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Phylogenetic Analysis of *Streptomyces* spp. Exhibited Different Antimicrobial Activities

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Abstract

Fourteen isolates were collected from a previous study and all were assigned to be *Streptomyces* genus, according to physiological and biochemical tests, however all the isolates varied morphologically and exhibited different antimicrobial activity. All 14 isolates were confirmed *Streptomyces* by *16S rRNA* PCR amplification. Six isolates with high antimicrobial activities were ascertained *Streptomyces* spp. by sequencing and phylogenetic analysis. Two isolates among the selected 6 isolates with antimicrobial activity against *E. coli* and *S. aureus* . It recommended to make a complete sequence for *16S* rRNA to detect the species that produce antimicrobial substances, also it's very necessary to identify the natural structure of these product.

Keywords: *Streptomyces*, phylogeny, antimicrobial activity and biomass

تحليل النسل لانواع الستربتومايسس المنتجة للمضادات الميكروبية ذات الفعاليات المختلفة

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الخلاصة

تم الحصول على 14 عزلة من دراسة سابقه والتي تم تشخيصها على انها جنس ال *Streptomyces*اعتمادا على الفحوصات الفسيولوجية والبايوكيميائية.اظهرت العزلات اختلاف بالشكل وبالفعالية المضادة للميكروبات. شخصت العزلات جزيئياباستخدام تقنية تفاعل انزيم البلمرة المتسلسل واكدت النتائج ان 14 عزله تابعه لجنس *Streptomyces*. ست عزلات اثبتت انها تابعه لجنس spp. *Streptomyces* ذات الفعالية المضادة للميكروبات باستخدام تحليل تسلسل القواعد النتروجينيه وتحليل النسل, كما اظهرت عزلتان من بين الست عزل فعالية عالية ضد بكتريا ال *E. coli* و*S. aureus* .

Introduction

The genus *Streptomyces* includes aerobic, Gram-positive, filamentous bacteria which produce well developed vegetative hyphae with branches and has a linear chromosome [1-3]. Streptomycetes are characterised by a complex secondary metabolism [4].

They produce over two-thirds of the clinically useful antibiotics of natural origin e.g. neomycin, cypemycin, grisemycin, bottromycins and chloramphenicol [5]. The now uncommonly used streptomycin takes its name directly from *Streptomyces*. Conventional taxonomy of *Streptomyces*, depend on characteristics of mycelial morphology, pigment production, and certain physiological properties. Taxonomic classification is available from the Global Biodiversity Information Facility showed that *Streptomyces* is the largest genus of Actinobacteria and the type genus of the family Streptomycetaceae [6]. International *Streptomyces* Project (ISP), proposed in 1964, an elaborate description of some strains of *Streptomyces* and their related taxa, which is dependent on a limited number of taxonomic characteristics selected from those accumulated in conventional taxonomic tests [7, 8].

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In 1983, a large-scale numerical phenetic survey of *Streptomyces* and related taxa was carried out. [9]. Over 500 species of *Streptomyces* bacteria have been described [10], species nomenclature are usually based on their color of hyphae and spores. *Streptomyces* are assigned to this class on the basis of their chemotaxonomy, their high G+C context and the similarities in the sequences of their 16S ribosomal encoding gene [11]. Molecular biology studies has increased for species classification, like ribosomal RNAs (*16S rRNA*s), which has been successfully applied to determine the phylogenetic relationships in bacteria [12]. Masakazu *et al*. [13] reported that in case of *Streptomyces*, part of their partial rDNA sequences are useful for the rapid identification of species. The aim of the work is to identify the *Streptomyces* spp. using the *16S rRNA* sequencing which previously tested to find their antimicrobial activities against multidrug pathogenic clinical isolates, the highest antimicrobial activities isolates were subjected to a phylogenic analysis, this due to imperative need for research and development of new antibiotics to resolve the problems which increase or contribute to resistance due to self-medication, overuse of antibiotics and reducing the lifetime of the antibiotic leading to infectious diseases.

Materials and Methods

Collection of samples

Fourteen isolates were obtained from a previous study, isolated from soil samples (data not shown), tested to find their antimicrobial activities against eight different clinical isolates : *P. aeruginosa, Candida* spp., *Serratia* spp. *E. coli, S. aureus, K. pneumonia* and *Proteus* spp. and *S. typhimurium* (All were obtained from the Al- Nahrain college of medicine and from biotechnology department\ College of Science).

Characterization of the *Streptomyces* isolates

Streptomyces colonies were characterized morphologically and physiologically according to the methods described by the International *Streptomyces* Project (ISP) [7].

Screening of isolates for antagonism

Shake flask fermentation was carried out in 250 ml shake flasks containing 30 ml of production medium (containing 20 grams of soybean flour and 20 grams of mannitol/ 1 L tap water) and shaken for 72h at 28ºC in pH 7.0 and inoculated by 1 ml of spore inoculums. Supernatant were collected from each isolates by centrifugation at 10,000 rpm\2. From each pathogenic bacteria 100 microliter was mixed with 20 ml of sterilized muller-hinton agar at 45C, mixed well, poured in a sterile petri dish. After solidification three to four well formed in each plate, 200 microliter of each supernatant was poured in each well, incubated at 37 C overnight [14].

DNA Extraction

Fourteen *Streptomyces* isolate were cultured in 30 mL of YMG medium (yeast extract 4 g/L, malt extract 10 g/L, glucose 4 g/L) supplemented with 0.5% (w/v) glycine for 46 h with shaking at 28°. Cells were harvested by centrifugation (5 min, 4000× *g*), washed [2× 10 mL of 10% (w/v) sucrose] as described by [15]. Genomic DNA extraction and purification carried out by HiPura (HiMedia), Lysozyme (10 mg) (Sigma, USA) was added to the bacterial suspension and incubated at 37°C for 20 min The pellet was resuspended with the lysis buffer and incubated at 37˚C for 30 min then loaded to extraction cartridge, DNA was eluted by 50 µl elution buffer (Provided by manufacturing company). The DNA samples measured for their concentration and purity using Microvolume UV Spectrophotometer (ACTGene, USA).

DNA sequencing and inseleco

The primers *16S rRNA* F: **5' TCACGGAGAGTTTGATCCTG 3'** and *16S rRNA* R: **5' GCGGCTGCTGGCACGTAGTT 3'** were used for identification of *Streptomyces* spp. as reported by Masakazu *et al*. [**13**]. PCR was performed in a 50 µl mixture containing 1× PCR buffer (10 mM Tris–HCl, 1.5 mM MgCl2, 50 mM KCl [pH 9]) (Merck, India), 100 µM (each) deoxynucleoside triphosphates, 1 U of Taq DNA polymerase (Merck, India), 10 pM each of forward and reverse primers, and 100 ng of templet DNA. The program for PCR included an initial denaturation 94°C for 5min, 30-40 cycles of denaturation at 97°C for 30s, annealing at 50°C for 1min, extension at 72°C for 1min and a final extension at 72 °C for 7min. The PCR products were loaded on a 1.5% agarose gel, stained with ethidium bromide (5ng/ml) and bands observed using a gel documentation system (ATTA, Japan). PCR products were sent for sequencing at Bioneer, Korea. The obtained sequence was compared for similarity with sequences present in the genomic database banks, using the "NCBI

Blast" program available at the ncbi.nlm.nih.gov web site and highest matching sequences downloaded.

Phylogenetic analysis

Phylogenetic analysed along with the sequences of closely related reference organisms (*S. ambofaciens*) retrieved from the EMBL/GenBank (https://www. ncbi.nlm.nih.gov/genbank/). The partial *16S rDNAs* sequence of selected isolates were aligned using the Multiple Sequence Alignment/ ClustalW2 program [16]. Phylogenetic analyses were performed using programs from the PHYLIP package and a phylogenetic tree was constructed by the neighbor joining (NJ) algorithm [17] using Kimura 10-parameter distance. The robustness of the inferred tree was evaluated by bootstrap (100 replications).

Results and Discussion

The emergence of antibacterial resistance is a serious problem worldwide and new antibiotics are necessary to fight multidrug resistant pathogens [18]. The bacterial resistance needs for the inquest of new antimicrobials instead of the ineffective antimicrobials [19]. Actinomycetes have been recognized as source of several secondary metabolites like antibiotics which make them useful as antagonistic agents against pathogens [20, 21]. In screening for *Streptomyces* production of the antimicrobial metabolites in shake flasks, the mycelial extract Figure-1 of all *Streptomyces* spp. isolates growth has been shown as small, colored pellets, six isolates were selected from 14 *Streptomyces* isolates, they showed high antimicrobial activities against 8 pathogenic isolates. Isolates S1and S4 showed high activities against both MDR Gram positive (*S. aureus*) and MDR Gram negative bacteria (*E.coli*) with zones of inhibition ranging between (11-24) mm. Figure-2, whears a study by Ashokkumar *et al.* [18] revealed that most of the active actinomycetes isolates were active against gram positive bacteria *S. aureus* than gram negative bacteria, and one actinomycetes isolate among 5 has the highest antibacterial activity against *S. aureus* and *E.coli*, this explained by the presence of clusters of antibiotic-biosynthetic genes in actinomycetes range in size from about 20 kb to 100kb. Even though a given strain can make several different antibiotics. However, many other genes are probably involved more indirectly in modulating antibiotic production [22].

Figure 1-Secondary metabolites production. Shake flask fermentation for *Streptomyces* isolates was carried out in 250 ml flasks containing 30 ml of production medium (SM broth /soybean flour and mannitol / pH 7) and shaken at 180 rpm for 72h at 28ºC.

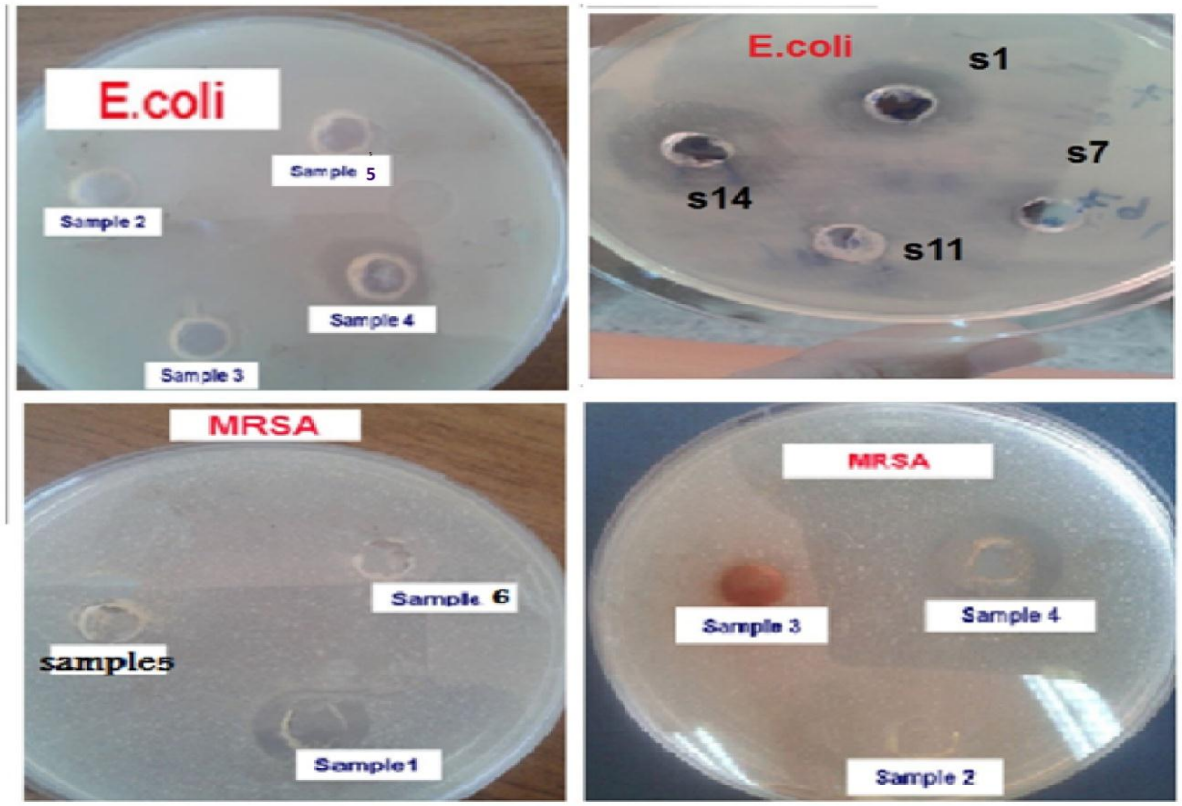

Figure 2- Detection the antimicrobial activities of *Streptomyces* spp. against pathogenic bacteria. (*E .coli* , *S. aureus*), cultured in muller-hinton agar at 37C for 18 hrs.

PCR technique and DNA sequencing

On the basis of the aerial and substrate mycelia, development of spiral spore chains and smooth spore surface of the isolates, they are placed under the genus *Streptomyces*. The taxonomic identity was ascertained as *Streptomyces* spp. by PCR and sequencing of *16S rDNA* as reported by Kandhasamy and Sun [23]. Hence the isolates were designated as *Streptomyces* spp. After recovering 500-bp from each of the 14 isolates by partial amplification of *16S rDNA*, variable region according to the *S. ambofaciens* as described by Masakazu *et al*., [16] Figure-3. In addition to sequences identity as shown in Figure-4. PCR and DNA sequence partially encompassing the *16S DNA* gene in species detection was also used by Xuechang *et al.* [24].

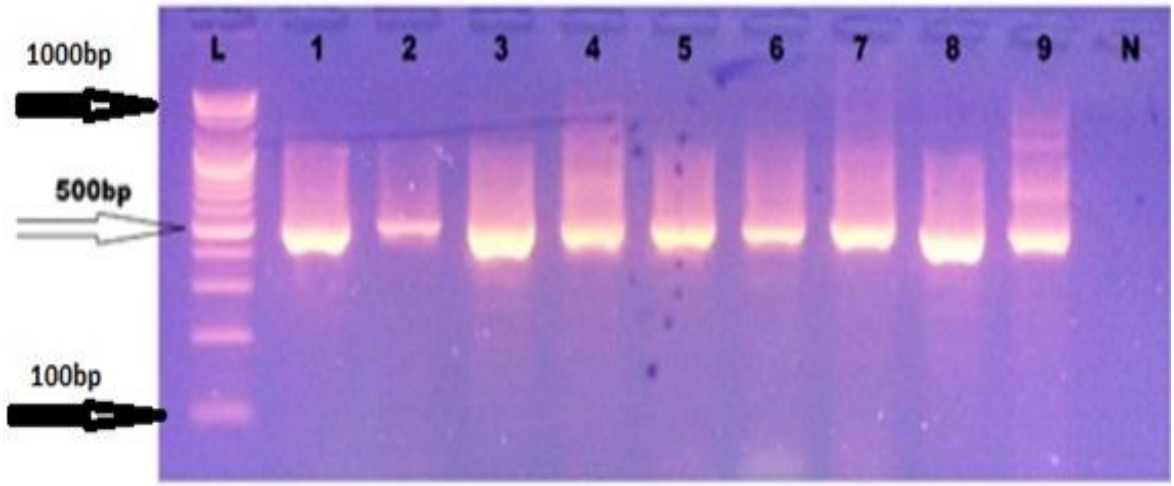

Figure 3- Agarose gel electrophoresis of 500bp specific PCR product for *16S rRNA* using 1.5% agarose gel at 90V for 1hr. in 1x TBE buffer, and visualized under transilluminator UV after staining by ethidium bromide. Lane L: 100 bp DNA ladder, lane (1,2,3,4,5,6,7,8, 9): positive result, lane N: negative control.

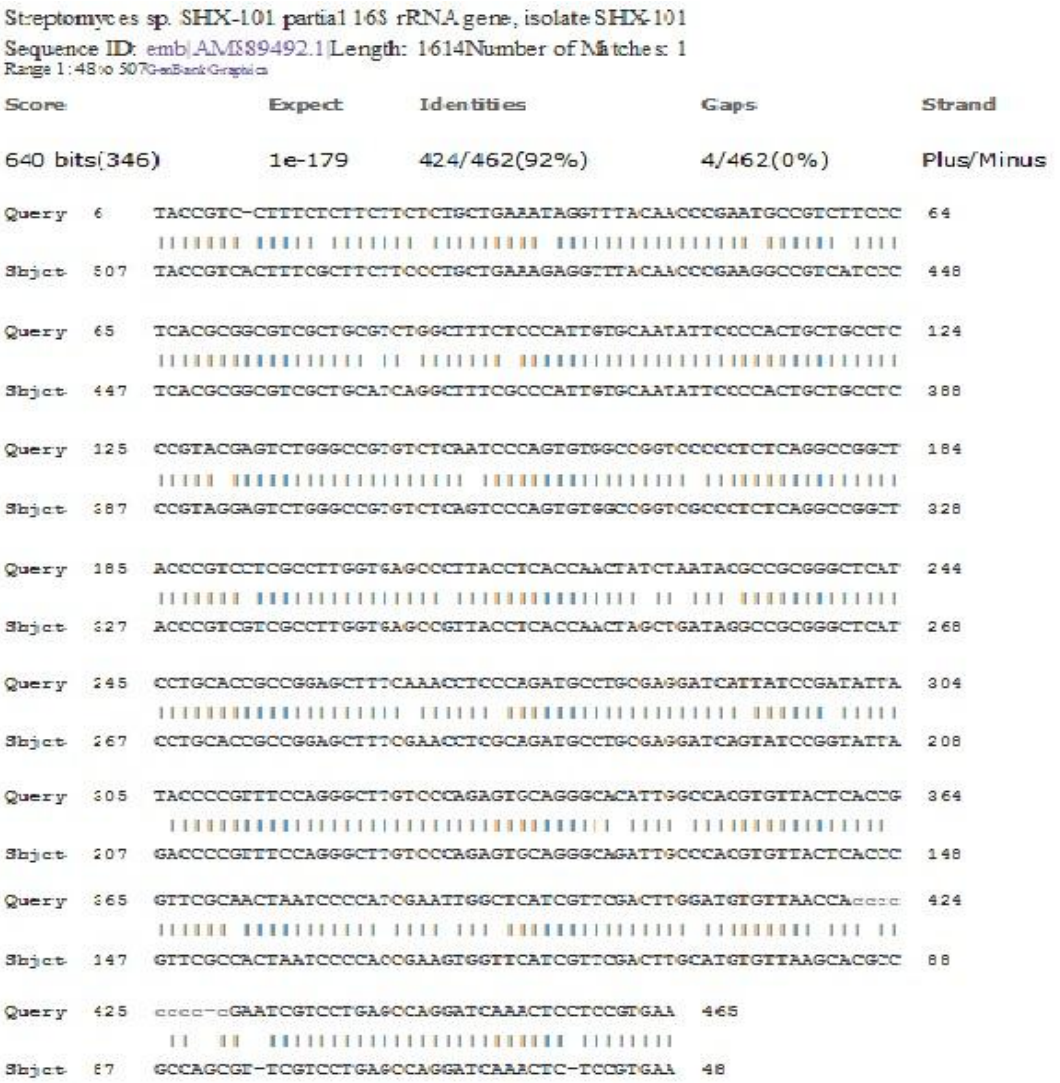

Figure 4- Sequences producing significant alignments.

Phylogenetic relationship through the partial sequence

To clarify the phylogenetic relationships of the six *Streptomyces* isolates among the isolates with high antimicrobial activities, the 500 bp sequences of the *16S rRNA* were subjected to phylogenetic tree analysis using ClustalW2 Figure-5, otherwise a study by Eqan *et al.*, [25] showed that using *trpB* (a housekeeping gene involved in tryptophan biosynthesis), also useful in determining intra-generic relationships within the genus *Streptomyces*. The Iraqi isolates found into 2 clusters (I and II). The phylogeographic grouping of isolates (1S, D1, 2S) was strongly associated with geographic origin of the strains in east Asian which aligned with separated cluster which contain strains from (Japan, China, Pakistan). The strains (D2, D3, D4) accumulate cluster II, showed more genetic variation than cluster I, this finding disagree with a study by [26], which reported that *Streptomyces* genes exhibited lower levels of *16S rRNA* sequence similarity with *Streptomyces* spp. The nineteen global strains in comparison to only six Iraqi isolates give a wide scale of area to show the exact position of the isolates in the phylogenic tree, like that even with one strain like a study by Kandhasamy and Sun [23] , they showed the position of one *Streptomyces* spp. (AM-S1) in comparison to huge related organisms in a *16S rRNA* gene tree.

From the present study, one can conclude that soil samples provided in vitro a good source of antagonistic activity actinomycetes against human pathogens. The morphological and *16S rDNA* sequencing and phylogeny, clearly demonstrated that all 14 isolates are *Streptomyces* spp. It recommend a further studies to the antibiotics producing genes and their relations within the lifestyle genes in Streptomyces spp. in addition to whole genomic sequence for the Iraqi isolates, further work

on optimization of these products, mechanism of action and evaluation to control infection especially for the six Streptomyces spp. which had the strongest inhibitory effects.

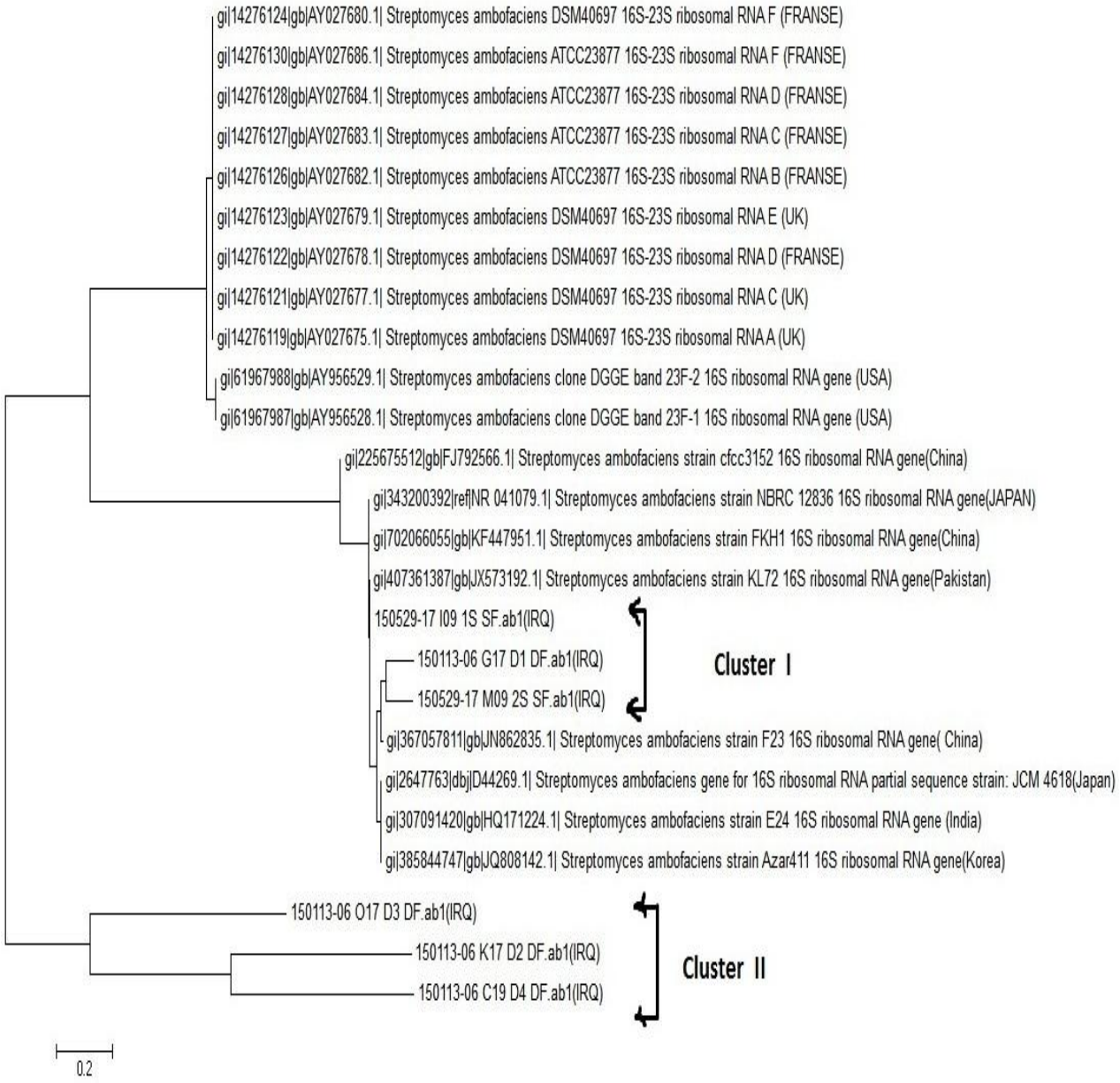

Figure 5- Neighbor – joining phylogenetic analysis based on the sequences of *16S rRNA* of *Streptomyces* spp. Isolates of Iraq and sequences of strains reported from different parts of the world available in public database GenBank (https://www. ncbi.nlm.nih.gov/genbank/).

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