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Phylogenetic analysis of *Actinomysetes* isolated from rhizospher of *Pisum Sativum* L.

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Abstract
In this study, *Actinomycetes spp* were isolated from soil samples collected from Allahabad. Three isolates of them were studied in detail and characterized. Their provisional identification was done following the criteria of Bergey s Manual of Systematic Bacteriology. The morphological and biochemical characterization and tentative identification methods were described. Further identification and phylogenetic study were done using molecular tools. 16S rRNA helped to determine three species as *AS1* (*Streptomyces lucensis gene*), AS2 *(Streptomyces griseus gene*) and AS3 (*Streptomyces olivaceusgene)*. Phylogenetic analysis done using bio-informatics tools CLUSTAL OMEGA and BLAST.

Keywords: actinomycetes, 16S rRNA, amplification, phylogenetic tree, evolutionary relatedness

Introduction
Actinomycetes are the well recognized as the richest source of bioactive compounds including clinically useful antibiotics, anti cancer agents and cell function modulators and hence of high pharmacological and commercial interest (Butler, 2008) [3]. They are widely distributed in various normal and extreme ecosystems, due to their unparalleled ability to degrade wide range of complex substrates and withstand extreme physico-chemical conditions (Berdy, 2005) [2]. Based on the hypothesis "poorly researched habitats can offer better prospects for discovering new natural products", actinomycetes from such habitats are currently in focus of considerable scientific interest (Arumugam, 2011) [1].

In recent years, intensive research had focused on identification of new natural antitumor agents derivedfrom various plants, marine organisms, animals and microorganisms. The distinct class of secondary metabolites obtained from actinomycete bacteria has enoughpotential, as well as scope for anticancerous activities. Cancer still represents one of the most serious human health problems despite the great understanding of itsbiology and pharmacology. The usual the rapeutics methods for cancer treatment are surgery, radiotherapy, immunotherapy and chemotherapy (Cocco *et al*., 2003). An analysis of the number of chemotherapeutic drugs and their sources indicated that over 60% of approved (Cragg, 2004), and many of them have been extracted from actinomycetes.

Actinomycetes are potential source of many bioactive compounds (Xu *et al*., 2005) [11], which have diverse clinical effects and important applications in medicine for treating various human diseases and disorders (Watve *et al.*, 2001) [9]**.** Actinomycete groups have been detected and characterized by their 16S rRNA sequences in cases where cultivation has proved unsuccessful (Rheims *et al*., 1996) [6]**.** The diversity analysis by ribo typing with 16S rRNA phylogenetic marker showed that a group of high GC rich Gram-positive bacteria (actinomycetes) are dominant in marine sediments (Urakawa *et al*., 1999) [8] for intending biomedical applications.16S rRNA sequencing method is being considered as the most important powerful tool to screen the isolated actinomycetes at molecular level (Wellington *et al*., 1994) [10].

As the frequency of novel bioactive compounds discovered from terrestrial Actinomycetes decreases with time, much attention has been focused on screening of Actinomycetes from diverse environments for their ability to produce new secondary metabolites. Studies have shown that Actinomycetes isolated from the marine environment are metabolically active and have adapted to lifein the sea. Streptomyces are especially prolific and can produce a great many antibiotics (around 80% of the total antibiotic production) and active secondary metabolites (Thenmozhi and Krishnan, 2011) [7]**.** More than 70% of our planet' ssurface is covered by oceans and life on Earth originated from the sea. In some marine ecosystems, such as the deep sea floor and coral reefs, experts estimate that the biological diversity is higher than that in the tropical rainforests. As marine environmental conditions are extremely different from terrestrial ones, it is summarized that marine Actinomycete shave different compound (Imada *et al*., 2007) [4].

Materials and Methods

Study Area

This study was conducted in the district during 2015/2016 and 2016/2017 The sample was collected from the Farm Department of Horticulture, Allahabad School of Agriculture, (SHIATS) Allahabad at of a latitude of 20^0 15^0 North and longitude of 60^0 3" East and at an altitude of 98 meters above mean sea level (MSL).Three samples of root nodules from field pea *(Pisam sativum L)* were collected randomly from different localities. Healthy root nodules were washed with tape water thrice before streaking on agar plate as describedby (Ben-Gweir if *et al.,* 2005). The nodules were sterilized externally using 95 % alcohol for 1-4 minute, followed by washing with calcium hypochlorite solution(10g/150ml distilled water) and crushing in a drop of sterile water. A loopful ground material was transferred to 5 ml of sterile water, of which 0.1 ml sample was spread onto the surface of Yeast Extract Mannitol Agar (YEMA). Plates were then incubated at 28°C for 48hours. Well-isolated typical single colonies were restreaked on freshly prepared YEMA plates in order toobtain pure cultures.

Purification and storage

Agar plates were incubated at 28°C for three to ten days. Individual colonies appearing over this period were re-streaked onto fresh Congo red YEMA plates to obtain pure Culture. For short-term storage sub-cultured onto YMA+Ca slopes in test tubes. For long-term storage broth culture suspended in 10% Glycerol and stored at -4°C.

Molecular characterization

The genomic DNA of actinomycetes strains were extracted using modified method described by Petersen and Scheie (2000). The DNA was pelleted using isopropanol and stored in TE Buffer at at -20°C. The PCR amplification of 16S ribosomal RNA gene was carried out by the method described by Gothwal *et al*. (2007) [12] using Thermal cycler. The Reaction mixture used include sterile water, 10x assay buffer, dNTPs mix (10mM each), Template DNA (20-30ng), Forward primer (100mM), Reverse primer (100mM), Taq poly (1U). The amplified 16s rRNA gene was run on a 1.2% agarose gel prepared in 1X TAE buffer. Then visualized under UV in a Gel documentation system.

Sequencing of 16s rRNA

Sanger Dideoxy chain Terminator sequencing was done in an automated DNA sequencer. Subjecting labeled ddNTP to a single capillary tube made the sequence detection. DNA fragments of different colours were seperated by their respective sizes in a single electrophoretic gel. The sequences were read by determining the sequence of the colours emitted from specific peaks as they pass the detector. Colour of each dye represents the different nucleotides. Computer converted the data of emitted light in the nucleotide sequences (Manikkam Radhakrishnan *et al.,* 2013) [5].

16S rRNA sequence analysis and multiple sequence alignment

Here the closely related homologs were identified through phylogenetic analysis by comparing the 16s rRNA gene sequence obtained with the hits of database for nucleotide sequences deposited at NCBI web server (www.ncbi.nlm.nih.gov), through Basic Local Alignment Tool (BLAST) program (http://www.ncbi.nim.nih.gov/blast/). Phylogenetic analysis and nucleotide conservation of the data set sequences were studied through multiple sequence alignment program *viz*., CLUSTAL OMEGA, phylogram was prepared using method of phylogenetic tree construction. Distances between the studied sequences help in understanding the evolutionary distance among the species (Radhakrishnan *et al.,* 2013) [5]**.**

Results and Discussion

The PCR amplification of 16s rRNA gene of three actinomycetes isolates yieldedrange of 1.3-1.5 base pair sequences. For these three isolates, BLAST analysis was done which showed similarity to *AS1 (Streptomyces lucensis gene),* AS2 *(Streptomyces griseus gene)* and AS3 *(Streptomyces olivaceusgene).* The agarose gel electrophoresis result of bacterial samples showed that genome size of all samples is more than 10 Kb. Their quantitative analysis showed that the DNA concentration of AS1 is 2.80µg/ml, DNA concentration of AS2 is 1.88 µg/ml and the DNA concentration of AS3 is 7.03 µg/ml.

The actinomycetes species are efficient in fixing nitrogen with the host legumes. 16S rRNA gene amplification of the isolates and their similarity search with the hits determined their genus as streptomyces. Similarity search using NCBI-BLAST, three species were identified as *Streptomyces lucensis gene, Streptomyces griseus gene* and *Streptomyces olivaceusgene.* A phylogenetic tree based on part of the amplified gene sequences ranged from 1.3 Kb to 1.5 Kb showed that the sequences of these genes in the three isolates were different. AS1 shared a similarity with *Streptomyces lucensis* gene, AS2 shared a similarity with *Streptomyces griseus* gene and AS3 shared a similarity with *Streptomyces olivaceus* gene.

We further characterized the actinomycetes strains via 16S rRNA gene amplification and sequencing in order to assign the strains to the closest species. Sequences were aligned with clustal OMEGA (Thompson *et al.,* 1994). The sequences of the three rhizobium isolates were submitted to Genbank and accession number for the isolates AS1 is LC176426.1 (*Streptomyces lucensis* Gene), AS2 is LC176427.1 (*Streptomyces griseus* Gene) and AS3 is LC176428.1 (*Streptomyces olivaceus* Gene) as shown in Table 2.

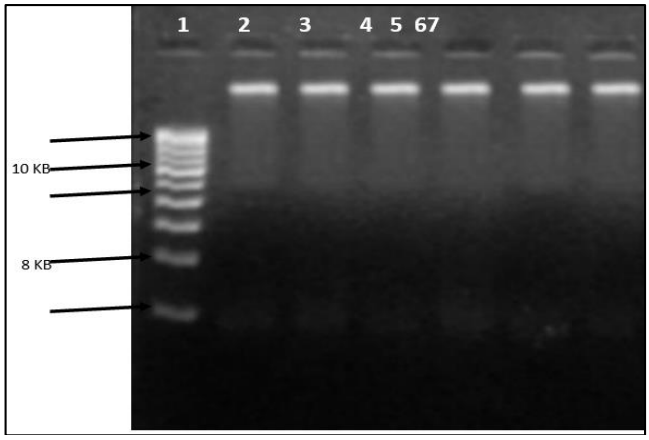

Fig 1: Agarose gel electrophoresis results of bacterial DNA

Table 1: Spectrophotometric analysis of genomic DNA of bacteria isolated from broth

S. No	Bacterial samples	Ratio	DNA Conc (µg/ml)
1	AS1	1.12	2.80
2	AS2	1.11	1.88
3	AS3	1.16	7.03

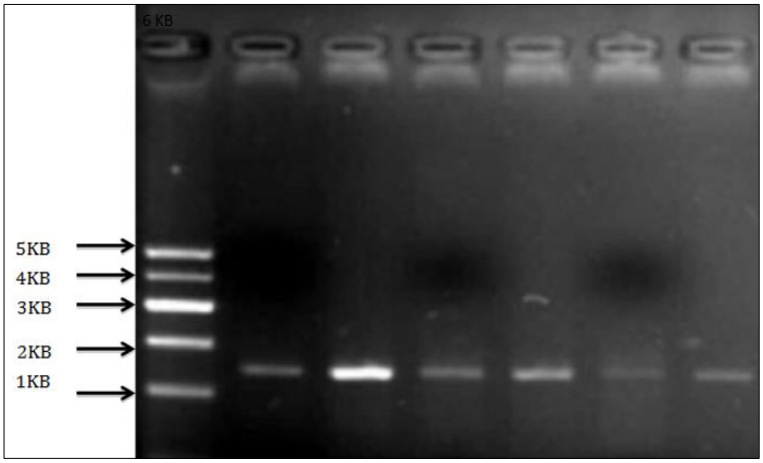

Fig 2: Agarose Gel Electrophoresis results of PCR product

Table 2: Analysis of the 16S rRNA gene sequences for comparison of isolates with other Actinomycetes spp using NCBI BLAST

S/No	Isolates	Type of *Actinomycetes*	Name of *Actinomycetes*	Gen Bank accession No Sequence ID	Query ID
1	AS1	*Streptomyces lucensis* gene	ABDR4	LC176426.1	2555
2	AS2	*Streptomyces griseus* gene	ABDR5	LC176427.1	142655
3	AS3	*Streptomyces olivaceus*gene	ABDR6	LC176428.1	167997

16S ribosomal RNA result for *Streptomyces lucensis*

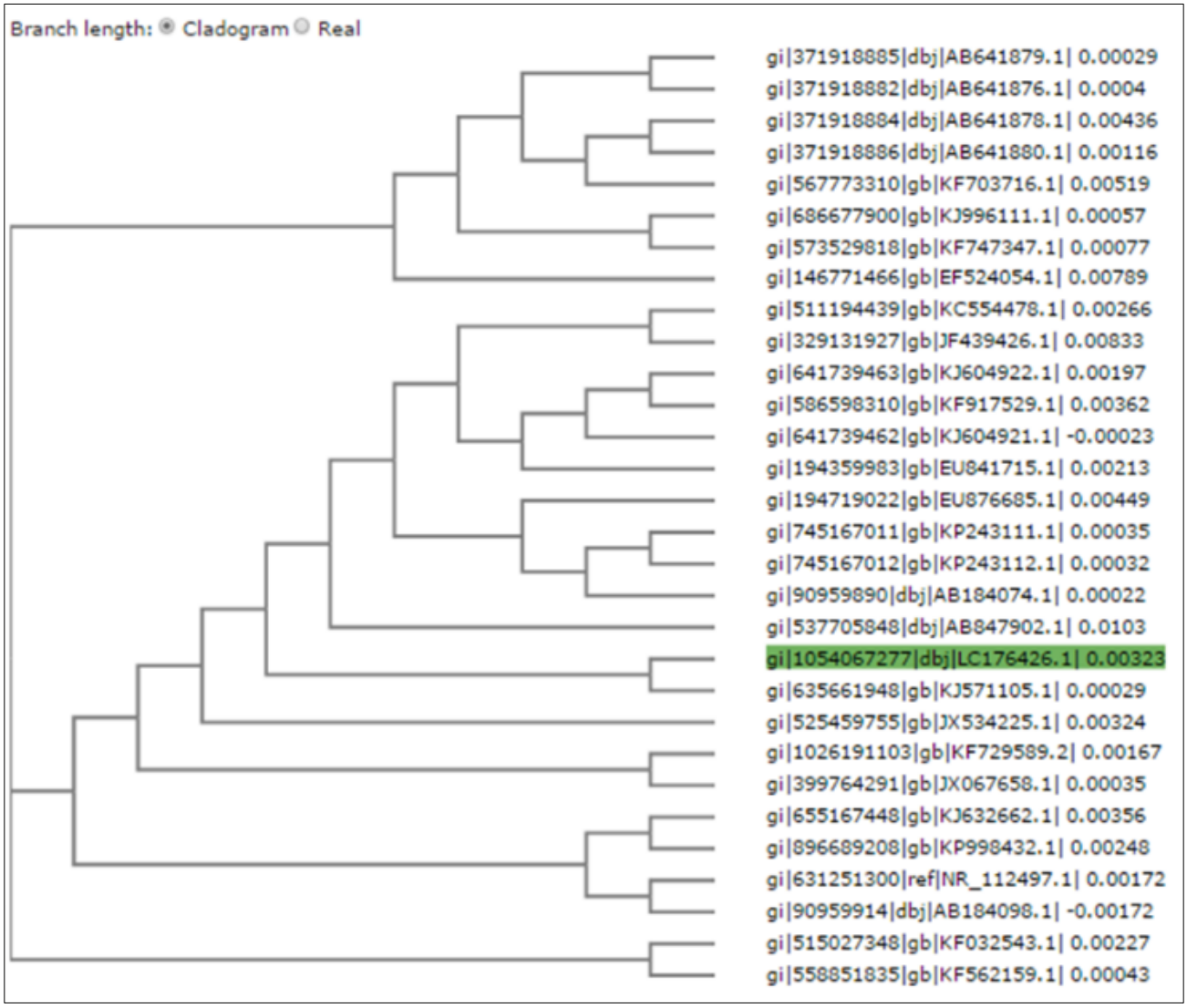

Fig 3: Phylogenetic relationship of the Actinomycetes and related taxa, based on 16s rDNA analysis. The evolutionary history was inferred using the Neighbor-Joining method

16S ribosomal RNA result for *Streptomyces griseus*

Fig 4: Phylogenetic relationship of the Actinomycetes and related taxa, based on 16s rDNA analysis. The evolutionary history was inferred using the Neighbor-Joining method.

16S ribosomal RNA result for *Streptomyces olivaceus*

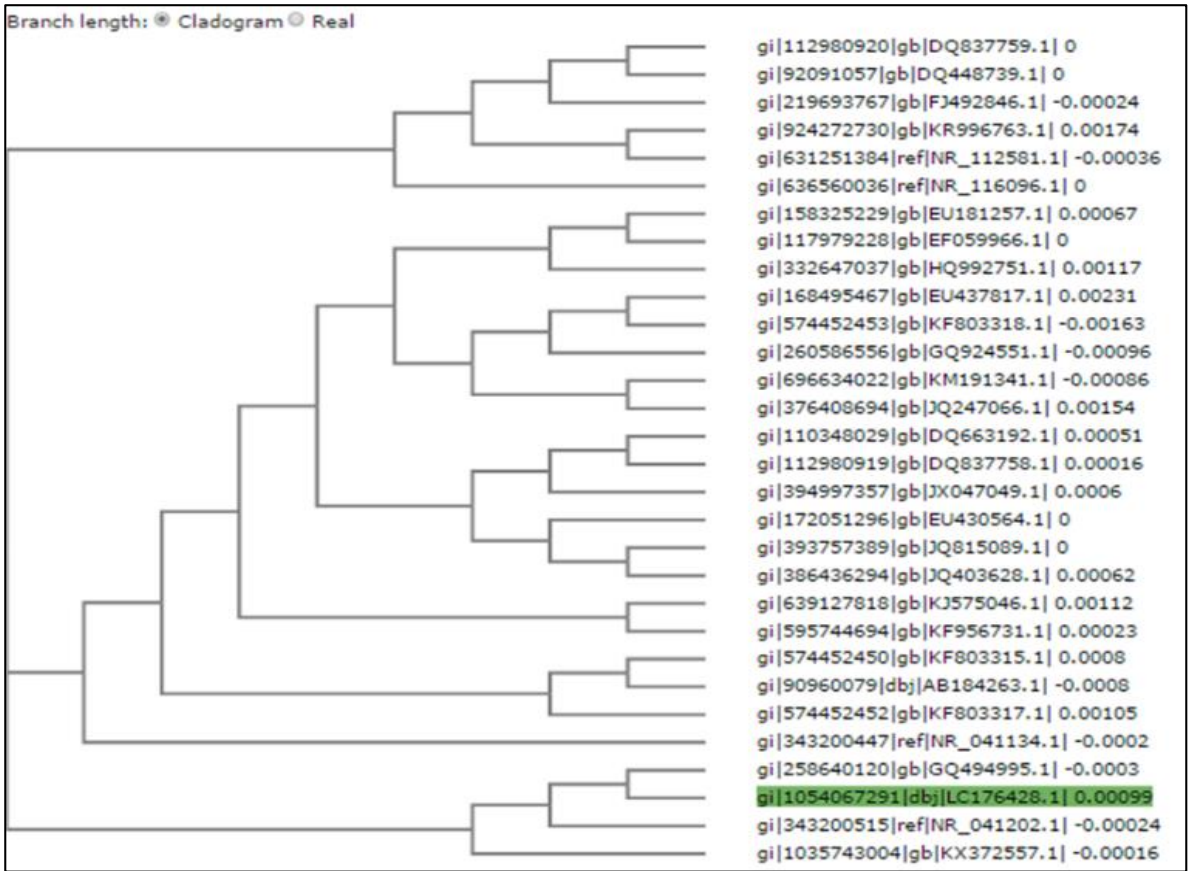

Fig 5: Phylogenetic relationship of the Actinomycetes and related taxa, based on 16s rDNA analysis. The evolutionary history was inferred using the Neighbor-Joining method.

Molecular tools are the most reliable and fast method for exploring the un-touched species of bacteria, they have agreatpotentialtoassistin isolating yet-uncultured bacteria with known rRNA sequences for further investigations (Teske *et al.*, 1996). One of these molecular tools is the PCR amplification of variable regions of the genes encoding 16S rDNA, by use of primers homologous to conserved regions of the gene. However, there are numerous reports based on 16S rRNA gene sequence data, which suggest that more than 99% of microorganisms in natural habitats are uncultured or uncultivable one (Whitman *et al*., 1998). In this present investigation, 16S rRNA amplified products were sequenced, which revealed that six strains belong to the members of the genus Streptomyces, which was the dominant actinobacterial genus. Ellaiah and Reddy (1987) isolated actinomycetes from natural habitats and found that the most common genus was Streptomyces.

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