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Genetic diversity among sixteen different *Actinomycetes* species isolated from Lonar Crater using ITS marker

Phylogenetic study of actinomycetes species isolated from Lonar Crater using ITS marker

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Abstract: Isolation studies of natural and ecologically enriched units help in achieving novel secondary metabolites rich in actinomycete species. The Lonar lake crater of the Buldhana district is one of the less explored naturally occurring alkaline ecosystems for the screening and discovery of a unique microbial community. The capability of marine *actinomycetes* to survive in extreme temperature and pH conditions enhances their bioactive system for the secretion of novel secondary metabolites. In the present study, we performed a study of sixteen- isolates of *actinomycetes* species isolated from Lonar lake, Buldhana District, Maharashtra. The isolation and the identification of the isolates were performed through molecular tools and phylogenetic analysis. . The phylogenetic relationship was determined by r-DNA-based phylogenetic markers ITS (Internal Transcribed Spacers) with the help of phylogenetic tools. The present study aimed to assess genetic variations among sixteen- isolates of *actinomycetes* isolated from Lonar lake by using ITS molecular marker. The dendrogram obtained from the data showed that hierarchical clustering separated the isolates into two groups. The results showed that the majority of the isolates belonged to *Streptomyces* species, 2 isolates of *Brevibacterium s*pecies and single isolates *of Kocuria, Conexibacter, Rubrobacter, Actinomadura, Micromonospora, Nocardiopsis,* and *Actinobiospora*. ITS can be used as an important molecular marker for phylogenetic study.

IndexTerms *- Actinomycetes*; Lonar lake; ITS, molecular; Phylogenetic study; Streptomyces.

I. INTRODUCTION

The majority of the *actinomycete* species possess the potential to produce bioactive secondary metabolites with biological applications including antibiotics, antifungal and antiviral agents, anticancer drugs, enzymes and immunosuppressants [1, 2]. Due to their peculiar characteristics around two-thirds of the commercially available antibiotics have been isolated from *actinomycetes* and still, new species of *actinomycetes* have been searched from unexplored terrestrial and marine environments for exploration of novel bioactive compounds [2, 3, 4]. Among the different *actinomycetes*, species studied *Streptomyces* species has been widely known as the largest antibiotic-producing organism [5]. The isolation and identification of *actinomycetes* formerly were performed through the study of morphological characters but this form of identification was inadequate for the differentiation of different species associated with different genera [6, 7]. Thus, the utilization of molecular and phylogenetic approaches has emerged as a boon for the classification of actinomycete species. With the development of molecular techniques, similar organisms that showed unintended placement in inappropriate groups received appropriate classification [7, 8]. The identification of the actinomycete phylogeny is executed through 16S rRNA and the usage of PCR for sequence analysis [5, 7]. Lonar lake or the Lonar crater is considered a national geographic heritage site situated in the Buldhana district, Maharashtra, India. Lonar crater is a naturally formed alkaline ecosystem and a desirable biome for the microbial community [9]. As the isolation of *actinomycetes* from the terrestrial environment has decreased for the screening of secondary metabolites much focus of the researchers has shifted towards the *actinomycetes* from the marine environment [10]. The present study also follows a similar path and investigates the saline water ecosystem of the Lonar crater for the screening of the antibiotic-producing actinomycete population based on molecular and phylogeny techniques. The present study also aimed to estimate the genetic diversity of 16 isolates of *Actinomycetes* species isolated from the saline water of Lonar lake studied by ITS r-DNA sequencing.

II. NEED OF THE STUDY

Lonar crater is a fertile ground for isolating *actinomycetes* which have varying cultural, morphological, biochemical and carbon source requirements. The microbial studies on the Lonar crater have reported the presence of aerobic and alkaliphilic bacteria also the *actinomycetes* population has been isolated from the place. The lake area is closed without any opening and showcases salinity, alkalinity, and unique biodiversity. Thus, the Lonar crater is a gold mine for novel organisms which need to be further confirmed by molecular markers. These diverse microorganisms could be used in commercial industries for the development of new products. The microbial population functions significantly in the biotechnological and pharmaceutical industries by secreting enzymes, antibiotics and other valuable products. The results obtained in the present study provide clear evidence that new/novel *Actinomycete* genera and species will lead to the discovery of significant bioactive secondary metabolites with biological activity with applications in drug discovery.

III. RESEARCH METHODOLOGY

3.1 Isolation and identification of *Actinomycetes*

Isolation and identification of *actinomycetes* from saline water of Lonar lake, Buldhana district, Maharashtra was published in our earlier study by Mendhe *et al.*, (2022) [11]. In the previous study, isolation and identification of 16 isolates of *actinomycete* species were performed through morphological and biochemical studies.

3.2 Identification of actinobacteria based on 16s RNA

3.2.1 Extraction of genomic DNA

The genomic DNA of each sample was extracted and purified by the AxyPrep Bacterial Genomic DNA Miniprep kit as directed by the supplied manufacturer's manual and the purification and quantification of the samples was estimated by Nanodrop. Universal 16S rRNA PCR forward primer (27f: AGAGTTTGATCMTGGCTCAG) and reverse primer (1492r: TACGGYTACCTTGTTACGACTT) were used for the amplification of 16S rRNA genes. PCR reaction was performed in a thermal cycler (Bio-rad) using the following conditions: initial denaturation of 5 min at 94°C, followed by 35 cycles consisting of 30 sec at 94°C (denaturation), 30 sec at 58°C (annealing) and 1 min at 72°C (extension) and final extension was 15 min at 72°C (Table 1). The PCR products were analyzed by 1.5 % agarose gel electrophoresis, amplified products were cut by clean scalpel, purified by Quigen quick PCR purification kit and amplicons were sequenced using the same universal primers. The obtained 16S rRNA sequences were further subjected to BLAST programme (http://www.ncbi.nlm.nih.gov/) to predict the organisms. ITS sequences after sequencing were pasted in the window at the online BLAST analysis tool which provided results in the form of maximum similarity of all available sequences in GeneBank.

3.2.2 Phylogenic analysis of *actinomycetes*

The evolutionary relationship of the *actinomycetes* was analyzed based on rDNA sequences using the neighborhood joining method. The percentage of replicate trees with associated data was clustered together in bootstraps (1000 replicates) and was shown next to branches [12]. Evolutionary analysis was done by MEGA version X 10.2.6. The pairwise distance and test of neutrality were calculated. Statistical method for evaluation of neutral mutation hypothesis needs only data on DNA polymorphism, namely genetic variation within the population at the DNA level. A statistical test proposed by Tajima is very effective in studying DNA polymorphism [13]. The results from Tajima's neutrality test were carried out in the study. Also, evolutionary divergence was analyzed using the Jukes-Cantor model [14]. The distances were calculated.

IV RESULTS AND DISCUSSIONS

4.1 Molecular identification and Phylogenetic analysis

Molecular identification was carried out based on 16S rRNA gene sequences [15]. The genomic DNA of 16 *Actinomycetes* isolates was extracted using a commercially available kit and the isolation procedure was performed according to the manufacturer's guide. The purity and DNA concentration was determined using Nanodrop. The DNA so obtained was pure and with a high concentration. These DNA samples of isolated *actinomycetes* species were subjected to further PCR amplification. Total DNA of 16 isolates was extracted and ITS regions were amplified using universal 16S rRNA PCR forward and reverse primer. The expected size of PCR product in ITS region was observed to be approximately 700 to 1500 bp on 1.5% Agarose gel after electrophoresis (Figure 6). The amplified products were cut by a clean scalpel, purified by Quigen quick PCR purification kit, and amplicons were sequenced using the same universal primers. All the isolated samples of *Actinomycetes* were sent for ITS region DNA sequencing. After complete authentication, all the above 16 *Actinomycetes* sequences were submitted to National Center for Biotechnology Information (NCBI) database through the online available submission policy. The NCBI acknowledged the submitted sequences and assigned a specific accession number to all the *actinomycete* isolates.

We received accession numbers for all the sequences in 15 days. The isolated *actinomycetes* species were ACM 01 *Kocuria rhizophila* (ON248525), ACM 02 *Conexibacter woesei* (ON377276), ACM 03 *Brevibacterium spp*., ACM 04 *Rubrobacter xylanophilus* (ON387757), ACM 05 *Streptomyces coelicolor* (ON387762), ACM 06 *Streptomyces clavuligerus* (ON388843), ACM 07 *Streptomyces spp*. (ON399110), ACM 08 *Brevibacterium spp.*, ACM 09 *Actinomadura citrea* (ON390800), ACM 10 *Saccharomonospora viridis* (ON390797), ACM 11 *Micromonospora echinospora* (ON390799), ACM 12 *Streptomyces rochei* (ON390798), ACM 13 *Nocardiopsis Spp.*, ACM 14 *Actinobiospora yunnansis*, ACM 15 *Streptomyces clavuligerus*, ACM 16 *Streptomyces spp.* (ON390801). The 16s rDNA ITS sequences obtained for all the *actinomycetes* species were added to the Basic Local Alignment Search Tool (BLAST) network service, which is available online free of cost.

The nucleotide sequences of isolated *Actinomycetes* spp. were compared with all the sequences available with nucleotide databases (e.g. NCBI). As per the availability of similarity tools in the software, the homology search was performed within the non-redundant databases of GeneBank using the BLAST analysis found at http://www.ncbi.nlm.nih.gov/BLAST/of NCBI. The BLAST results

reported for all sequences of the present study revealed that each sequence of *Actinomycetes* species had homology or similarity with nucleotide sequences of the same species present in the databases at different similarity level which ranges between 99-100%. For each sequenced amplicon, sequences put in the window given for the test need to be in FASTA (Fast Alignment Sequence test for application) format and aligned using the online BLAST search tool for nucleotides in NCBI databases. The output of the resulting window showed the best similarity matches with the sequences available in databases. For phylogenetic analysis or evolutionary relationship of these 16 isolates, initially, multiple sequence alignment was carried out using ClustalW software which is available in Mega X software. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version X 10.2.6 [15].

The file obtained after multiple sequence alignment was used for the construction of a dendrogram or phylogenetic tree using the neighborhood joining tool of the phylogenetic analysis program. The evolutionary relationship was analyzed based on rDNA sequences using the neighborhood joining method. The sequences were aligned using ClustalW algorithms. The percentage of replicate trees in which associated data is clustered together in bootstraps (1000 replicates) was shown next to branches. The evolutionary data was computed by Kimura 2-parameter method and units of the number of base substitutions per site [16]. Pairwise Distance Calculation and Test of Neutrality Statistical methods to evaluate the neutral mutation hypothesis need only data of DNA polymorphism, namely genetic variation within the population at DNA level. A statistical test proposed by Tajima's is very effective in studying DNA polymorphism [13]. The results from Tajima's neutrality test were carried out (Table 2). Also, evolutionary divergence was analyzed using Jukes-Cantor model [17]. The distances were calculated (Table 3).

The tree was drawn to scale, with branch length in the same units considered as those of evolutionary distances in the phylogenetic tree. The reliability and accuracy of the phylogenetic tree was checked by using Tajima's neutrality statistical test. The evolutionary relationships between sequences were analyzed from the number of base substitutions per site among sequences. The genetic relationship was calculated in the form of a similarity coefficient from the dendrogram. The diversity was studied through the phylogenetic tree. The dendrogram was mainly divided into two clades *Nocardiopsis* spp. in one separate clade and the other 15 isolates in other clades. The first clade was divided into two clades with internodes 99 and 70. ACM 09 *Actinomadura citrea* (ON390800), ACM 10 *Saccharomonospora viridis* (ON90797), ACM 11 *Micromonospora echinospora* (ON390799), ACM 12 *Streptomyces rochei* (ON 390798) and ACM 1 *Kocuria rhizophila* (ON248525), ACM 02 *Conexibacter woesei* (ON377276), ACM 03 *Brevibacterium spp.*, ACM 04 *Rubrobacter xylanophilus* (ON387757), ACM 05 *Streptomyces coelicolor* (ON387762), ACM 06 *Streptomyces clavuligerus* (ON388843), ACM 07 *Streptomyces spp.* (ON399110), ACM 08 *Brevibacterium spp*., ACM 13 *Nocardiopsis Spp.*, ACM 14 *Actinobiospora yunnansis*, ACM 15 *Streptomyces clavuligerus*, ACM 16 *Streptomyces spp*. (ON 390801). The detailed diversity and similarity of *actinomycetes* species have been shown in the phylogenetic tree (Figure 1). These results were in resemblance to results demonstrated by Sharma *et al.*, [18]. According to Zhi *et al.*, [19], actinobacteria are distinguishable from other bacterial taxa by their distinctive gene organization patterns, whichgives them their branch on the 16S rRNA gene tree [20]. Ilkunr *et al.*, [21] suggested that there are few investigations in the sediments of the Eastern Mediterranean Sea, particularly in connection to environmental factors, despite the significant morphological and phylogenetic diversity, biotechnological, and economic value of *actinomycetes*. The *actinomycetes* were isolated from deep basins of the Eastern Mediterranean Sea, with regional variation (72–1235 m depths) during the study. With the help of commercial testing kits, it was discovered that they used proteins rather than carbs. Highly varied *Streptomycetes* strains were discovered by the investigation of the 16S rRNA gene, two of which represented novel taxa as well as the genera *Nocardiopsis* and *Pseudonocardia.*

V. CONCLUSION

Molecular markers undoubtedly prove to be a successful tool for the identification of microorganisms. The primary identification of the isolates can be carried out using morphological methods as it is influenced by environmental conditions but the confirmatory study for the identification of *actinomycetes* at the species level should be performed through a molecular marker. The sequences obtained in the above study were compared with sequences available in the GeneBank database and the best match was chosen which supported our preliminary study of morphological identification. In this study molecular identification, BLAST analysis and phylogenetic analysis were carried out, and also the degree of genetic variability among isolates of *actinomycetes* from Lonar lake based on ITS r-DNA sequencing was evaluated. ITS marker was found to be a powerful tool for the analysis of genetic variation among the *actinomycetes*. We suggest that the ITS marker may be used as one reliable alternative for the determination of genetic variation among the different *actinomycetes* species.

Figure Captions

Figure 1: Phylogenetic tree based on 16S rRNA gene sequences of the *actinomycete* strains isolated from Lonar crater.

Table Captions

Table 1.0 Details of PCR programme
Table 2: Results from the Tajima's test for 3 Sequences
Table 3: Distance matrix

Table 1: Details of PCR programme

Step	Temperature (°C)	Time	No. of cycles
Initial denaturation	94	5 min	1
Denature	94	30 sec	35
Annealing	58	0 sec	
Extension	72	1 min	
Final extension	72	15 min	1

Table 2: Results from the Tajima's test for 3 Sequences

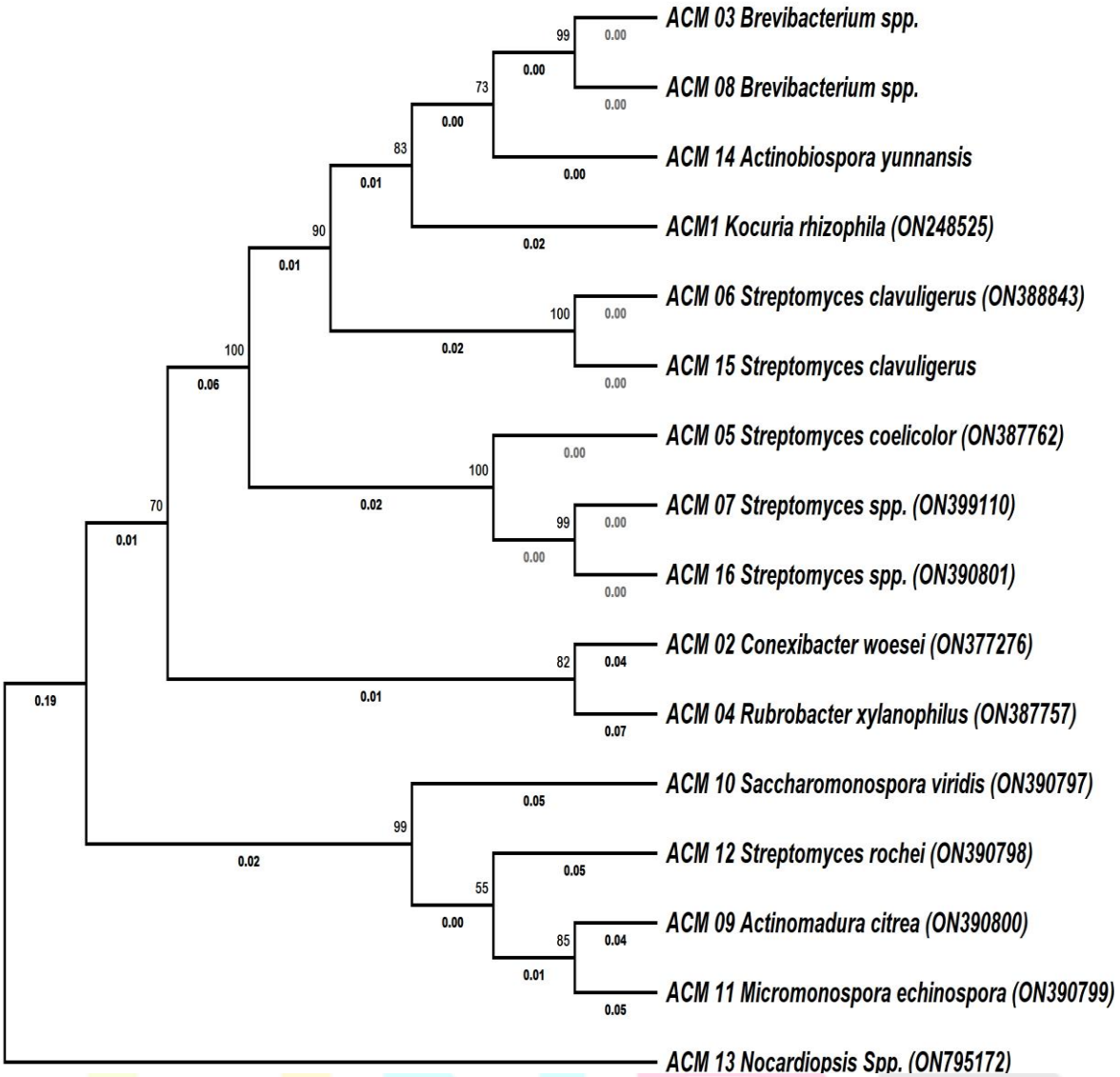
Configuration	Count
Identical sites in all three sequences	828
Divergent sites in all three sequences	17
Unique differences in Sequence A	26
Unique differences in Sequence B	144
Unique differences in Sequence C	15

The equality of evolutionary rate between sequences **A** (*ACM1 Kocuria rhizophila (ON 248525)*) and **B** (*ACM 02 Conexibacter woesei (ON 377276)*), with sequence **C** (*ACM 03 Brevibacterium spp.*) used as an outgroup in Tajima's relative rate test (Tajima *et al.*, 1993). The χ^2 test statistic was 81.91 (P = 0.00000 with 1 degree[s] of freedom) P-value less than 0.05 is often used to reject the null hypothesis of equal rates between lineages. This analysis involved 3 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 1030 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

Table 3: Distance matrix

ACM1_*Kocuria_rhizophila*_(ON_248525)															
ACM_02_*Conexibacter_woesei*_(ON_377276)	0.14														
ACM_03_*Brevibacterium*_spp.	0.02	0.14													
ACM_04_*Rubrobacter_xylanophilus*_(ON_387757)	0.17	0.11	0.17												
ACM_05_*Streptomyces_coelicolor*_(ON_387762)	0.05	0.13	0.04	0.16											
ACM_06_*Streptomyces_clavuligerus*_(ON_388843)	0.04	0.15	0.02	0.17	0.04										
ACM_07_*Streptomyces*_spp._(ON_399110)	0.05	0.13	0.04	0.16	0.00	0.04									
ACM_08_*Brevibacterium*_spp.	0.02	0.14	0.00	0.17	0.04	0.02	0.04								
ACM_09_*Actinomadura_citrea*_(ON_390800)	0.17	0.15	0.17	0.16	0.17	0.17	0.17	0.17							
ACM_10_*Saccharomonospora_viridis*_(ON_390797)	0.17	0.138	0.16	0.16	0.17	0.17	0.17	0.16	0.11						
ACM_11_*Micromonospora_echinospora*_(ON_390799)	0.18	0.15	0.18	0.15	0.19	0.19	0.19	0.18	0.09	0.10					
ACM_12_*Streptomyces_rochei*_(ON_390798)	0.18	0.14	0.17	0.15	0.17	0.17	0.17	0.17	0.09	0.10	0.11				
ACM_13_*Nocardiopsis*_spp.	0.58	0.55	0.57	0.56	0.58	0.57	0.58	0.57	0.55	0.55	0.56	0.56			
ACM_14_*Actinobiospora_yunnansis*	0.02	0.14	0.00	0.17	0.03	0.03	0.038	0.00	0.17	0.16	0.18	0.17	0.58		
ACM_15_*Streptomyces_clavuligerus*	0.04	0.15	0.026	0.17	0.04	0.00	0.043	0.02	0.17	0.17	0.19	0.17	0.57	0.03	
ACM_16_*Streptomyces*_spp._(ON_390801)	0.05	0.13	0.040	0.16	0.00	0.04	0.00	0.04	0.17	0.17	0.19	0.17	0.58	0.03	0.043

Figure 1: Phylogenetic tree based on 16S rRNA gene sequences of the *actinomycete* strains isolated from Lonar crater.



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