Finally out of 8 samples, four sequences were confirmed as *Escherichia coli* family, two sequences were *Vibrio cholerae* family, and other two sequences were *Pseudomonadeceae* family. From the comparative study of phylogenetic tree these microorganisms belongs to Volume 2, The Proteobacteria, Class III gamma (γ) proteobacteria.

CONCLUSION

Classification of microorganisms on the basis of traditional microbiological methods create a imprecise mind about their taxonomic status. Hence, 16srRNA sequencing is preferred for the identification and classification of microorganisms which are difficult to classify by their phenotypic properties. But this is not an easy task and computers plus fairly complex mathematics must be employed to minimize the number of gaps and mismatches in the sequences being compared.

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