MALAYSIAN JOURNAL OF BIOCHEMISTRY & MOLECULAR BIOLOGY

The Official Publication of The Malaysian Society for Biochemistry & Molecular Biology (MSBMB)
http://mjbmb.org

# MOLECULAR PHYLOGENETIC ANALYSIS USING 16S RIBOSOMAL RNA AND MULTIPLE HOUSEKEEPING GENES OF A BIOACTIVE *Streptomyces* sp. ISOLATED FROM MOSUL, IRAQ

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History
Received: 12th July 2019
Accepted: 22nd December 2019

Keywords:

*Phylogenetic; 16S rRNA gene; housekeeping genes; Streptomyces* sp.

**Abstract**

The genus of *Streptomyces* is the most well known candidate producer of antibiotics and many pharmaceutically important secondary metabolites. Multilocus sequence analysis (MLSA) has shown to be a promising method for streptomycetes taxonomy. In this study, a new bioactive *Streptomyces* sp. designated TMM19 was isolated from Mosul city, Iraq. The isolate grown strong on all International *Streptomyces* Project (ISP) media and exhibited different colours of aerial mycelia. *S.* sp. TMM19 strain displayed bioactivity against *Staphylococcus aureus* (ATCC 43300), *Enterococcus faecalis* (ATCC 51299) and *Bacillus subtilis* (ATCC 23857) pathogens. The results of phylogenetic analyses showed that *S.* sp. TMM19 was most related to *Streptomyces* sp. ZFG47 (accession no. CP030073.1) and *S. actuosus* (accession no. CP029788.1) based on the 16S rRNA genes tree, and to *S.* sp. Z022 (accession no. CP033073.1) and *S. puniciscabiei* (accession no. CP017248.1) based on MLSA of three protein-coding genes (*atpD, recA and rpoB*). The merged 16S rRNA and the three housekeeping genes phylogenetic tree showed that *S.* sp. TMM19 was again most related to sp. ZFG47 and *S. actuosus*, this might be explained by the highly conserved of 16S rRNA gene among *Streptomyces* strains. The 16S rRNA gene of *Streptomyces* sp. TMM19 strain was deposited in the GenBank database (NCBI) under the accession no. MN062265.1

## INTRODUCTION

The genus of *Streptomyces*, which currently contains 848 validly described species (LPSN, 2019), belong to the Gram-positive phylum of actinomycetes. The genus is primarily found in soil, and as such has a vast array of genes which enable its survival in a changeable environment (Goodfellow et al., 1983). Its production of bioactive secondary metabolites is well documented, consisting of compounds such as antibiotics, anticancer and immunosuppressive drugs (Alvarez et al., 2012). Currently, two-thirds of the clinically important antibiotics are produces by streptomycetes (Newman et al., 2003). 95% of antibiotic-producing actinobacteria belong to the genus *Streptomyces* of the family *Streptomycetaceae* (Bérdy 2005). Actinomycin and streptomycin where the first antibacterial metabolites to be identified from *Streptomyces antibioticus* and *Streptomyces griseus*, respectively (Schatz et al., 2005).

Complex organic media specific for *Streptomyces* commonly contain malt extract, yeast extract or dextrose. During the 1960s, the International *Streptomyces* Project (ISP) commenced due to collaboration of studies of about 450 species of *Streptomyces*, and established a series of general media for enrichment and characterisation of bacteria within the genus, for large and small scale isolation procedures (Shirling & Gottlieb 1966).

Molecular approaches like comparative sequencing appears to offer the highest phylogenetic resolution for characterization of actinobacteria (Bansal and Meyer, 2002). The most common method used for prokaryotic classification of relationships of higher taxa is the analysis and comparison of sequences of the gene that codes for the 16S ribosomal RNA, however, this procedure is not always helpful for species differentiation and certainly not for delineation of strains within the same species (Guo et al., 2008). Single protein-encoding genes, on the contrary, yield higher resolution for species relatedness compared to 16S rRNA gene-based methods. For instance, concatenation of the sequence of the gene coding for the β-subunit of DNA gyrase *gyrB* showed 98 to 100% similarities of strains of 18 species (Hatano et al., 2003).

Currently, the widely used method MLSA results in the most reliable method for streptomycetes taxonomy. This involves the analysis and comparison of three to six partial sequences of conserved housekeeping genes which code for proteins with essential functions, such as the *atpD*, *gyrB*, *recA*, *rpoB* and *trpB* gene (Labeda et al., 2012) that are thought to be present in one single copy within the whole genome, in contrast to 16S rRNA genes (Chakravorty et al., 2007).

## MATERIALS AND METHODS

### Sampling and isolation

A soil sample at depth of 10 cm was collected aseptically from grassland area belonging to the campus of Mosul University located at Mosul city, Iraq. The soil sample was air-dried at 70 °C for 15 min in a hot air oven and then cooled to room temperature. Soil sample was crushed, mixed thoroughly and large debris were removed to obtain fine soil particles. For the isolation of *Streptomyces* sp. TMM19, serial dilution plate technique described by El-Nakeeb and Lechevalier (1963) was applied and Starch Casein Agar (SCA) as a selective medium (Kuster and Wiliams, 1964) was used. International *Streptomyces* Project (ISP) media: yeast malt-extract medium (ISP2), oatmeal agar (ISP3), inorganic salts-starch agar (ISP4), glycerol asparagine medium (ISP5), peptone yeast iron medium (ISP6) and tyrosine medium (ISP7) (Shirking and Gottlieb, 1966) were used for the growth of *S.* sp. TMM19. Aerial mycelia and soluble pigments colours were described based on RAL K7 Classic (edition 2012, Germany).

### Bioactivity screening

The isolate was tested against the bacterial strains *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 43300), *Klebsiella pneumonia* (ATCC 700603), *Acinetobacter baumannii* (ATCC 19606), *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus faecalis* (ATCC 51299) and *Bacillus subtilis* (ATCC 23857). The pathogenic strains were grown overnight in 5 ml of LB liquid media at 37°C, (*B. subtilis* at 30°C). Cultures were diluted 1:100 in 20 ml of soft nutrient agar and poured on Petri dishes containing 25 ml of LB agar. Plugs of *Streptomyces* sp. TMM19 culture grown on ISP2, ISP3, ISP4, ISP5, ISP6 and ISP7 at 30°C for two weeks plus a control plug containing only agar of each medium, were taken under sterile conditions using a cork borer, and placed over the layer of soft nutrient agar containing the tested pathogen. The plates were incubated overnight at 37°C, with the exception of *B. subtilis* at 30°C. The next day, zones of inhibition were noticed and recoded.

### DNA extraction and amplification of 16S rRNA, *atpD*, *recA*, and *rpoB* genes

The TMM19 strain was grown at 30°C for 7 days on a rotary shaker (250 rpm) in a 50 ml falcon tube containing 20 ml of ISP1 broth medium. A pre-lysis lysozyme-based cell wall digestion step was included by harvesting and resuspending the mycelium in 500 µl of lysozyme buffer (50 mg/ml) and incubating at 37°C for 90 min with occasional vortexing. Then, the protocol of Wizard® Genomic DNA Purification Kit (Promega, USA) was followed for DNA extraction. Polymerase chain reaction (PCR) amplification of genes 16S rRNA, *atpD*, *recA*, and *rpoB* from genomic DNA of *S.* sp TMM19 isolate was performed following the Promega PCR Master Mix protocol, using 1µl of DNA template, 2µl of 10µM of the primers listed in **Table 1**, 25µl PCR Master Mix (2X) including 1µl of Dimethyl Sulfoxide Solution (DMSO) for a total volume of 50µl. PCR amplification consisted of initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 45 seconds, annealing at 55°C for 1 min, extension at 72°C for 1 min, ending with a final extension at 72°C for 10 min.

**Table 1**. List of primers utilised for PCR amplification indicating the gene, sequence and reference.

| Gene | Primer | Sequence | Reference |
|---|---|---|---|
| 16S rRNA | 27F<br>1492R | 5'-AGAGTTTGGATCMTGGCTCAG-3'<br>5'-CGGTTACCTTGTTACGACTT-3' | Goodfellow et al., 2007 |
| *atpD* | atpD PF<br><br>atpD PR | 5'-GTCGGCGACTTCACCAAGGGC AAGGTGTTCAACACC-3'<br><br>5'-GTGAACTGCTTGGCGACGTGGGTG TTCTGGGACAGGAA-3' | Guo et al., 2008 |
| *recA* | recA PF<br><br>recA PR | 5'-CCG CRC TCG CAC AGA TTG AAC GSC AAT TC-3'<br><br>5'-GCS AGG TCG GGG TTG TCC TTS AGG AAG TTG CG-3' | Guo et al., 2008 |
| *rpoB* | Srpo F<br>rpoB PR | 5'-TCG ACC ACT TCG GCA ACC GC-3'<br>5'-CCT CGT AGT TGT GAC CCT CCC ACG GCA TGA-3' | Labeda et al., 2012 |

### Sequencing of 16S rRNA, *atpD*, *recA* and *rpoB*, and construction of phylogenetic trees

Gel electrophoresis (1% agarose, 90V for 45 min, 1kb DNA Step Ladder (Promega, USA)) was run and the corresponding 16S rRNA, *atpD*, *recA* and *rpoB* bands removed from the gel by visualising with UV transilluminator. The fragments were weighed (400mg), purified (Wizard® SV Gel and PCR Clean-Up System, Promega) quantified with NanoDrop, then sent for sequencing along with primers used in PCR amplification (Eurofins Genomics, Germany). The 16S rRNA, *atpD*, r*ecA* and *rpoB* gene sequences were returned in FASTA format. The sequences were used in a nucleotide BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1990) search ('blastn' algorithm, default parameters). The top 19 homologous sequences (whole genome sequences) to *atpD*, *recA* and *rpoB* of *Streptomyces* sp. TMM19 were retrieved from the National Center for Biotechnology Information (NCBI) public database in FASTA format (**Table 2**).

**Table 2.** List of the atpD, recA and rpoB gene sequences that showed similarity with *Streptomyces* sp. TMM19

| Gene | Strain | Accession No. | Identity / 16S |
|---|---|---|---|
| *atpD* / 16S | *S.* sp. ETH9427 | CP029624.1 | 98.57% / 98.06% |
| | *S.* sp. 4F | CP013142.1 | 98.24% / 98.21% |
| | *S.* sp. WAC 01438 | CP029601.1 | 98.54% / 98.06% |
| | *S. glaucescens* | CP009438.1 | 97.79% / 98.58% |
| | *S. fungicidicus* | CP023407.1 | 97.53% / 98.06% |
| | *S. pactum* | CP019724.1 | 96.58% / 97.61% |
| | *S.* sp. CB09001 | CP026730.1 | 96.36% / 97.76% |
| | *S.* davawensis | HE971709.1 | 96.25% / 95.96% |
| *recA* / | *S.* sp. KPB2 | CP034353.1 | 97.06% / |

| | | | |
|---|---|---|---|
| 16S | | | 97.76% |
| | *S.* sp. CCM_MD2014 | CP009754.1 | 96.94% / 97.76% |
| | *S. olivaceus* strain KLBMP | CP016795.1 | 96.23% / 97.61% |
| | *S. actuosus* strain ATCC 25421 | CP029788.1 | 96.11% / 98.13% |
| | *S. parvulus* strain 2297 | CP015866.1 | 95.88% / 97.61 |
| *rpoB* / 16S | *S. leeuwenhoekii* | LN831790.1 | 97.22% / 98.21% |
| | *S. cyaneogriseus* strain NMWT 1 | CP010849.1 | 97.22% / 98.21% |
| | *S. puniciscabiei* strain TW1S1 | CP017248.1 | 96.49% / 97.48% |
| | *S.* sp. ZFG47 | CP030073.1 | 95.52% / 98.73% |
| | *S.* sp. Z022 | CP033073.1 | 95.16% / 97.63% |
| | *S. nigra* strain 452 | CP029043.1 | 94.92% / 98.06% |

To obtain the allele sequences corresponding to the 16S rRNA, *atpD*, *recA* and *rpoB* genes, the 19 whole genome sequences were then uploaded to the MLST 2.0 web server (www.genomicepidemiology.org). The allele sequences for each related strain were retrieved from MLST 2.0 web tool in FASTA format and aligned using MEGA7 software (Tamura, et al., 2013) with MUSCLE (Codons) alignment algorithm. A neighbour-joining phylogenetic tree for each gene or combined genes was constructed using MEGA7 tool from the aligned sequences (Hall, 2013). Parameters used for construction included 'bootstrap' test of phylogeny (1000x replicates), *p*-distance model/method, and 'complete deletion' for Gaps/Missing Data Treatment. All other parameters remained as default.

## RESULTS AND DISCUSSION

### Characterisation with ISP media

The International *Streptomyces* Project (ISP) depicts a series of standardised media for use in the characterisation of novel *Streptomyces* isolates (Shirling & Gottlieb, 1966). ISP media ISP2, ISP3, ISP4, ISP5, ISP6, and ISP7 were inoculated with *Streptomyces* sp. TMM19 and the phenotype recorded (**Figure 1**). All the media presented considerable growth with abundant sporulation, with ISP2, ISP3 and ISP4 being the most prolific media. The isolate exhibited different colours of aerial mycelia that varied from antique pink (RAL 3014) on ISP2, brown red (RAL 30110) on ISP3 to bright red orange (RAL 2008) on ISP5. Moreover, soluble night blue (RAL 5022) pigment was observed on ISP4 medium. No melanin production was observed on both ISP6 and ISP7 media. The cultural characteristics of the *S.* sp. TMM19 were consistent with description of the genus *Streptomyces* reported in previous studies (Shirling & Gottlieb, 1966; Zhao et al., 2006; Antony-Babu et al., 2010). These studies also stated that ISP2, ISP3, ISP4 and ISP5 culture media were especially valuable for colour determination of *Streptomyces* isolates.

The ISP description allows distinction between our isolate and other strains of *Streptomyces*. For example, the recently described species *Streptomyces humi* was found to have positive growth on all ISP standard media ISP2-ISP7 except ISP4 (Zainal, *et al*. 2016). Another study described a novel species of *Streptomyces*, *S. glaucescens* NEAE-H, which was able to produce melanin pigment on ISP6 and ISP7 (El-Naggar & El-Ewasy 2017). In comparison, *S.* sp TMM19 was found to have strong growth on all ISP media with no melanin production on ISP6 and ISP7.

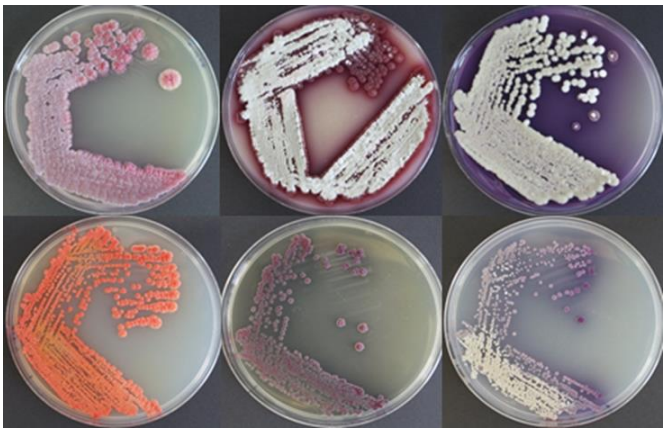

**Figure 1**. ISP media inoculated with *Streptomyces* sp. TMM19. Top Row: ISP2, ISP3 and ISP4. Bottom Row: ISP 5, ISP6 and ISP7

### Antimicrobial bioactivity

The *S.* sp. TMM19 strain displayed a different bioactivity depending on the media they were grown on. As illustrated in **Table 3**, TMM19 strain presented activity against *S. aureus*, *E. faecalis* and *B. subtilis* when grown on ISP2, while was active against *S. aureus*, *E.* and *B. subtilis* when grown on ISP3, ISP4 and ISP5. At the same time no activity was observed when the isolate grown on ISP6 and ISP7. The inhibition of the growth of three different species of bacteria (*S. aureus*, *E. faecalis* and *B. subtilis*), could suggest that the TMM19 strain produce either more than one antibiotic compounds, or one compound with several microbial targets. However, the fact that the three positive indicator microorganisms are Gram-positive bacteria, and no Gram-negative bacteria were inhibited, may imply a common mechanism of action towards this type of organisms. Previous findings noted that Gram-positive bacteria are significantly more sensitive to metabolites expressed by streptomycetes than Gram-negatives (Scherrer & Gerhrdt 1971; Bouras et al., 2013; Aouiche et al., 2014).

**Table 3**. Antimicrobial activity of *Streptomyces* sp. TMM19 against tested Gram positive and Gram negative bacteria.

| | **A** | **B** | **C** | **D** | **E** | **F** | **G** |
|---|---|---|---|---|---|---|---|
| ISP-2 | - | + | - | - | - | + | + |
| ISP-3 | - | + | - | - | - | - | + |
| ISP-4 | - | + | - | - | - | - | + |
| ISP-5 | - | + | - | - | - | - | + |
| ISP-6 | - | - | - | - | - | - | - |
| ISP-7 | - | - | - | - | - | - | - |

A=*E.coli*, B=*S. aureus*, C=*K. pneumonia*, D=*A. baumannii*, E=*P. aeruginosa*, F=*E.faecalis*, G=*B. subtilis*

The isolates was observed to express different inhibition patterns of bioactivity depending on the media that was cultured in, fact that is consistent with previous findings stating the impact of the cultivation conditions, like varying sugar (carbon) sources, on the

antibiotic production (Bode et al., 2002; Christian et al., 2005; Chai et al., 2012). In this regard, TMM19 strain was found to produce a bioactive secondary metabolite when grown on ISP2, ISP3, ISP4 and ISP5.

**Phylogenetic trees**

A Neighbour-Joining phylogenetic tree was constructed for *S.* sp. TMM19 using its 16S rRNA sequence to demonstrate its homology to other *Streptomyces* 16S rRNA sequences that retrieved from the whole genome sequences. *Streptomyces* sp. ZFG47 and *Streptomyces actuosus* strains were most phylogenetically related to *S.* sp. TMM19 strain based on the 16S rRNA genes tree (**Figure 2**).

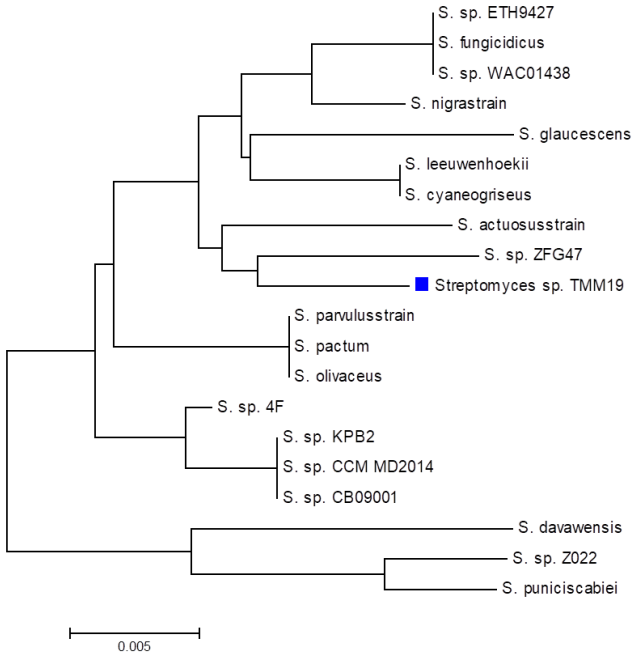


**Figure 2.** Neighbour-Joining phylogenetic tree (1000 bootstrap) constructed using MEGA7 tool from 19 16S rRNA sequences homologous to that of *Streptomyces* sp. TMM19 (indicated by blue square).

A multi locus sequence analysis (MLSA) was performed using the nucleotide sequences of the protein-coding genes genes *atpD, recA* and *rpoB*, in order to study the phylogenetic relationship among S. sp. TMM19 and the 19 *Streptomyces* strains. Multiple housekeeping gene sequences were merged and aligned to form a neighbour-joining phylogenetic tree illustrated in **Figure 3**. The tree showed that *Streptomyces* sp. Z022 and *Streptomyces puniciscabiei* strains were the most related strains to *S.* sp. TMM19 isolate.

The phylogenetic tree constructed from the concatenated sequences of the 16S rRNA and the three protein-coding genes genes *atpD, recA* and *rpoB* showed that S. sp. TMM19 was again most related to *Streptomyces* sp. ZFG47 and *Streptomyces actuosus* strains along with *Streptomyces nigra, Streptomyces glaucescens, Streptomyces leeuwenhoekii and Streptomyces cyaneogriseus* strains (**Figure 4**).

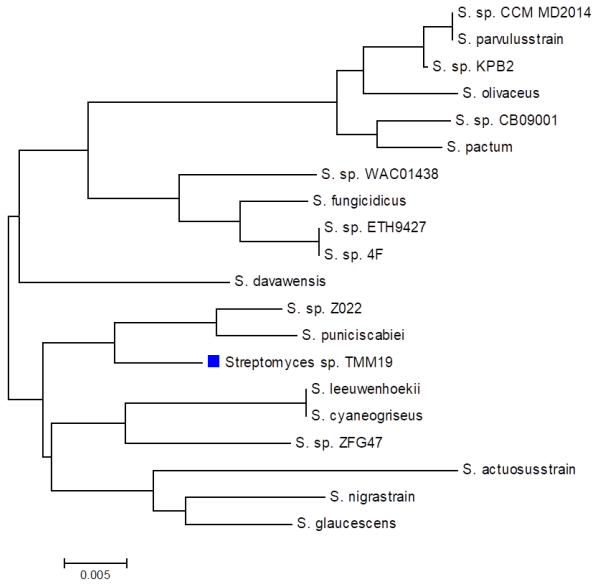


**Figure 3**. Neighbour-Joining phylogenetic tree (1000 bootstrap) constructed using MEGA7 tool from 19 merged nucleotide sequences of *atpD, recA* and *rpoB* genes homologous to that of Streptomyces sp. TMM19 (indicated by blue square).

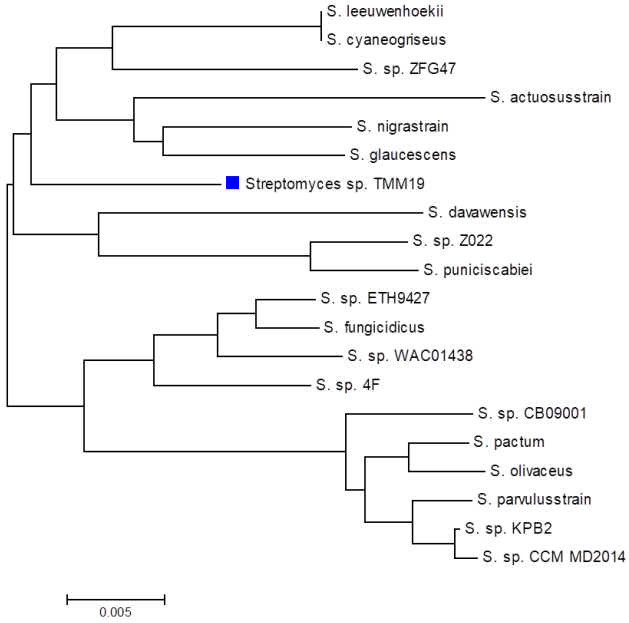


**Figure 4.** Neighbour-Joining phylogenetic tree (1000 bootstrap) constructed using MEGA7 tool from 19 merged nucleotide sequences of genes 16S rRNA, atpD, recA and rpoB homologous to that of Streptomyces sp. TMM19 (indicated by blue square).

It can be seen that although the 16S rRNA analysis tree did not correlate with the results obtained from the MLSA tree as *S.* sp. TMM19 was close to *Streptomyces* sp. ZFG47 and *Streptomyces actuosus* strains in the 16S gene tree while it was close to *Streptomyces* sp. Z022 and *Streptomyces puniciscabiei* strains in the MLSA tree, *Streptomyces* sp. TMM19 was well separated in both the 16S rRNA and the protein-coding gene trees. In contrast,

the 16S rRNA tree and the merged 16S rRNA and the three protein-coding genes tree showed a tree classification correlation, this might be explained by the close relation of the isolates since strains with a DNA similarity of 70% or greater tend to exhibit more than 97% of 16S rRNA gene sequence similarity (Stackebrandt & Goebel 1994). However, MLSA genes yield higher resolution for intra-species relatedness compared to 16S rRNA gene-based methods. For instance, concatenation of the sequence of the gene coding for the β-subunit of DNA *gyrB* showed 98 to 100% similarities of strains of 18 species (Hatano et al., 2003). Moreover, researchers have involved MLSA results, such as the *atpD, gyrB, recA, rpoB* and *trpB* genes, as a new and a reliable method for streptomycetes taxonomy (Rong et al., 2010; Rong and Huang, 2012; Labeda et al., 2012). Currently, MLSA is widely used method to infer bacterial phylogeny (Tsang et al., 2016; Ferres and Iraola, 2018).

Nevertheless, the bacterial 16S ribosomal RNA gene is the most conserved DNA that shows considerable sequence diversity among different bacteria (Chakravorty, 2007). Also, 16S rRNA gene has been demonstrated to be a robust molecular tool for positioning and clustering of the streptomycetaceae family and illustrating their diversities within the phylogenetic tree (Chun & Goodfellow, 1995; Labeda & Kroppensted, 2000; Kumar et al., 2007; Labeda et al., 2012). However, this procedure is not always helpful for species differentiation and certainly not for delineation of strains within the same species (Guo et al., 2008).

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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