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Genetic Diversity among Plant Pathogenic *Streptomyces* Strains from Potato Fields in Northwest of Iran

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ABSTRACT

Different species of *Streptomyces* are common in most types of soil. Some certain species are plant pathogenic especially on potato. This study was conducted to evaluate genetic diversity among some local *Streptomyces* spp. strains isolated from soil and infected tubers in potato farms in northwest of Iran. *Repetitive DNA elements* (BOX, ERIC and REP) as genetic markers were used for diversity studies and distance matrix was performed by UPGMA method. The cluster analysis showed 8, 5 and 7 distinct clusters with 85% similarity levels in ERIC, BOX and rep markers, respectively. Genetic clusters were moderately geographical region-depended but no significant correlations were found between clusters and other characteristics such as strains virulence or potato cultivars. Also, a dendogram was constructed from combined results of all three markers which showed four distinct groups at 80% similarity level. A close correlation was observed between strains virulence and these genetic groups. DNA sequencing followed by morphologic assay were used for detection of distinct species. The results demonstrated the existence of a considerable genetic diversity among collected plant pathogenic *Streptomyces* strains in the region. Also, the results suggest that combined results from all three ERIC, BOX and REP markers could be more useful and reliable than from each individual marker.

1. Introduction

Streptomyces species are gram-positive, filamentous soil inhabitant bacteria that are well known for their abilities to produce antibiotics and other secondary metabolites (Loria et al., 2006; Crawford et al., 1993).Common scab is an important disease of potato and taproot vegetables including beets and radishes which are caused by *Streptomyces* species. *Streptomyces scabies* is a dominant pathogenic species causing potato scab (Wanner, 2006). Two other *Streptomyces* species, *S. acidiscabies* and *S. turgidiscabies* can produce similar but weaker symptoms whereas *S. ipomoea* causes soil rot of sweet potatoes (Labeda and Lyons, 1992). Raised, superficial or pitted necrotic lesions on tuber surface are characteristic symptoms of common scab of potato (Loria et al., 1992). It reduces the marketable yield by affecting the exterior quality of the tubers and leads to economic losses (Hiltunen et al., 1992). Conventional methods which are commonly used to study the variability of these bacteria include morphological and physiological approaches.

Molecular techniques are now a rapid and robust avenue to perform species identification

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and genetic diversity studies (Rene´e et al., 2008). One of the most commonly used molecular technique for diversity studies is repetitive PCR (rep-PCR) which uses DNA primers corresponding to repetitive extragenic palindromic (Stern et al., 1984), enterobacterial repetitive intergenic consensus (Hulton et al., 1991), and repetitive BOX (Martin et al. 1992) sequences. The patterns of DNA fingerprinting produced by rep-PCR could be a very useful tool in rapidly determining strain identify and specific genetic diversity (Sadowsky et al., 1996). *rep*-PCR DNA fingerprints of bacterial strains is relatively unique, stable, and reproducible (Sadowsky et al., 1996).

This study was conducted to determine the genetic diversity among the plant pathogenic strains of *Streptomyces* spp. isolated from different locations of the East Azarbaijan and Kurdistan provinces as the major potato-producing regions in the Iran. The aim of this research was to assess the genetic diversity of *Streptomyces* spp. strains for epidemiological and ecological studies in East Azarbaijan and Kurdistan provinces.

2. Materials and Methods

2.1. Sample collection

Plant and soil samples were collected from various potato-producing regions of East Azarbaijan and Kurdistan provinces during 2010-2012 (Fig 1). Bacterial strains were isolated from soil and necrotic tissue of tubers as described by Schaad and co-workers (2001). Potato tubers were washed and surface sterilized in 10% sodium hypochlorite solution. Scab lesions were carefully excised and ground with a Teflon pestle in 1.5mL tubes containing 600 µL sterile water. The soil and tuber extracted suspensions were streaked onto yeast malt extract agar (YME), NPPC water agar (containing nystatin, polymyxin, penicillium, cycloheximide) and Peptone/yeast-extract/iron (PYI) agar (schaad et al. 2001). Plates were incubated at 27°C for 8–10 days and characteristic powdery *Streptomyces* colonies were then picked. Pure cultures were obtained by three repeated cultures of a single colony on yeast malt extract. Isolates were maintained at -20°C as spore suspensions in 20% glycerol**.**

2.2. Pathogenicity assay

To determine pathogenicity and aggressiveness of collected strains, pathogenicity test was performed for all strains according to the method described previously by Loria and co-workers (1992). Tubers were washed and surface sterilized in 10% sodium hypochlorite solution and cores (1.5cm diameter) of pith tissue were removed from the center of the tuber. Cores were sliced and placed on moist sterile filter paper in petri-dishes. Two to four replicate tuber disks were used per treatment. Cultures of strains were grown on Oat Meal Agar (OMA) for 5-7 days at 28°C from the time sporulation. Colonies were inverted onto the tuber disks. All tuber slice assays were repeated three to four times. The tuber slice assay was used to assess the necrotizing ability of *Streptomyces* strains used in media production experiment. Slices were observed for necrosis and collapse of tuber cells under and surrounding the disks.

Pathogenicity on radish seedlings was performed for all strains according to the method previously described (Schaad et al., 2001). Radish seeds were surface disinfected with 0.5% sodium hypochlorite solution for 3 min, and then rinsed several times in sterile distilled water and incubated on 1% water agar to germinate at room temperature. Seeds are then inoculated with 0.1-0.5 ml of *Streptomyces* old cultures grown for 4-6 days in Oatmeal Agar (OMA). Seedlings were grown for 6-10 days at room temperature. Pathogenic strains were caused brown to black lesions and stunting of the seedling and cell hypertrophy. The experiment was performed in duplicate.

2.3 Morphological and physiological test

S. scabies strains were identified according to Schaad and co-workers (2001).YME agar was used to assess colony and spore color. PYI agar was used to determine the production of dark, diffusible melanoid pigments and brownish halos around colonies which were considered as positive results. YME and NPPC water agar cultures were used to determine spore-chain morphology by direct microscopic examinations as described by Shirling and Gottlieb (1996). Spore color and aerial hyphae form and color were used for differentiation of the collected strains.

2.4. DNA extraction

Genomic DNA was extracted from all strains by a modified method of Li and De Boer (1995). For bacterial DNA extraction, the strains were grown for 1-3 days in nutrient broth at 26°C. 1.5ml of cultured media was used to harvest the cells by centrifugation of the broth culture at 3000 g for 3 min and the pellet was suspended in 400 µL sterile distilled water. Then, 400µl of 2X extraction buffer (50mM Tris-HCL, 25mM EDTA, 1% SDS, 10µg/ml proteinase K) was added and incubated at 55°C for 3h, followed by adding 400µl ammonium acetate (7.5 M) and centrifugation at 12500g for 10 min before 750 µL of the supernatant was transferred to another tube. To precipitate DNA, 700 µL of cold absolute isopropanol was added and kept at -20°C overnight. Then, the sample was centrifuged at 12500g for 30 min at 4°C and 1ml 70% ethanol the was added to the resultant pellet and centrifuged to precipitate the DNA. Then, the DNA pellet was dried at room temperature and re-suspended in 100µL of deionized water. Five to 10ng of genomic DNA was used in each PCR (polymerase chain reaction).

2.5. Identification of the strains by PCR

The primer pair StrepF⁄StrepB and ASE3⁄scab2m (Wanner, 2006) were used to identify bacterial strains at genus and species levels, respectively (Table 1). The PCR amplifications were performed using the following cycles: one initial denaturation at 95°C for 2 min, annealing at 50°C for 30 seconds and extension at72°C for 1 min and a final extension at 72°C for 10 min. PCR were carried out in 25µL reaction solution. The samples were electrophoresed on 1.5% agarose gels, stained with ethidium bromide and photographed under UV light.

2.6. Rep, ERIC and BOX-PCR diversity study

To assess genetic diversity among the strains of *S. scabies*, genomic DNA was extracted and used in rep-PCR (include BOX, ERIC and REP molecular marker) analysis (table 1). BOX, REP (REP1 and REP2) and ERIC (ERIC 2F and ERIC 1R) (Rene´e et al., 2008) primers were synthesized by Takapozist company. PCR were carried out in 25µL reaction. PCR amplification was performed with a thermal cycler programmed for the following cycles: one initial denaturation step at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C (except for REP primers, which 40°C was used) for 1 min, and extension at 65°C for 4 min. Then, each amplification (25 µl) was analyzed by running the sample in agarose gel (1%w/v). Gels documentation was carried out by using a charge coupled device camera imaging system.

The comparisons of DNA fingerprint patterns were performed by measurement of band positions of PCR products following electrophoretic separation. The presence or absence of an intense reproducible band at each location within the gel was scored (1=presence, 0=absence) and used to produce a two-dimensional rectangular data matrix of binary values for all strains of interest. The data were normalized, and a similarity matrix was generated using the simple matching similarity coefficient and Unweighted Pair Group Method with Arithmetic (UPGMA) clustering. Similarity matrix of the strains by UPGMA method was performed using NTSYS-pc version 2.1e. In addition, cluster analysis was performed on the combined REP, ERIC, and BOX-PCR banding patterns, then a single dendogram was drawn for all three markers.

2.7. DNA sequencing

After clustering bacterial strain using single and combined results of DNA markers, one type strain from each cluster was selected and the PCR products were sent to sequencing by Takapozist Co., Iran using Sanger sequencing method for further analysis. The results of DNA sequencing were then submitted to NCBI GenBank. Phylogenic relationship between sequenced strains and other bacteria taxa was evaluated using MAGA 6 software.

3. Results

3.1. Bacterial isolation and morphological assay

In total, 42 suspected *Streptomyces* spp. isolates were collected from necrotic lesions of potato tubers and soil (Table 2). All of collected strains showed typical aerial pseudo-hyphae in microscopic observations which mostly had type-II simple and filamentous aerial hyphae with short chain while few strains had long. Colony on YME agar medium has gray and dark yellow pigments (Fig 2).

Table 1. The sequences of used primers in this study for analysis of *Streptomyces* strains

Primer name	Sequence (5'-3')	Reference
StrepF	ACGTGTGCAGCCCAAGACA	Wanner, 2006
StrepB	ACAAGCCCTGGAAACGGGGT	
ASE3	AACGGCCAGAGATGGTCGC	
scab2m	TTCGACAGCTCCCTCCCTTAC	
BOXA1R	CTACGGCAAGGCGACGCTGAC	Rene'e et al., 2008
REP1	IIIICGICGICAICIGGC	
REP2	ICGTCTTATCIGGCCTAC	
ERIC 2F	AAGTAAGTGACTGGGGTGAGCG	
ERIC 2R	CTACGGCAAGGCGACGCTGAC	

Fig 1. Sampling areas shown inside Iran map; 1: East Azarbaijan, 2: Kurdistan

3.2. Pathogenicity and virulence assay

Pathogenicity assay performed on potato slices and radish seedlings resulted in positive reactions in just 35 strains. Most of these positive strains had pathogenicity ability on both potato and radish while few strains showed pathogenicity only on potato or radish. Based on the severity of symptoms, the strains could be divided into three groups; virulent and non-virulent and low virulence (Table 2).

3.3. PCR results

The strains were identified by using specific primers StrepF and StrepB where all the collected strains produced an expected band of 1027bp in agarose gel electrophoresis. Thirty out of forty-two *Streptomyces* strains in East Azarbaijan and Kurdistan gave the product size of 475bp using the primer pair ASE3⁄scab2m in electrophoresis which was molecularly identified as *Streptomyces scabies* (Fig 3 and 4).

3.4. BOX, ERIC and rep-PCR tying

All of the strains were type-able by BOX-PCR, ERIC and rep-PCR fingerprinting method and in total 19 reproducible bands, ranging from 100 to 2000bp, were scored and used for analysis. The similarity of patterns varied from 60 to 95% in BOX-PCR, 70-98% in ERIC and 65-96% in rep-PCR. The genetic clustering showed 8, 5 and 7 distinct groups at 85% similarity level in ERICs, REPs and BOX markers, respectively. Somehow, a close correlation could be seen between these genetic groups, sampling area and virulence though no distinct group could be detectable. But, the constructed dendogram based on the combined results of all three markers showed four distinct groups at 80% similarity level (Fig 5).

Group 1 and 2 were included plant pathogenic *Streptomyces* strains which were detectable with StrepF/StrepB and ASE3⁄scab2m primers confirming that they belong to *Streptomyces scabies*. Group one was totally composed of non-virulent or very weak pathogenic strains while group two –except one strain (GH1)- comprised of only virulent strains of *S. scabies*. DNA sequencing was done on the representative strain of this group (SA2), and the result was submitted to NCBI GenBank under the accession number KT025828. Phylogenetic tree drawn by MEGA6 software for this strain revealed a close relation (99%) between this strain and *S. scabies* strains (Fig 6).

All members of group three were low virulent *Streptomyces* strains which were just detectable by *Streptomyces* universal primers (StrepF/StrepB) but could not produce any band with S. *scabies*-specific primers. Members of group three had common characteristics in possessing very long and flexuous aerial hyphae and lack of any pigments in media which could be indicative of *S. turgidiscabies* though more examination are needed. Strain with code name (B2) was selected as representative for this cluster and its sequence was submitted to NCBI under the accession number KT025829. Phylogenetic clustering also indicated that this selected strain has 99% similarity with *S. turgidiscabies* (Fig 6).

Group four comprised seven totally nonpathogenic strains of *Streptomyces* without any virulence on potato or radish tubers. Some had been collected from soil and some were endophytic to potato tubers. Group four strains had a various types of aerial hyphae from very short to long spore chains. One strain (named H3) was selected from this group and, after sequencing (accession number KT025830), it was shown that it had 99% similarity to *Streptomyces acrimycini* (Fig 6).

4. Discussion

Genetic diversity was studied among some native strains of *Streptomyces* species which were collected from potato farms in northwest of Iran. The results showed a considerable and significant genetic diversity among the collected samples. Several previous studies have also shown that despite the similarities in pathogenicity, strains of *Streptomyces scabies* and other closely- related species are genetically diverse (Doering-Saad et al., 1992; Takeuchi et al., 1996). St-Onge and co-workers (2008) showed that the strains of *S. scabies* in eastern Canada were very diverse and could be separated into 10 identical genetic groups. Those groups were identically related to geography of sampling area but no distinct group was found for each sampling site.

In this study, three ERIC, BOX and rep-PCR genetic markers were used to evaluate the genetic variation among collected strains. Previously, it was demonstrated that repetitive intergenic DNA elements (ERIC, BOX and rep) are very powerful tools for detecting genomic diversity among many types of bacteria and for understanding the ecology and phylogeny of the microorganism (Judd et al., 1993; Versalovic et al., 1994). Likewise, in earlier reports, these three DNA markers have been used for differentiation of *Streptomyces* species (Flores-Gonzalez et al., 2008; Clark et al., 1998). In this study, all ERIC, BOX and rep-PCR analysis were found useful for genetic clustering of *Streptomyces* strains and were partially able to distinguish DNA fingerprinting of strains based on location of isolation of the strains and their virulence. Previous studies have also shown that these markers are able to differentiate *Streptomyces* strains based on their collection area, but no distinct group was distinguishable (St-Onge et al., 2008). Clark and co-workers (1998) also showed rep-PCR is a suitable method for clustering bacteria on the basis of sampling site. In current study, also we used combined results of three markers for data interpretation instead of using them individually. As such, the results were more useable and interpretable so that the *Streptomyces* strains were divided into four separate groups based on their pathogenicity and virulence.

Conclusion

This study identified clear genetic differences between the *Streptomyces* strains by the use of the markers ERIC, BOX and rep-PCR. Results from this study opens up a new avenue for further detailed investigations on the differences which exist between the strains. Because this bacterium is an important plant pathogen and because severity of the related disease usually depends on the type of strain, results from such studies can be very useful as to the control of the incited diseases.

Table 2. Results of biochemical and biological assay on *Streptomyces* strains

Strain No.	Code	Source	Region[1]	Primer 1[2]	Primer 2[3]	Pathogenicity[4]	Virulence[5]
1	SA1	Soil	1	√	-	+	1
2	SA2	Tuber	1	√	√	+	2
3	SA3	Tuber	1	√	√	+	2
4	SA4	Tuber	1	√	√	+	2
5	SA5	Tuber	1	√	√	+	2
6	SA6	Soil	1	√	√	+	1
7	SA7	Soil	1	√	√	+	1
8	SA8	Tuber	1	√	√	+	2
9	SA9	Tuber	1	√	√	+	1
10	B1	Tuber	1	√	√	+	2
11	B2	Tuber	1	√	-	+	1
12	B3	Soil	1	√	√	+	1
13	B4	Soil	1	√	√	+	2
14	B5	Tuber	1	√	√	+	2
15	B6	Tuber	1	√	√	+	2
16	B7	Tuber	1	√	√	+	1
17	B8	Soil	1	√	-	-	0
18	B9	Soil	1	√	√	+	1
19	B10	Tuber	1	√	√	+	2
20	H1	Tuber	1	√	√	+	2
21	H2	Tuber	1	√	-	+	1
22	H3	Soil	1	√	-	-	0
23	DE1	Tuber	2	√	√	+	1
24	DE2	Tuber	2	√	-	+	1
25	DE3	Tuber	2	√	√	+	2
26	DE4	Tuber	2	√	√	+	2
27	DE5	Soil	2	√	-	+	1
28	DE6	Soil	2	√	-	-	0
29	DE7	Tuber	2	√	√	+	2
30	DE8	Soil	2	√	√	+	1
31	DE9	Tuber	2	√	√	+	1
32	DE10	Soil	2	√	-	-	0
33	GH1	Tuber	2	√	√	+	2
34	GH2	Tuber	2	√	√	+	2
35	GH3	Tuber	2	√	√	+	2
36	GH4	Tuber	2	√	√	+	2
37	GH5	Tuber	2	√	√	+	2
38	GH6	Tuber	2	√	√	+	2
39	GH7	Soil	2	√	√	+	1
40	GH8	Soil	2	√	-	-	0
41	GH9	Soil	2	√	-	-	0
42	GH10	Soil	2	√	-	-	0

1. Collected regions; 1= East Azerbaijan, 2= Kurdistan
2. Primer set 1= StrepF/StrepB
3. Primer set 2= ASE3/Scab2m
4. Pathogenicity on potato, radish or both
5. 0=no virulence, 1= low or weak, 2=severe

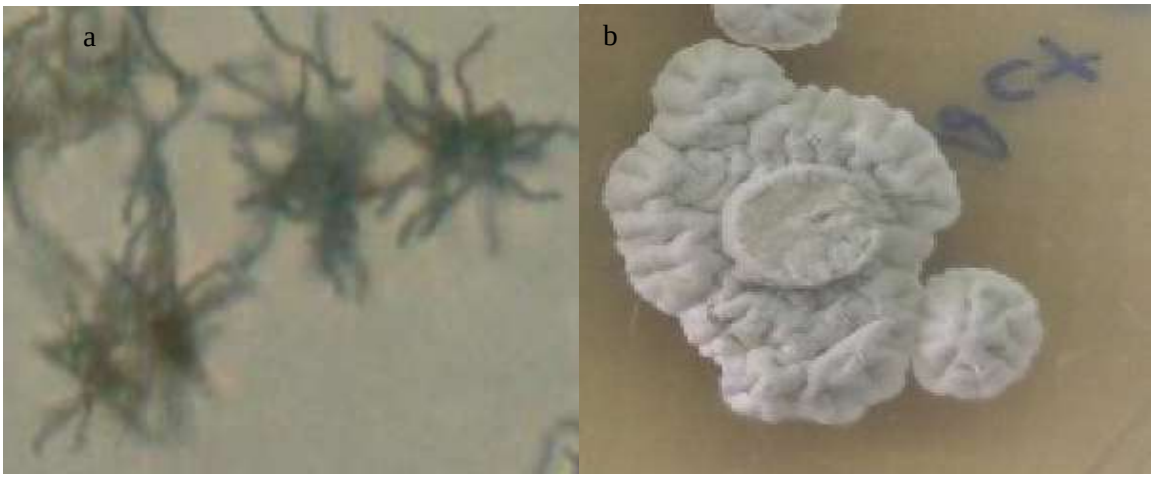

Fig 2. Flexible aerial pseudo-hyphae in the collected strains (a), gray and raised colonies (b)

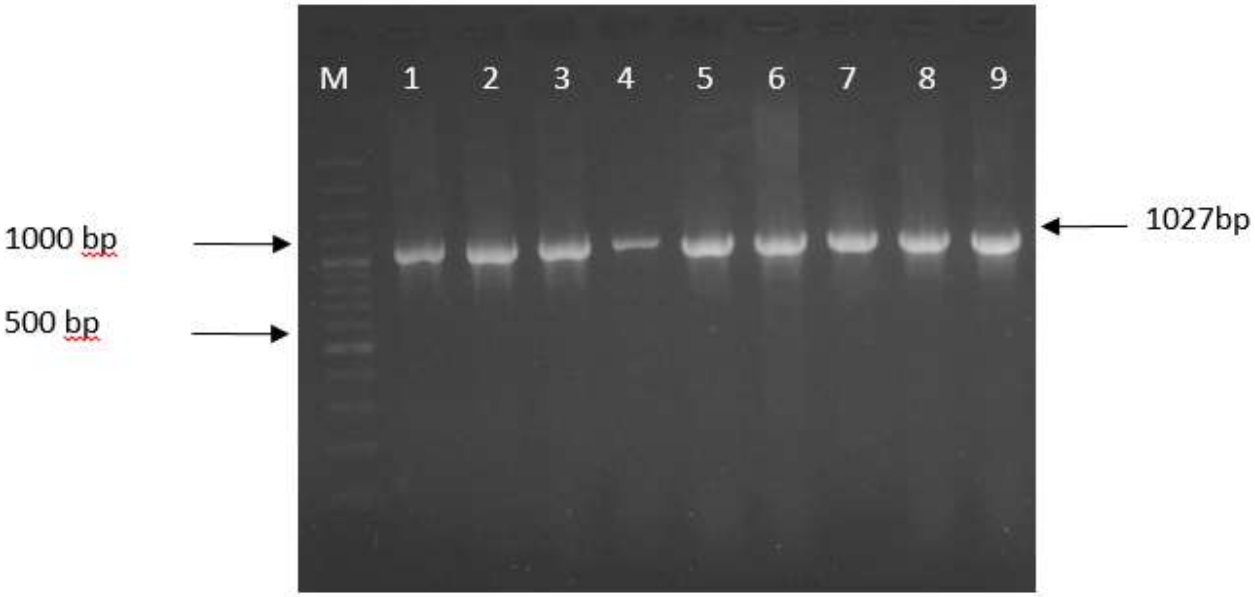

Fig 3. Electrophoresis results for PCR products with StrepF/StrepB primers; M=Molecular Marker, 1-9 wells isolates No. SA2, SA3, GH1, GH2, B1, B3, DE3, DE7 and DE10

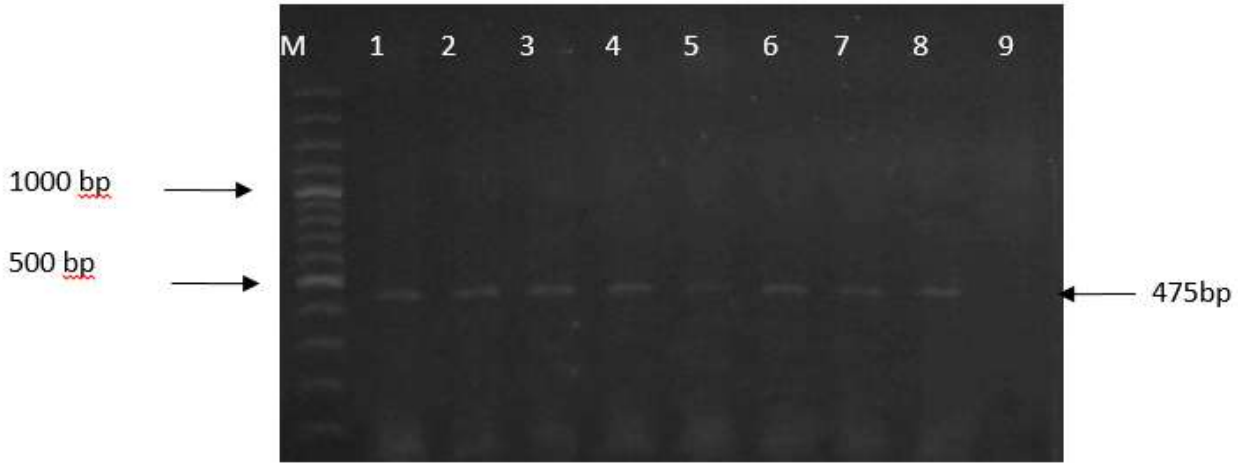

Fig 4. Electrophoresis results for PCR products with ASE3⁄scab2m primers; M=Molecular Marker, 1-9 wells isolates No. SA2, SA3, GH1, GH2, B1, B3, DE3, DE7 and DE10

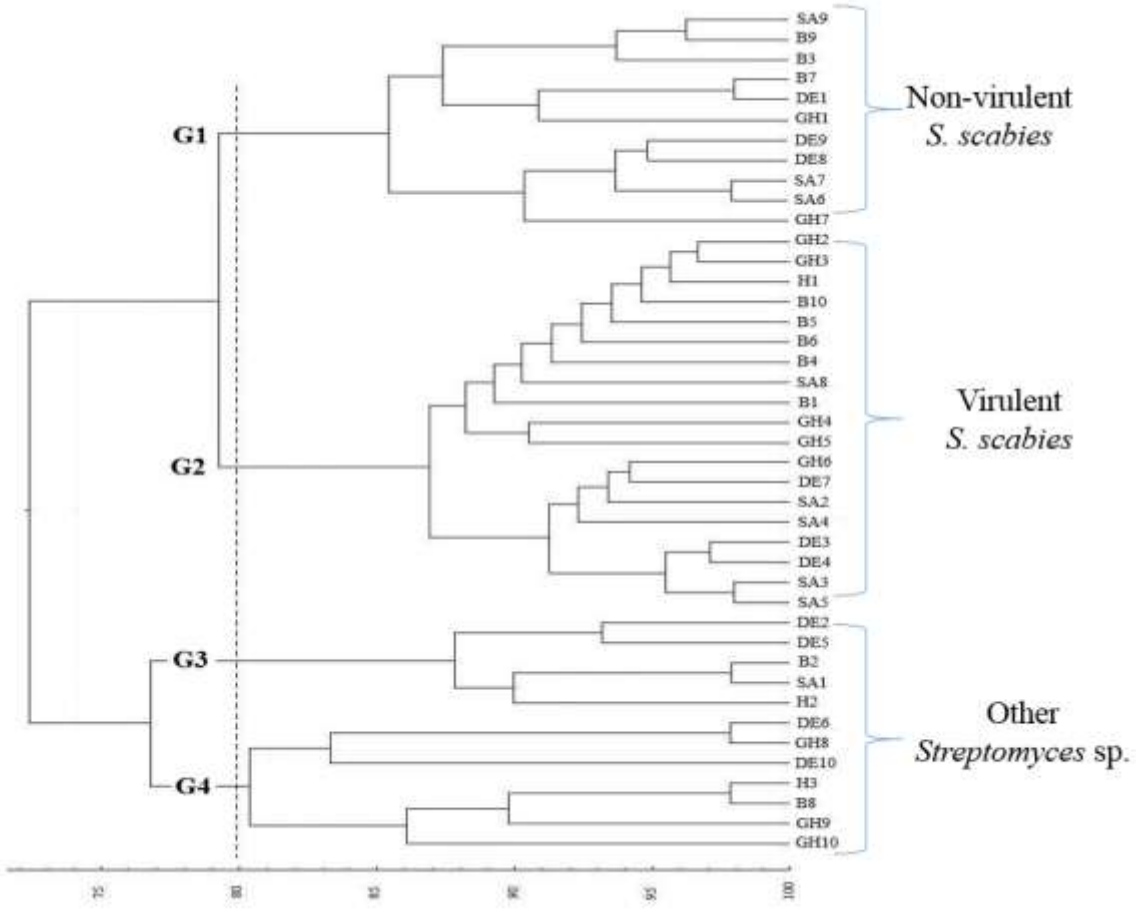

Fig 5. Combined results of ERIC, BOX and rep-PCR on *Streptomyces* strains using of NTSYS-pc (ver. 2.1) software. See Table 2 for isolates names.

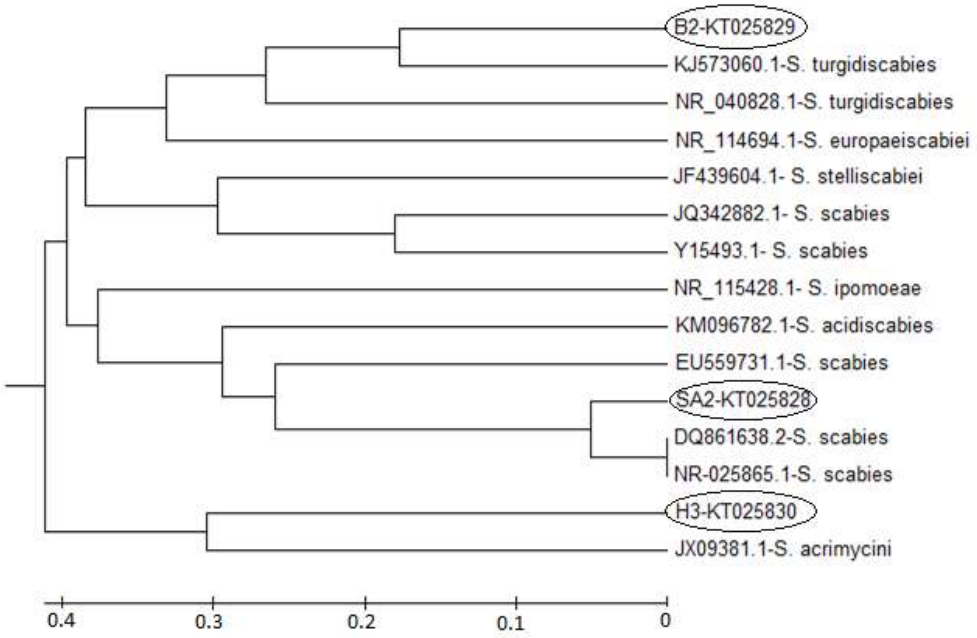

Fig6. An UPGMA phylogenetic tree obtained from the 16s RNA gene sequence data from *Streptomyces* strains. Each isolate is shown by its GenBank accession number and strain name.

Refereces

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