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Polyphasic Characterisation and Phylogenetic Affiliation of Beta Glucanase Producing Actinomycetes Strains TBG-MR17 and TBG-AL13, Isolated from Western Ghats Soil, Kerala, India

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Abstract

Soil is a natural reserve encompassing the diversity of microorganisms, with amazing production of useful metabolites. Over the past few decades, an enormous number of actinomycetes species have been isolated from soil samples. The isolation and taxonomical description of actinomycetes with the novel metabolites production from under-exploited or unexplored environments is tremendously crucial, which unlocks exhilarating avenues in biotechnology research. The polyphasic taxonomic approach exploiting morphological, cultural, biochemical, physiological, and molecular characteristics, assist the taxonomists to develop universal eloquent actinomycetes taxonomic identification system. This approach effectively identifies actinomycetes isolates from the genus level and fairly extends up to the species level. In this study, the cumulative result of polyphasic characterization elucidates the taxonomic position of efficient β-glucanase producing Western Ghats actinomycetes isolates TBG-MR17 and TBG-AL13 respectively as *Streptomyces althioticus* TBG-MR17 and *Streptomyces cinereoruber* sub sp. *cinereoruber* TBG-AL13.

The major part of the earth's natural biodiversity is occupied by microbes which plays beneficial roles in diversified environments. Conversely, microbial taxonomy is developing in a very slow manner, accordingly, not more than 1% of the microbial population has been identified until now. The foremost problem related to taxonomy was the lack of reliable, uniform, and advanced methodologies for microbial systematics and identification studies[6]. The established traditional and conventional methods of microbial classification mainly depend on morphological, biochemical and physiological aspects that are insufficient to produce a flawless image relating to the taxonomical status[23]. It necessitates certain additional elucidation regarding accurate taxonomy.

Nowadays with the advancements in genomics, the microbial taxonomical complexity is more comprehensible. The addition of genotypic approaches in classification delivered an understanding of the phylogenetic interactions of organisms up to the subspecies level. Molecular genomics, cultural, biochemical, physiological and chemotaxonomic studies collectively constitute the taxonomic position of microorganisms is named as 'polyphasic taxonomic approach'[34], which helps to determine the species-level taxonomy within the genus[2]. Currently, this approach is more popular in bacterial classification and provides new insights into the organism's species and genus level sortings. Accurate taxonomical identification is obligatory for each novel microbial isolates from unusual environmental habitats.

Actinobacteria is one of the largest taxonomic entities among the currently recognized major lineages in the Bacteria domain[15]. The genera within the phylum exhibit massive diversity in terms of morphological, physiological and metabolic competencies. The taxonomy of Actinobacteria has significantly developed gradually with the accumulation of understandings. Though it remains rather confused and the species level definition becomes an incomplete task because of the variability in cultural, morphological, physiological and biochemical characteristics observed at both inter-and intra-species level[2]. Consequently, the polyphasic taxonomic approach is more advisable in actinomycetes taxonomy. The key characteristics used to define the taxonomy of Actinobacteria into genus and species levels includes morphological studies based on microscopy, cellular chemistry-based chemotaxonomy, cultural studies mainly on growth mycelia formation and pigments, biochemical-physiological characteristics and finally molecular genotypic studies based on 16s rRNA gene sequencing[1,3]. Accordingly, the polyphasic taxonomic approach can encounter the challenges of classifying unknown strains, this study explicates the characterization and taxonomical identification of β-glucanase producing potent Western Ghats actinomycetes isolates TBG-MR17 and TBG-AL13 by using the possibilities of polyphasic approach.

Morphological characterizations :

The potent isolates were inoculated into starch casein agar media (SCA) and incubated at 28°C for 7 days. Colony appearance, texture, shape, odour, oxygen relationships and motility were examined. Spore chain morphology was studied using the inclined coverslip culture method[4] and observed using a light microscope (Nikon Eclipse Ci-E) under high power (1000x) magnification. Spore surface morphology was observed using a scanning electron microscope (SEM) after fixing with 1% paraformaldehyde in 0.2 M Sorensen phosphate buffer (pH 7.2-7.4) and dehydrated using different concentrations of ethanol (30%, 50%, 70%, 90% and 100%)[27]. The specimen was loaded into CPD Critical Point Drying (CPD) apparatus (HCP-02, Hitachi), dried with liquid carbon dioxide as a transition fluid and was made electrically conductive by sputter coating with a thin film of gold-palladium using sputter coater (Model E-101, Hitachi), under vacuum. Observation and photography were done at suitable magnifications using scanning electron microscope (FEI QUANTA 200, Netherland).

Cultural characterizations :

Growth, presence of soluble pigments, aerial mycelium and substrate mycelium colour (reverse colony colour) were noted in 7 to 14 days old culture on different culture media as described by International Streptomyces Project (ISP) viz. tryptone-yeast extract agar (ISP-1), yeast extract-malt extract dextrose agar (ISP-2), oatmeal agar (ISP-3), inorganic salts starch agar (ISP-4), glycerol asparagine agar (ISP-5), peptone yeast extract agar (ISP-6), tyrosine agar (ISP-7) and Sabouraud's dextrose agar (SDA) (Shirling and Gottlieb, 1966). The colour of mycelia and soluble pigments, if any, were noted and compared with the ISCC-CNBS colour charts[11].

Biochemical characterizations :

Biochemical tests like Gram's staining, hydrogen sulphide production, nitrate reduction, degradation of tyrosine (0.5% w/v), xanthine (0.4% w/v), and aesculin (0.1% w/v) and various enzyme activities such as gelatinase, catalase, protease, amylase, chitinase, xylanase, lipase and cellulase were carried out. Utilization of Carbon Sources viz., D-arabinose, D-fructose, meso-inositol, D-mannitol, raffinose, L-rhamnose, sucrose, cellulose, D-xylose (1% w/v) were carried out according to Williams *et al.*,[37].

Physiological characterizations :

Growth on various temperatures (10°C to 50°C) and pH (4 to12), NaCl tolerance (0.5, 1, 3, 5, 7 and 10% of w/v) and resistance to antibiotics such as chloramphenicol ($5\mu g.mL^{-1}$), ampicillin ($2\mu g.mL^{-1}$), kanamycin ($2\mu g.mL^{-1}$) and tetracycline ($2\mu g.mL^{-1}$) were analysed[37].

Molecular- Taxonomical characterization:

Genomic DNA was isolated according to Murray and Thompson[20]. PCR amplification of 16S ribosomal RNA (rRNA) gene was done using universal primers 8-27F (5'-AGAGTTTGATCCTGGCTCAG- 3') and 1495R (5'-CTACGGCTACCTTGTTACGA-3') (Weisburg et al., 1991). PCR reaction was carried out in a final volume of 25µL containing 10µL of 20-50ng of template DNA, 1µL of 10pmol forward primer (8-27F), 1µL of 10pmol of reverse primer (1495R), 12.5µL of GoTaq® G2 Hot Start Green Master Mix (2X). The amplification was done in Bio-Rad thermal cycler (S1000TM) with the following conditions: 95°C for 2 min, 35 cycles of 95°C for 1 min, 58°C for 30 sec and 72°C for 1.30 sec and a final extension of 72°C for 5 min. The purified PCR products were sequenced in ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using BigDye® Terminator v3.1 Cycle Sequencing Kit from Applied Biosystems based on Sanger's dideoxy chain termination method. The sequencing PCR was set up in a final volume of 10µL containing 2µL of DNA, 1µL of Primer and 7µL of ready Reaction Mix. The reaction was performed following the standard PCR conditions: Initial denaturation 95°C for 2 min followed by 25 cycles of denaturation of 95°C for 10 sec, annealing at 55°C for 5 sec and extension at 60°C for 4 min.

Phylogenetic Analysis :

The sequence quality of 16s rDNA sequences was checked using Sequence scanner software v5.2 (Applied Biosystems). Sequences were assembled using BIOEDIT v7.2.5. The presence of chimeric artefacts within the obtained sequence was analysed using DECIPHER GPL v3.0 [38]. Phylogenetic neighbours were identified using the EzBioCloud server (https:// www.ezbiocloud.net/identify) along with homology search was performed using BLAST search algorithm[39]. Alignment of similar sequences and nucleotide similarity values were calculated using the CLUSTAL MUSCLE programme[7]. The evolutionary history was inferred using the Neighbor-Joining (NJ) method[24]. The evolutionary distances were computed using the Maximum Composite Likelihood method[23]. The tree topologies were evaluated by performing bootstrap analysis based on 1,000 replications[8]. Evolutionary analyses were conducted in MEGA X [13].

The study was conducted to identify the taxonomic affiliation of β-glucanase producing potent soil actinomycetes strains, TBG-MR17 and TBG-AL13 using polyphasic taxonomic approaches such as morphological, cultural, biochemical, physiological, chemo-taxonomical and molecular basis. Preliminary confirmation of taxonomy was done based on the morphological, cultural, biochemical and physiological features such as appearance, texture, spore chain morphology, colour, pigmentation, growth and utilization of various conditions etc. This classical approach explained within the identification keys by Nonomura[21], the ninth edition of Bergey's Manual of Determinative Bacteriology[10] and Laboratory Manual for Identification of Actinomycetes[4].

The strains TBG-MR17 and TBG-AL13 produced grey round, dry powdery colonies with geosmin (earthy) odour. Starch casein agar (SCA) has been identified as good media for growing actinomycetes strains[18] and suppressed the fungal and bacterial contamination. The detected powdery texture, earthy odour of geosmin and spore chains on aerial mycelium on SCA facilitated the categorisation of our actinomycetes strains, TBG-MR17 and TBG-AL13, in the family *Streptomycetaceae*. The colony formation, mycelia colour, structure of spore chains are the important features of Streptomyces identification[14,35,36].

Light microscopic examination of spore chain topography revealed the strain TBG-MR17 has spirals (S) spore chains (Figure 1.a) and scanning electron microscope observation of spore surface morphology confirmed it is spiny (Figure 1.b). Individual spore was oval-rod shaped, measuring around 690 nm wide and 720 nm long. Whereas, TBG-AL13 has Rectiflexibiles (RF) spore chains (Figure 1.c) with smooth spore surface topology (Figure 1.d). The spores were oblong and the average spore size measuring around 800 nm in width and 1.5 nm long. Both isolates showed well sporulated aerial and substrate mycelia on starch casein agar media. Identification of spore chain morphology by SEM is another significant characteristic for *Streptomyces* identification and used widely

in actinomycetes research[12]. Considering the total soil actinomycetes, more than 95% of the filamentous actinomycete population comprises the genus *Streptomyces*[5].

Aerial and substrate mycelium colour notified in different ISP media is considered as an important character in actinomycetes identification. Both potential β-glucanase producing isolates propagated well on different ISP agar media and exhibited typical characteristics of *Streptomyces* species. Strain TBG-MR17 produced good growth on ISP1, ISP2, ISP3, ISP4, ISP6 and ISP7 media. The poor growth was observed on ISP5 and SDA and developed a grey to yellow aerial mycelium on all tested media and showed different colour gradations (Figure 2). TBG-AL13 showed good growth on ISP1, ISP2, ISP3, ISP4, ISP5 and ISP7 with well-developed aerial and substrate mycelia and produced very negligible growth on ISP6 and SDA. The aerial mycelium showed dark grey, yellow to white colour gradations. Substrate mycelium produced brown, black, green and yellow to white colours on tested media. A blackish yellow soluble melanin pigment was produced in ISP6 media (Figure 3). Melanin production has been also considered as the other significant factor in the taxonomical identification of actinomycetes[28].

Several biochemical and physiological parameters were widely used in actinomycetes identification[9,17]. Current results indicated that both isolates produced catalase, amylase, chitinase and cellulase. But individually they produce other compounds such as protease, xylanase, lipase *etc*. The degradation and production of many compounds by actinomycetes isolates especially *Streptomyces* sp. have been reported so far[19,26,36]. Carbon sources utilization confirmed the identity of *Streptomyces* spp.[25]. Both strains showed good growth in a wide range of pH and very less salt tolerance. The biochemical and physiological characteristics of strains TBG-MR17 and TBG-AL13 are listed in Table-1.

Comparison of morphological, cultural, physiological and biochemical characteristics of strains TBG-MR17 and TBG-AL13 with Nonomura Key for classification (1974) and ISP descriptions[28-32] revealed that both strains come under genus *Streptomyces*. Although, it produces imprecise and unauthenticated interpretations especially in species-level identification. The ambiguity in species definition can overcome by molecular taxonomy study of genomic characteristics.

Molecular taxonomic characterization of the isolates TBG-MR17 and TBG-AL13 were done by extracting genomic DNA (Figure 4.a) followed by PCR amplification of 16s rRNA gene (Figure 4.b), its sequencing and sequence analysis. The amplification of hypervariable regions in 16s rDNA delivers a precise signature, which frequently describes the identification of new actinomycetes strains[22]. 16S rDNA gene has fewer alterations and changes throughout the evolution[16]. The assembly and alignment of forward and reverse 16s rRNA contigs of TBG-MR17 and TBG-AL13 obtained nearly full-length sequences *ie*., 1372 bases and 1448 bases respectively for strain TBG-MR17 and TBG-AL13. Similarity-based searches in the EzBioCloud server confirmed both sequences have all the signature nucleotides which labelled

Table-1. Biochemical and physiological characteristics of strains TBG-MR17 and TBG-AL13

Biochemical Characteristics		
Characteristics	TBG-MR17	TBG-AL13
Gram staining	+	+
H_2S production	-	+
Nitrate reduction	-	+
Degradation Tests		
Gelatin hydrolysis	-	+
Catalase	+	+
Protease	-	+
Amylase	+	+
Chitinase	+	+
Xylanase	+	-
Lipase	-	+
Cellulase	+	+
Aesculin	+	-
Tyrosine	-	+
Xanthin	-	-
Carbon Source Utilization		
D-arabinose	±	+
D-fructose	±	++
Meso-inositol	+	-
D-mannitol	-	-
Raffinose	-	-
L-rhamnose	±	±
Sucrose	+	++
Cellulose	-	+
D-xylose	+	+
Physiological characteristics		
Temperature range (°C)	20 - 40	20 - 40
pH range	4 -10	4 -12
NaCl (%)	0.5, 1.0, 3.0 and 5.0	0.5, 1.0 and 3.0
Antibiotic Resistance		
Kanamycin	-	-
Ampicillin	+	+
Tetracycline	+	+
Chloramphenicol	-	-

++ (strongly positive utilization); + (positive utilization); ± (utilization doubtful)
- (utilization negative)

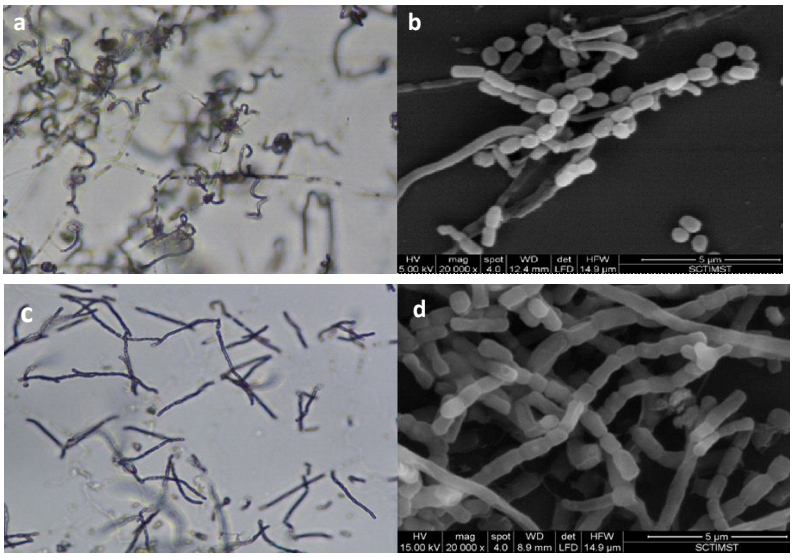

Fig 1. Microscopic studies of actinomycetes strains (a) Light microscopic view of TBG-MR17 showed spirales (S) spore chains (b) Scanning electron microscopic view of TBG-MR17 showed oval-rod shaped spores with spiny surface topography (c) Light microscopic view of TBG-AL13 showed the stain has Rectiflexibiles (RF) spore chains. (b) Scanning electron microscopic view of TBG-AL13 showed oblong spores with a smooth surface.

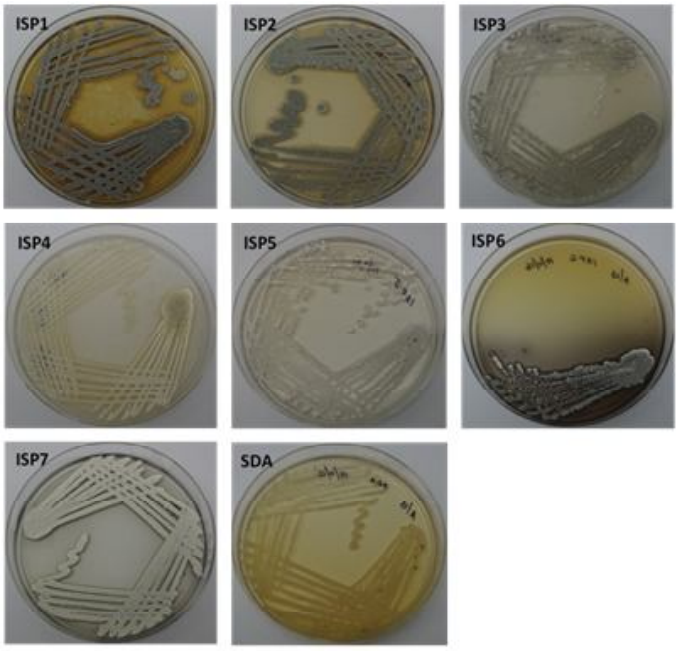

Fig 2. The growth of strain TBG-MR17 on different ISP media

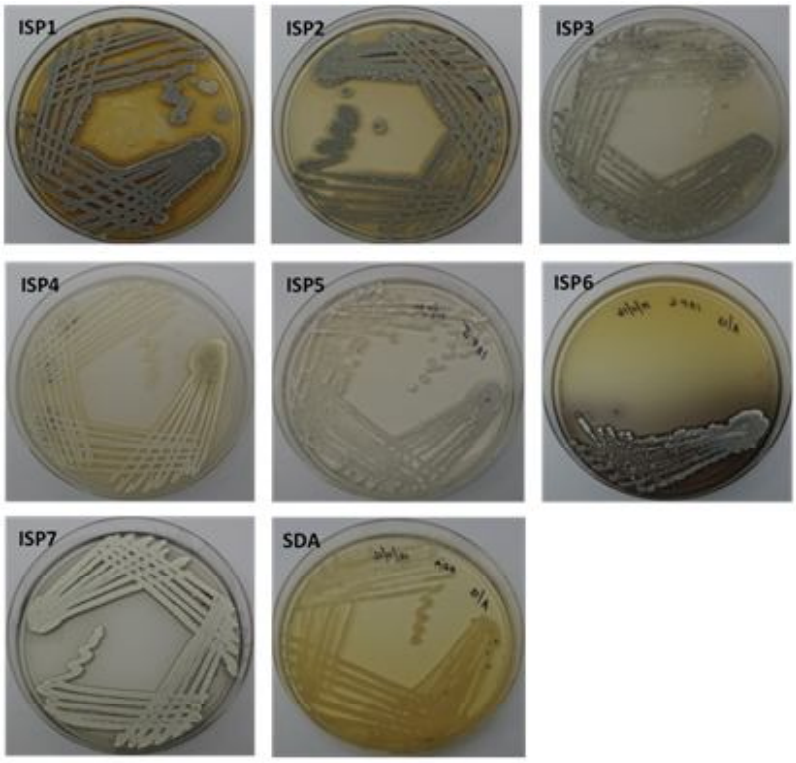

Fig 3. The growth of strain TBG-AL13 on different ISP media

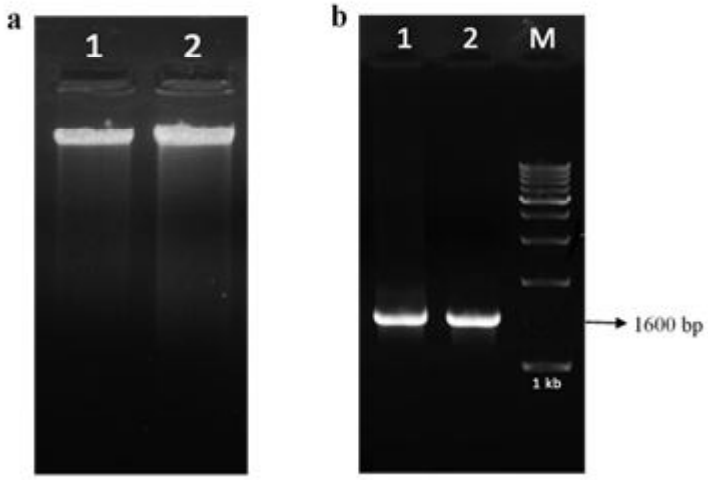

Fig 4. Genomic DNA and 16s rRNA gene amplicons of actinomycetes strain TBG-MR17 and TBG-AL13.

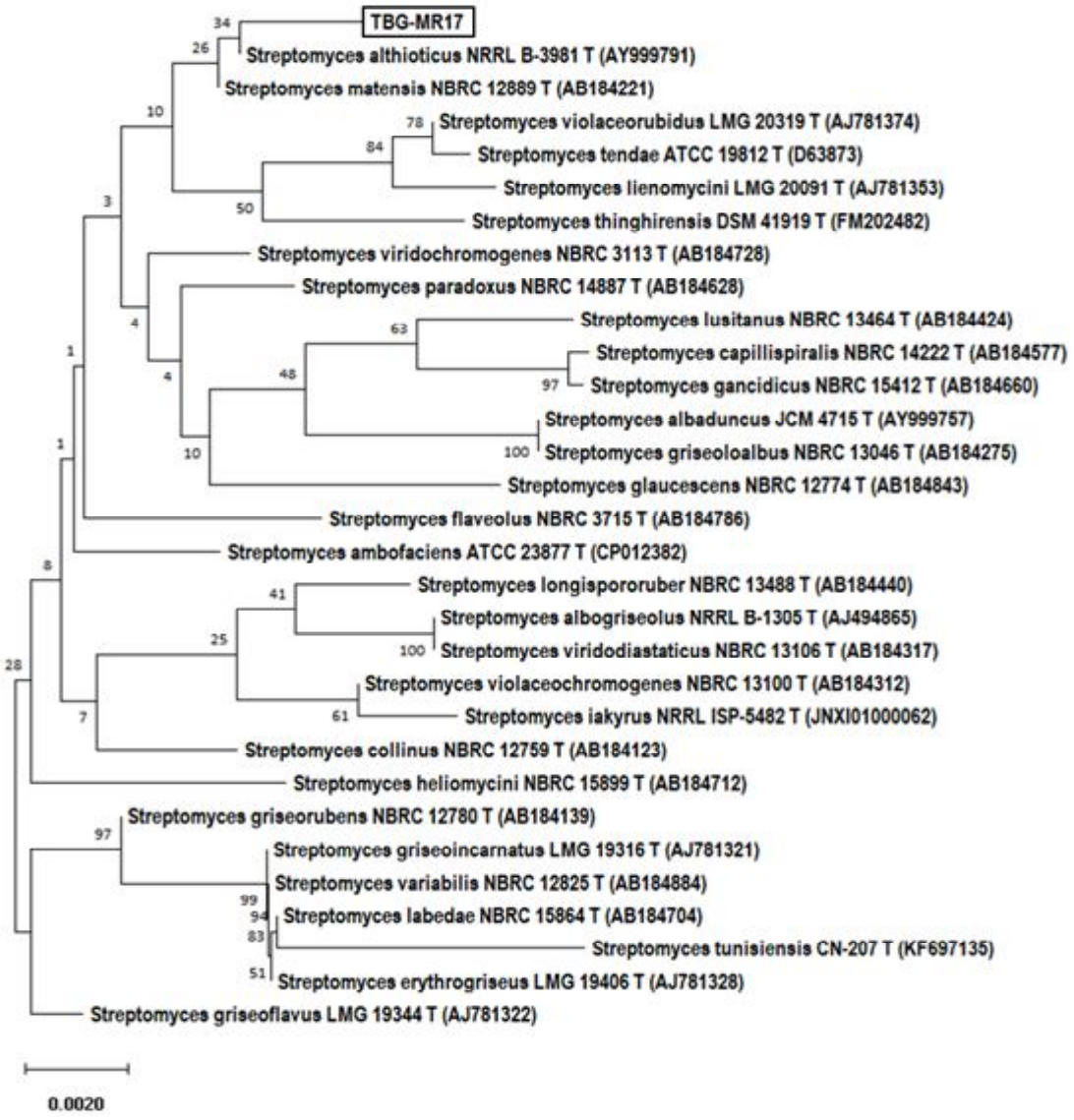

Fig 5. Evolutionary relationships of TBG-MR17. The optimal tree with the sum of branch length = 0.08637777 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 31 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 1455 positions in the final dataset.

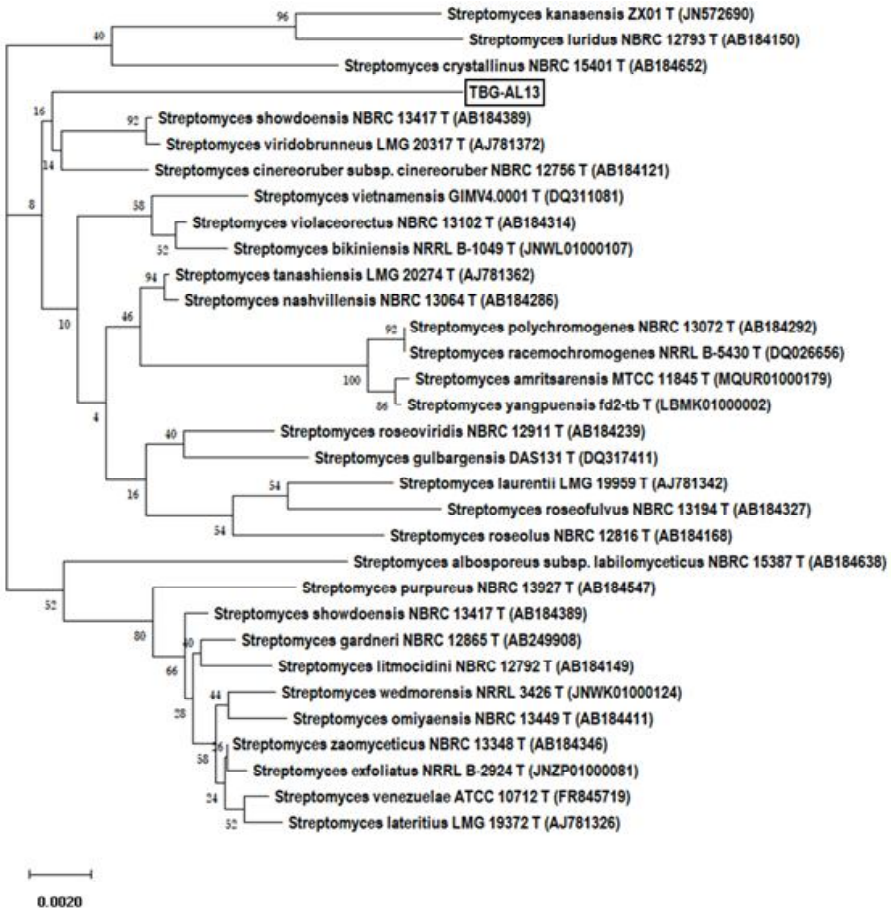

Fig 6. Evolutionary relationships TBG-AL13. The optimal tree with the sum of branch length = 0.13152078 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 32 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 1463 positions in the final dataset.

the genus *Streptomyces*. The sequence TBG-MR17 closely related with *Streptomyces althioticus* NRRL B-3981T (99.85%). The pairwise similarity value of TBG-MR17 with its closely related members of 30 Streptomyces species are between 98.7 to 99.85 %. The Neighbor-Joining (NJ) phylogenetic tree of TBG-MR17 indicated that the strain placed in genus *Streptomyces* and forms a distinct clade with *Streptomyces althioticus* NRRL B-3981T (Figure 5). EzBioCloud similarity search of TBG-AL13 16s rRNA gene sequence revealed its close relationship with *Streptomyces cinereoruber* subsp. *cinereoruber* NBRC

12756T (98.39%). 32 closely related Streptomyces species with pairwise similarity value between 96.93 to 98.39 % were selected for phylogenetic tree construction. The Neighbor-Joining (NJ) tree of TBG-AL13 confirmed its close relation with *Streptomyces cinereoruber* subsp. *cinereoruber* NBRC 12756T (Figure 6). 16s rDNA sequence-related phylogenetic studies provided conclusive shreds of evidence that confirmed the strain TBG-MR17 as '*Streptomyces althioticus* TBG-MR17' and TBG-AL13 as '*Streptomyces cinereoruber* subsp. *cinereoruber* TBG-AL13'.

The 16s rRNA gene sequence of strain TBG-MR17 and TBG-AL13 were deposited in GenBank under the accession number KY458759 and MF686453 respectively.

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