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Genetic Characterization of Three Soil *Streptomyces* Isolates Exhibiting Antiviral Activity Against TMV; Insights from 16S rRNA Gene Sequencing and RAPD Analysis

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ABSTRACT

In the present study, each of the 16S ribosomal RNA (rRNA) gene sequences and random amplified polymorphic DNA (RAPD) method were used to confirm the biological classification of three *Streptomyces* isolates having antiviral activities against Tobacco mosaic virus (TMV). The strongest anti-TMV effect with an inhibition percentage of 97.5 was recorded for *S. rochei*-IS-02 followed by *S. caeaoi*-IS-03 (95.5%) and *S. aureocirculatus*-IS-01 (92.5%) at dilution ½. Lengths of 771, 663 and 693 nts were determined for the partial nucleotide sequences of 16SrRNA gene of *S. aureocirculatus*-IS-01, *S. rochei*-IS-02 and *S. caeaoi*-IS-03**,** respectively. Sequences producing significant alignments showed identities ranging from 98.41 to 100%. The phylogenetic relationship confirmed the identification of the *Streptomyces* isolates. A total of 85 DNA fragments (81 polymorphic and 4 monomorphic) were amplified in RAPD assay of the three *Streptomyces* isolates using eight random primers and contained unique DNA fragments as positive DNA markers. These DNA fragments were distributed as 48, 41 and 42 for the three isolates. The eight primers produced a total amplified DNA fragments of 85 distributed as follows 8, 10, 13, 12, 12, 10, 11 and 9 for the eight oligonucleotide primers, respectively. The similarities between the three *Streptomyces* species based on RAPD-PCR ranged from 66.332 to 76.158%. The phylogenetic tree showed that *S. aureocirculatus*-IS-01 and *S. rochei*-IS-2 were found together in the same cluster, while, *S. caeaoi*-IS-03 was found in a separate cluster. As a conclusion 16SrRNA and RAPD-PCR methods were confirmed to be more than suitable tools to differentiate between the Streptomyces species.

INTRODUCTION

Streptomyces were successfully isolated, purified and characterized from different soils worldwide including Egypt (Mohamed *et al.*, 2001, Mohamed *et al.*, 2012, Shori *et al.*, 2012, Alvarez-Mico *et al.*, 2013, Kamjam *et al.*, 2019 and Bo *et al.*, 2019).

According to previous investigations, it was proved that the secondary metabolites extracted from actinomycetes were important in controlling plant diseases including plant viruses (Chen *et al*., 2019). They also reported that the Streptomyces strain STZ isolated from the soil of Tianzhu Mountain in Shenyang, China exhibited antiviral activity against TMV. Belonging to 65 families 885 plant species were susceptible to infection of TMV (Roossinck 2015, Fraile and García-Arenal, 2018).

It is very difficult to rely on the morphological identification and cultural and physiological characteristics of actinobacteria including strepomycete (Kim *et al.,* 1999). This is due to the high similarity between them particularly in the color of aerial mycelium, type of spore chain, production of melanoid pigments and spore surface.

The use of molecular biology methods such as 16S ribosomal RNA (16SrRNA) gene could be considered an ideal tool to confirm biological identification (Anderson and Wellington 2001). 16SrRNA and 18SrRNA were applied to identify four bacterial (*Bacillus* sp. MSLB-1, *Bacillus* sp. MSLB2, *Bacillus* sp. MSLB3, *Escherichia coli* MSL-19) and three fungal isolates (*Aspergillus niger* MLSAs1, *Aspergillus* sp. MLSAs2, *Penicillium* sp. MLSP1), respectively, isolated from soil up to strains (El-Masry *et al.,* 2019).

16SrRNA was applied to identify four bacterial strains (*Bacillus* sp. MSLB-1, *Bacillus* sp. MSLB2, *Bacillus* sp. MSLB3, *Escherichia coli* MSL-19) isolated from soil up to strains (El-Masry *et al.,* 2019). Sequence data of 16SrRNA revealed that it could provide the basis for proposals to unify the genus *Streptomyces*, in addition to other phylogenetic investigations that sought to determine the intrageneric structure of a genus (Stackebrandt *et al.,* 1991, Mehling *et al.,* 1995, Kim *et al.,* 1996 and Takeuchi *et al.,* 1996).

Biswas *et al.* (2017) used 16S ribosomal RNA (rRNA) gene sequence to confirm the characterization and identification of some streptomycetes (*Streptomyces labedae, S. variabilis, S. erythrogriseus* and *S. griseoincarnatus*) isolated from sediment of Sundarbans mangrove forest in India, previously classified based on medium 2 in International *Streptomyces* Project (ISP), the isolates characterized by a yellowish brown to red aerial hyphae, retinaculum-apertum chain spore with spiny-surface.

El-Masry *et al.* (2021) isolated, purified and identified some bioagent streptomycetes having antimicrobial activities from the soil in Egypt followed by molecular identification up to strains based on 16SrRNA gene.

Physiological characteristics represented using sugars as a sole carbon source and the production of enzymes cannot be relied upon absolutely in the identification of such microorganisms. Therefore, it is necessary to use the recent techniques that can differentiate between them such as random amplified polymorphic DNA (RAPD) (Malkawi *et al.,* 1999, Gharaibeh *et al.,* 2003, and Hussein *et al.,* 2006), inter simple sequence repeat (ISSR) (Bornet and Branchard 2001, Bornet *et al.,* 2002, Fedrigo *et al.,* 2016 and Abd El-Aziz *et al.,* 2023) among DNA fingerprinting of streptomycetes.

RAPD-PCR technique was applied in several investigations to differentiate between streptomycetes isolated from different sources, *i.e.*, soil and marine ecosystems (Hussein *et al.*, 2006, Luo *et al.,* 2010, Mohamed *et al*., 2012, Shori *et al.,* 2012, Mohamed, *et al.,* 2013, Maleki et al. 2013, Biswas et al. 2017, Qattan and Khattab 2019 and Shoukry et al. 2019).

Malkawi *et al.* (1999) concluded that RAPD-PCR method was efficient in discriminating and evaluating the genetic

variation between the *Streptomyces* isolates. Martin *et al.* (2000) evaluated six oligonucleotides presenting a high G+C content as RAPD primers were selected to compare 12 strains of six *Streptomyces* species. Therefore, RAPD-PCR technique allowed the rapid, sensitive and specific evaluation of genetic diversity among *Streptomyces* species.

This study was designed to use each of 16SrRNA gene and RAPD marker to confirm and differentiate between three soil-Streptomyces isolates having antiviral activities against TMV.

MATERIALS AND METHODS

Source of *Streptomyces* Isolates:

The three *Streptomyces* isolates under investigation were kindly provided by the Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, Cairo, Egypt. These isolates were biologically identified as *S. aureocirculatus, S. rochei* and *S. caeaoi*, and coded as IS-01, IS-02 and IS-03, respectively.

Preparation of Streptomycetes Suspension:

Two spore discs of the three *Streptomyces* isolates were separately added to 250 mL flasks containing 50 mL of ISP3 broth medium as recommended by Chen *et al.* (2019) followed by incubation for a week at 28°C under shaking in a rotary shaker at 180 rpm. Low-speed centrifugation at 8000 rpm for 20 min was done, and the pellets were discarded, and the filtrates were stored at 4 °C till use.

Antiviral Activities of *Streptomyces* Strains against TMV:

An indicator host (*Nicotiana glutinosa*) inducing necrotic local lesions (NLL) with TMV and containing 4-5 leaves was used. The infectious TMV sap was prepared according to the method of El-Ahdal *et al*. (1984). The crude TMV extract was diluted by adding an equal volume of 0.01 M phosphate-buffered saline (PBS), pH 7.0 before use followed by preparation of two-fold dilutions up to 1/16. Antiviral activities of the three streptomyces isolates were against TMV were determined against TMV using the diluted infectious sap of TMV by the half-leaf method as described (Gooding and Hebert 1967). The inoculated leaves were washed with sterile distilled water after 30 min from inoculation and cultivated in a glass greenhouse at 26 ±2°C. Four/five days post inoculation the number of NLL was recorded. The percentage of inhibition was calculated based on the following formula: 100- (Average number of NLL with diluted filtrate treatment ÷ No. of NLL at control leaf × 100).

16SrRNA Gene Sequencing and Analysis:

The three *Streptomyces* isolates which are biologically classified as *S. aureocirculatus, S. rochei* and *S. caeaoi* were sent to Macrogen® (908 world Meridian venture center, #60-24, Gasan-dong, Geumchun-gu, Seoul 153-781, Korea) for determining the nucleotide sequences of 16SrRNA gene for confirmation of the biological identification. Two universal primers called 785F: GGA TTA GAT ACC CTG GTA 3'; 907R: 5' CCG TCA ATT CMT TTR AGT TT3' were applied in the presence of DNA extracts of the three isolates which were prepared according to the method of Mohamed *et al.* (2012). Using the protocol supplied by the manufacturer, the nucleotide sequences were performed using the ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits, ABI PRISM 3730XL Analyzer (96 capillary type) sequencer (Applied Biosystems), MJ Research PTC-225 Peltier Thermal Cycler, DNA polymerase (FS enzyme, Applied Biosystems). The nucleotide sequences were aligned with the most similar strains documented in GenBank, using Standard Nucleotide BLAST, National Library of Medicine, NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM).

RAPD DNA Fingerprinting:

DNA extracts of the three *Streptomyces* isolates were prepared according to the method of Mohamed *et al.* (2012) and used as templates for PCR

amplification in the presence of eight random oligonucleotides belonging to different groups: P-01 (5'AGG GGT CTT G3'), P-02 (5'CAATCGCCGT3'), P-03 (5'GTG AGG CGT C3'), P-04 (5'CCG CAT CTA C3'), P-05 (5'TGA GTG GGT G3'); P-06 (5'-CTT CCC CAA G-3'), P-07 (5'TCA GGG AGG T3'), P-08 (5'GGT GCA CGT T3'), following the PCR protocol described by Mohamed *et al.* (2013). DNA polymorphisms were scored as I (Present) and 0 (Absent), and each genetic similarity, pairwise combination and genetic distance were recorded (Hassanien *et al.* 2004) which was used to determine population relationships using the unweighted pair group method of analysis (UPGMA) (Sneath and Sokal 1973). On PCR the amplified products were visualized by electrophoresis (Sambrook *et al.*, 1989) in a 1.2% agarose gel at 60 v for 2.5 h with 1X TBE buffer. DNA fragments appeared in the gel after UV light transilluminator examination.

RESULTS AND DISCUSSION

In the present study, two *Streptomyces* isolates (*S. rochei*-IS-02, *S. caeaoi*-IS-03) belonging to the grey series and one isolate belonging to the white series (*S. aureocirculatus*-IS-01) were used. They carry spore chains of RF and Spiral with smooth surfaces (Figure 1). *S. caeaoi*-IS-03 was producing Melanoid pigment and they varied in their ability to grow on Czapek's medium. Ten sugars (D-Fructose, D-Galactose, D-Glucose, D-Mannitol, D-Xylose, i-Inositol, L-Arabinose, L-Rhamnose, Raffinose and Sucrose) were used as a sole source of carbon by *S. caeaoi*-IS-03 in the growth medium. *S. aureocirculatus*-IS-01 and *S. rochei*-IS-02 can't utilize Raffinose. *S. rochei*-IS-02 was not able to utilize sucrose as a sole source of carbon. A similar was noted by *S. aureocirculatus*-IS-01 as it was not able to utilize L-Arabinose and L-Rhamnose, while *S. rochei*-IS-02 can't use Raffinose.

In 1943, Waksman and Henrici proposed the *Streptomyces* genus as a spore-forming, aerobic gram-positive bacteria. These bacteria induced extensive branching substrates and aerial mycelia and were characterized with DNA with a high content of cytosine and guanine which ranged from 69 to 73 l% (Williams *et al.*, 1983, Williams *et al.*, 1989 and Korn-Wendisch and Kutzner, 1992). This type of bacteria is also known for the presence of LL-diaminopimelic acid and the absence of characteristic sugars in the cell wall (Lechevalier and Lechevalier, 1970).

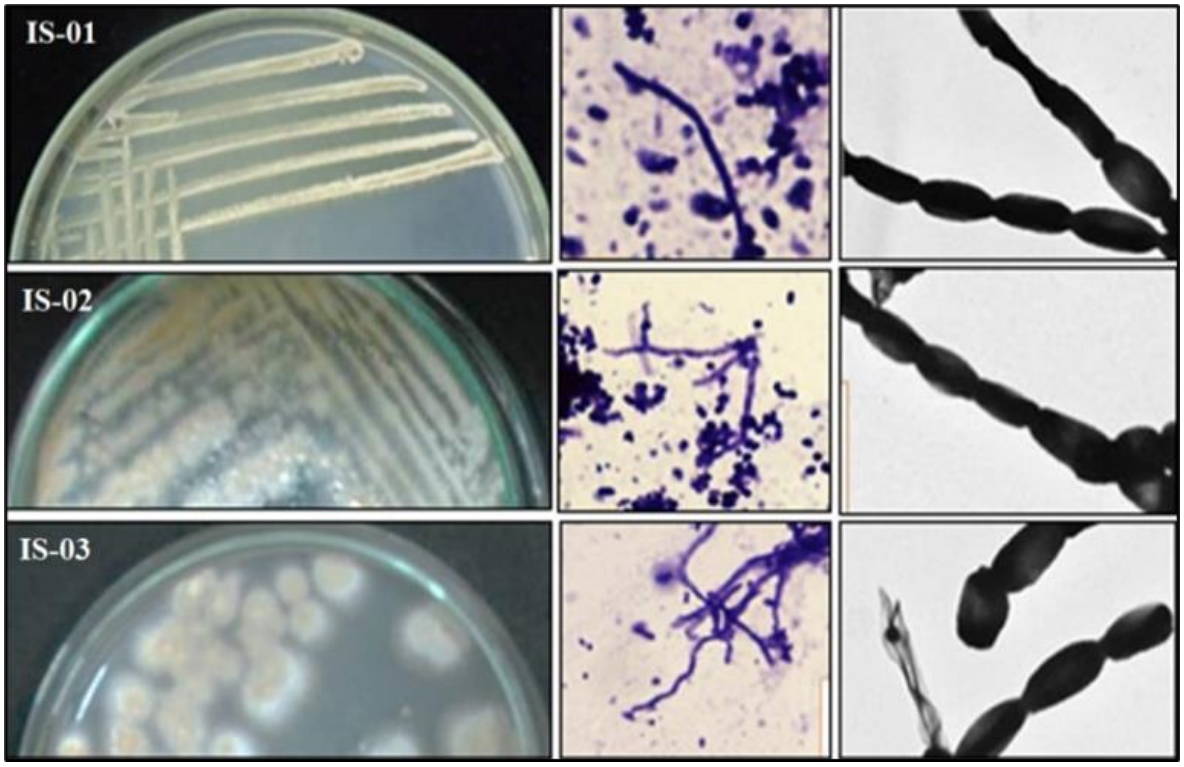

Fig.1: Color of aerial mycelia (White, Grey, Grey), type of spore chains (RF, RF, Spiral) and spore surfaces (Smooth) of three Streptomyces isolates IS-01, IS-02, and IS-03, respectively.

Antiviral Activities of *Streptomyces* against TMV:

Recently microbial metabolites have been considered as a potential alternative for chemical insecticides. This is due to the advantages of these metabolites with respect to good environmental compatibility, low mammalian toxicity and a unique mode of action (Seiber 2011). Chen *et al*. (2019) showed that based on the cultural, morphological, physiological and biochemical properties the isolate having antiviral activity against TMV was identified as *Streptomyces hygroscopicus* and molecularly confirmed by 16S rRNA sequences. Yeo *et al*. (1998) tested the antiviral of an identified isolate of *Streptomyces* coded B25 against TMV under field conditions. They showed that TMV infection decreased from 95.3% up to 58.3% two weeks post-treatment.

Scholthof *et al.* (2011) reported that plant diseases caused by viruses were considered an economic pathogen inducing yearly losses in the production of horticulture and cereal worldwide. They also showed development and commercialization of highly effective antiviral substances are urgent because chemical insecticides and pesticides were not effective enough to control plant virus disease.

In this study inhibition rates of the three streptomycete isolates against TMV were assessed by the half-leaf method using *N. glutinosa* indicator host as mentioned in the materials and methods. Results in Table (1) and illustrated by Figures (1-5) showed that the strongest anti-TMV effect with an inhibition percentage of 97.5 was recorded for *S. rochei*-IS-02 followed by *S. caeaoi*-IS-03 (95.5%) and *S. aureocirculatus*-IS-01 (92.5%) at dilution ½, and by increasing the dilutions the inhibitory activities were decreased and reached 37.5% at dilution 1/16. The experimental results were in harmony with that of Zhu (2011) who found that the fermented filtrate extracted and purified from *Streptomyces parvus* Yn168 inhibited the activity of TMV by reduction of the number of NLL in which the percentage of inhibition reached up to 84.2% compared to control.

Also, the experimental results could be supported by some investigations, *i.e.*, Han *et al.* (2014) showed that a number of bio-agents isolated from the fermentation broth of *Streptomyces noursei* var. *xichangensis* were able to inhibit assembly of virions among inhibiting the polymerization of TMV coat protein (CP) and induced pathogenesis-related proteins (PRs), and Han *et al.* (2015) who purified a novel glycoprotein (GP-1) from *Streptomyces kanasensis* exhibited extensive inhibitory effect on TMV infection. In addition, Han *et al*. (2021) showed that the bioactive glycoprotein compound named GP-1 enhanced systemic resistance with a significant reduction in NLL induced by TMV on the leaves of the indicator host.

Table 1: Antiviral activities against TMV of the three *Streptomyces* isolates on *N. glutinosa* indicator host**.**

***Streptomyces* isolates**	**No. of NLL and % of inhibition against TMV**							
	1/2		1/4		1/8		1/16	
	NLL	% Inhibition	NLL	% Inhibition	NLL	% Inhibition	NLL	% Inhibition
S. aureocirculatus-IS-01	3	92.5	6	85.0	9	77.5	25	37.5
S. rochei-IS-02	1	97.5	4	90.0	9	77.5	18	55.0
S. caeaoi-IS-03	2	95.5	5	87.5	9	77.5	21	47.5
Control (untreated)	40							

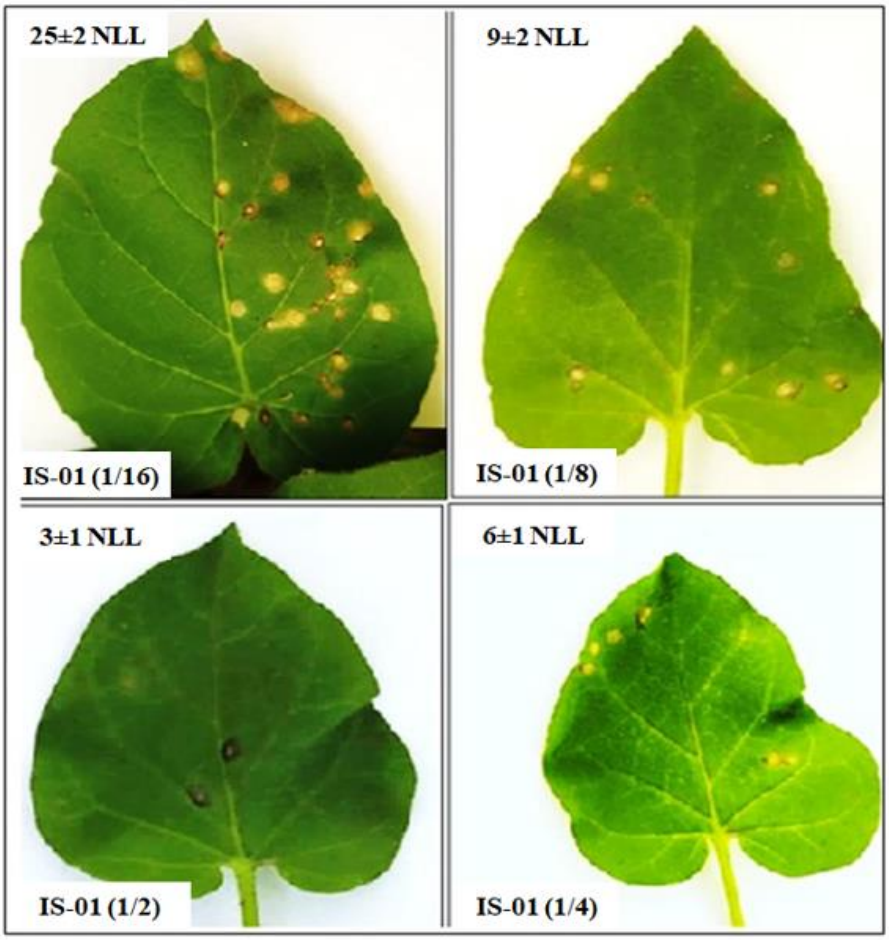

Fig.2: Antiviral activity of *S. aureocirculatus*-IS-01against TMV on *N. glutinosa* indicator host.

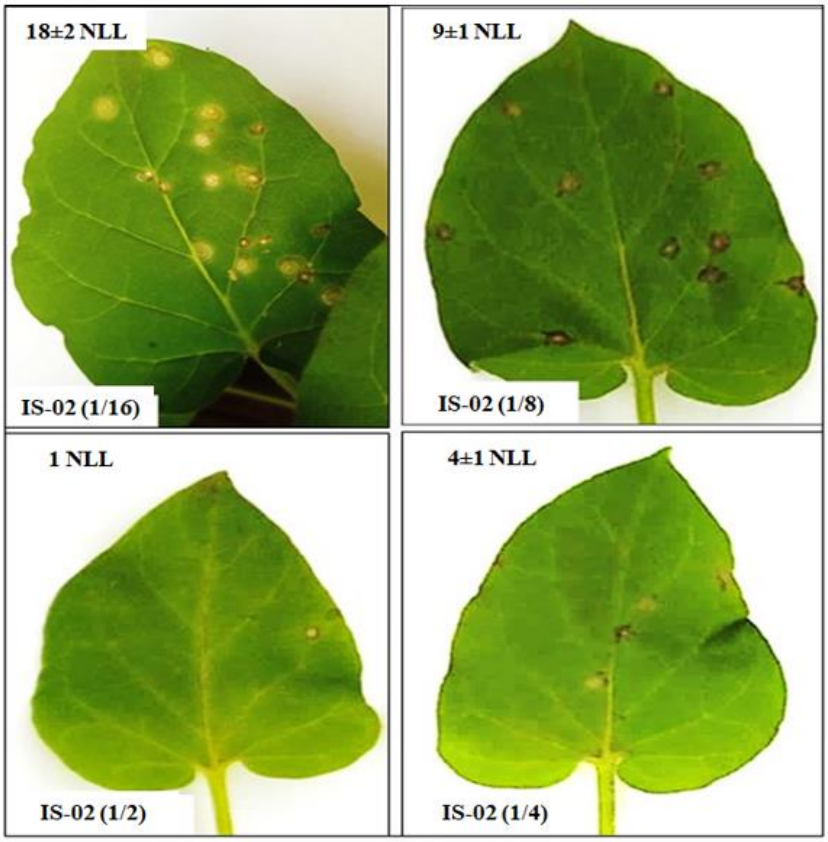

Fig. 3: Antiviral activity of *S. rochei*-IS-02 against TMV on *N. glutinosa* indicator host.

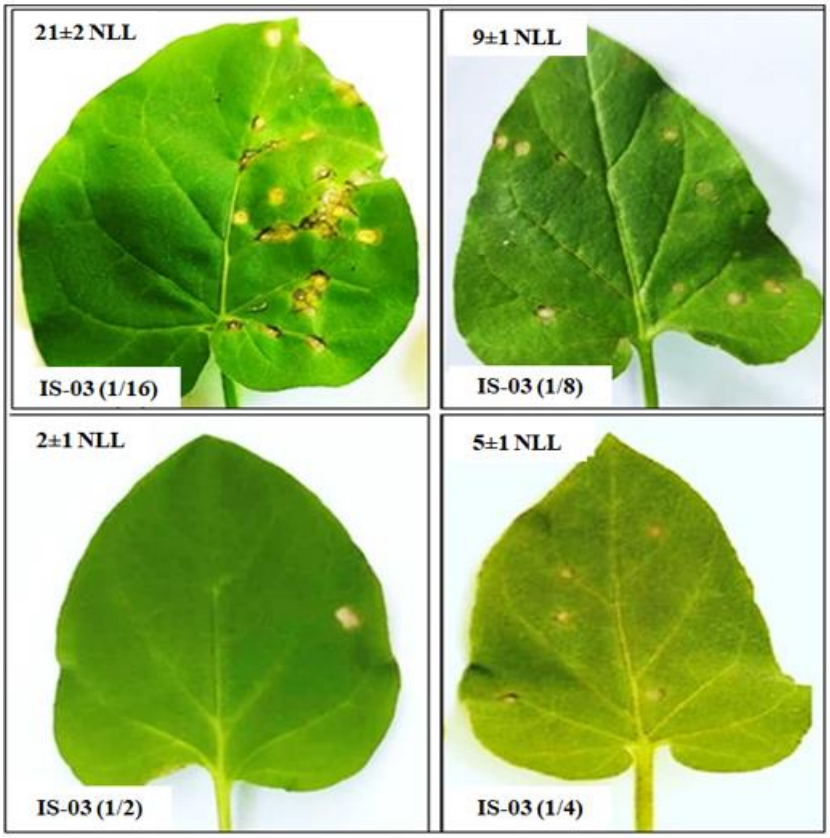

Fig. 4: Antiviral activity of *S. caeaoi*-IS-03 against TMV on *N. glutinosa* indicator host.

Fig.5: *N. glutinosa* indicator host inoculated with TMV as a control (untreated).

16SrRNA Gene Sequencing and Analysis:

16SrRNA gene was considered a gold taxonomy tool based on the determination of the phylogenetic relationship between the bacteria. In the case of actinobacteria, the large size of *Streptomyces* members makes this tool insufficient to distinguish the closely related species of *Streptomyces* (Law *et al.* (2018). Based on these findings, the need for an alternative phylogenetic gene marker plus 16SRNA gene is an important issue such as RAPD, ISSR, SSR and SCoT methods. DNA fingerprinting of streptomyces was studied *via* different molecular tools including RAPD-PCR (Mahfouz and Mohamed, 2002; Abdel-Fattah, 2005; Saleh *et al.*, 2011; Shash, 2011; Mohamed *et al.*, 2012; Shori *et al.*, 2012 and Mohamed *et al.*, 2013).

In this study, data in Figures (6), (7) and (8) show the numbers 771, 663 and 693 nts represent the partial nucleotide sequences of the 16SrRNA genes of the three streptomycetes, i.e., *S. aureocirculatus*-IS-01, *S. rochei*-IS-02 and *S. caeaoi*-IS-03, of this study, respectively. These isolates were documented in GenBank as strains under the accession numbers LC784317.1, LC784318.1 and LC784319.1, respectively. Results of sequences producing significant alignments showed that the query cover percent between the partial nucleotide sequences of the three *Streptomyces* species and the most five similar strains in GenBank ranged from 96 to 100 for *S. aureocirculatus*-IS-01 (Table 2), 99% for *S. rochei*-IS-02 (Table 3) and 100 for *S. caeaoi*-IS-03 (Table 4).

```
   1 ttagtggcga acgggtgagt aacacgtggg caatctgccc tgcactctgg gacaagccct
  61 ggaaacgggg tctaataccg gataatactt ctgctctcct gagcagaggt tgaaagctcc
 121 ggcggtgcag gatgagcccg cggcctatca gcttgttggt gaggtaatgg ctcaccaagg
 181 cgacgacggg tagccggcct gagagggcga ccggccacac tgggactgag acacggccca
 241 gactcctacg ggaggcagca gtggggaata ttgcacaatg ggcgaaagcc tgatgcagcg
 301 acgccgcgtg agggatgacg gccttcgggt tgtaaacctc tttcagcagg gaagaagcga
 361 aagtgacggt acctgcagaa gaagcgccgg ctaactacgt gccagcagcc gcggtaatac
 421 gtagggcgca agcgttgtcc ggaattattg ggcgtaaaga gctcgtaggc ggcttgtcac
 481 gtcggttgtg aaagcccggg gcttaacccc gggtctgcag tcgatacggg cgagctagag
 541 tgtggtaggg gagatcggaa ttcctggtgt agcggtgaaa tgcgcagata tcaggaggaa
 601 caccggtggc gaaggcggat ctctgggcca atactgacgc tgaggagcga aagcgtgggg
 661 agcgaacagg attagatacc ctggtagtcc acgccgtaaa cggtgggcac taggtgtggg
 721 caacattcca cgttgtccgt gccgcagcta acgcattaag tgccccgcct g
```

Fig. 6: Partial nucleotide sequences (771 nts) of 16SrRNA gene of *Streptomyces aureocirculatus*-IS-01.

```
   1 gtcgaaagct ccggcggtgc aggatgagcc cgcggcctat cagctagttg gtgaggtaat
  61 ggctcaccaa ggcgacgacg ggtagccggc ctgagagggc gaccggccac actgggactg
 121 agacacggcc cagactccta cgggaggcag cagtggggaa tattgcacaa tgggcgaaag
 181 cctgatgcag cgacgccgcg tgagggatga cggccttcgg gttgtaaacc tctttcagca
 241 gggaagaagc gaaagtgacg gtacctgcag aagaagcgcc ggctaactac gtgccagcag
 301 ccgcggtaat acgtagggcg caagcgttgt ccggaattat tgggcgtaaa gagctcgtag
 361 gcggcttgtc acgtcggttg tgaaagcccg gggcttaacc ccgggtctgc agtcgatacg
 421 ggcaggctag agttcggtag gggagatcgg aattcctggt gtagcggtga aatgcgcaga
 481 tatcaggagg aacaccggtg gcgaaggcgg atctctgggc cgatactgac gctgaggagc
 541 gaaagcgtgg ggagcgaaca ggattagata ccctggtagt ccacgccgta aacggtgggc
 601 actaggtgtg ggcaacattc cacgttgtcc gtgccgcagc taacgcatta agtgccccgc
 661 atg
```

Fig. 7: Partial nucleotide sequences (663 nts) of 16SrRNA gene of *Streptomyces rochei*-IS-02**.**

```
   1 cgttgggctc taggtgtggg cgacattcca cgttgtccgt gccgcagcta acgcattaag
  61 tgccccgcct ggggagtacg gccgcaaggc taaaactcaa aggaattgac gggggcccgc
 121 acaagcggcg gagcatgtgg cttaattcga cgcaacgcga agaaccttac caaggcttga
 181 catacaccgg aaaactctgg agacagggtc ccccttgggg tcggtgtaca ggtggtgcat
 241 ggctgtcgtc agctcgtgtc gtgagatgtt gggttaagtc ccgcaacgag cgcaaccctt
 301 atcctgtgtt gccagcatgc ctttcggggt gttggggact cacgggagac cgccggggtc
 361 aactcggagg aaggtgggga cgacgtcaag tcatcatgcc ccttatgtct tgggctgcac
 421 acgtgctaca atggccggta caatgagctg cgataccgcg aggtggagcg aatctcaaaa
 481 agccggtctc agttcggatt ggggtctgca actcgacccc atgaagtcgg agtcgctagt
 541 aatcgcagat cagcattgct gcggtgaata cgttcccggg ccttgtacac accgcccgtc
 601 acgtcacgaa agtcggtaac acccgaagcc ggtggcccaa ccccttgtgg gagggagctg
 661 tcgaaggtgg gactggcgat tgggacgaag tcg
```

Fig. 8: Partial nucleotide sequences (693 nts) of 16SrRNA gene of *Streptomyces caeaoi*-IS-03.

Table 2: Identities (%) and Query Cover (%) with E-value (0%) of partial nucleotide sequences of 16SrRNA gene of *Streptomyces aureocirculatus*-IS-01 compared to the five most similar strains documented in GenBank.

Description	Query Cover (%)	Identities (%)	Accession
Streptomyces aureocirculatus strain DSM 40386T 16S ribosomal RNA gene, partial sequence	100	98.56	KF772674.1
Streptomyces aureocirculatus strain NBRC 13018 16S ribosomal RNA, partial sequence	100	98.56	NR_112344.1
Streptomyces aureocirculatus strain CSSP728 16S ribosomal RNA, partial sequence	98	98.54	NR_043371.1
Streptomyces aureocirculatus strain P6F55 16S ribosomal RNA gene, partial sequence	96	98.51	MN421297.1
Streptomyces aureocirculatus strain YQX2-5m 16S ribosomal RNA gene, partial sequence	96	98.50	OM534621.1

Table 3: Identities (%) and Query Cover (%) with E-value (0%) of partial nucleotide sequences of 16SrRNA gene of *Streptomyces rochei*-IS-02 compared to the five most similar strains documented in GenBank.

Description	Query Cover (%)	Identities (%)	Accession
Streptomyces rochei strain PU-KB10-5 16S ribosomal RNA gene, partial sequence	99	100.00	MN813140.1
Streptomyces rochei strain NEAE-25 16S ribosomal RNA gene, partial sequence	99	100.00	HQ889312.2
Streptomyces rochei strain WR15 16S ribosomal RNA gene, partial sequence	99	99.85	MT659661.1
Streptomyces rochei strain SA1019 16S ribosomal RNA gene, partial sequence	99	99.85	MT355863.1
Streptomyces rochei ANH gene for 16S ribosomal RNA, partial sequence	99	99.85	LC537844.1

Table 4: Identities (%) and Query Cover (%) with E-value (0%) of partial nucleotide sequences of 16SrRNA gene of *Streptomyces caeaoi*-IS-03 compared to the five most similar strains documented in GenBank.

Description	Query Cover (%)	Identities (%)	Accession
*Streptomyces cacao*i gene for 16S rRNA, partial sequence, strain: NBRC 12837	100	98.56	AB184183.1
Streptomyces cacaoi strain DSD2595 16S ribosomal RNA gene, partial sequence	100	98.56	MW217135.1
Streptomyces cacaoi strain NBRC 12748 16S ribosomal RNA, partial sequence	100	98.56	NR_041061.1
Streptomyces cacaoi strain SCSIO 68063 16S ribosomal RNA gene, partial sequence	100	98.41	OP482267.1
Streptomyces cacaoi strain Z1-1 16S ribosomal RNA gene, partial sequence	100	98.41	KJ571100.1

The identities between strains and those documented in GenBanl reflect the query cover percent as it ranged from 98.50 to 98.56% between the sequences of *S. aureocirculatus*-IS-01 and those in GenBank represented by accession numbers of KF772674.1, NR_112344.1, NR_043371.1, MN421297.1 and OM534621.1. In the case of *S. rochei*-IS-02 the identities ranged from 99.85 to 100% Between MN813140.1, HQ889312.2, MT659661.1, MT355863.1 and LC537844.1. Regarding *S. caeaoi*-IS-03 the identities compared to AB184183.1, MW217135.1, NR_041061.1, OP482267.1 and KJ571100.1 ranged from 98.41-98.56.

The resulting dendrograms shown in Figures (9), (10) and (11) showed the genetic relationship of partial nucleotide sequences of 16SrRNA gene of the three isolates and that of the five most similar strains documented in GenBank. Results confirmed that these isolates could be strains of *S. aureocirculatus*, *S. rochei* and 100 for *S. caeaoi*.

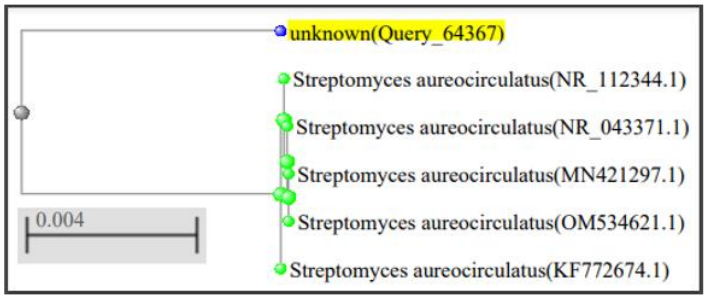

Fig. 9: Dendrogram shows the genetic relationship of partial nucleotide sequences of 16SrRNA gene of *Streptomyces aureocirculatus*-IS-01 compared to the five most similar strains documented in GenBank.

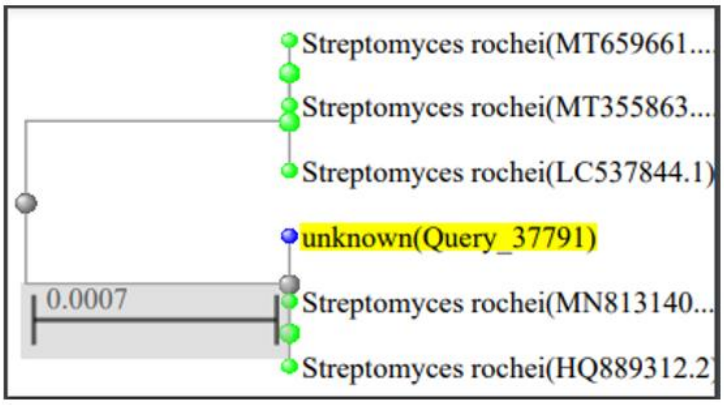

Fig. 10: Dendrogram shows the genetic relationship of partial nucleotide sequences of 16SrRNA gene of *Streptomyces rochei*-IS-02 compared to the five most similar strains documented in GenBank.

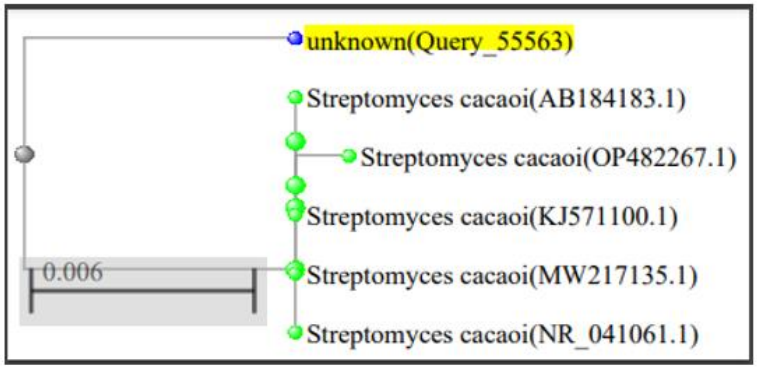

Fig. 11: Dendrogram shows the genetic relationship of partial nucleotide sequences of 16SrRNA gene of *Streptomyces caeaoi*-IS-03 compared to the five most similar strains documented in GenBank.

RAPD DNA fingerprinting

RAPD-PCR technique allowed the rapid, sensitive and specific evaluation of genetic diversity among *Streptomyces* species (Martin *et al.* 2000). Gharaibeh *et al.* (2003) used the RAPD-PCR technique to evaluate the relatedness of 73 *Streptomyces* isolates producing antibiotics isolated from different soil habitats in Jordan. A number of three monomorphic DNA fragments with sizes of 2777, 800 and 250 pb were common in 95% of streptomycete strains. They confirmed that DNA fingerprinting produced *via* RAPD-PCR successfully differentiated between the isolates which were characterized with different cultural properties.

In the present study, data in Tables (5), (6), (7) and (8) show the scoring of DNA polymorphisms produced by the applied eight oligonucleotides (P-01, P-02, P-03, P-04, P-05, P-06, P-07 and P-08) used for RAPD-PCR to determine the genetic variation between the three Streptomyces

(*S. aureocirculatus*-IS-01, *S. rochei*-IS-02 and *S. caeaoi*-IS-03) of this study. These primers produced a total amplified fragments of 85 distributed as follows 8, 10, 13, 12, 12, 10, 11 and 9 for the eight oligonucleotide primers, respectively (Table 9 and Figs. 12-15). The three *Streptomyces* species appeared 48, 42 and 42 DNA fragments representing percentages of 56.47, 49.41 and 49.41 when the DNA extracts of *S. aureocirculatus*-IS-01, *S. rochei*-IS-02 and *S. caeaoi*-IS-03, respectively, were used as templates in RAPD-PCR test.

Results in Table (10) revealed the type of the 85 amplified DNA fragments, which were classified as four monomorphic fragments and 81 polymorphic fragments. These fragments contained unique DNA fragments as positive DNA markers distributed between the three *Streptomyces* species as follows: 16 (19.75%), 18 (22.22%) and 14 (17.28%), respectively.

The similarities between the three *Streptomyces* species based on RAPD-PCR DNA fingerprinting were 76.158 between *S. aureocirculatus*-IS-01 and *S. rochei*-IS-02; 66.332 between *S. aureocirculatus*-IS-01 and *S. caeaoi*-IS-03; 73.485 between *S. rochei*-IS-02 and *S. caeaoi*-IS-03 (Table 11). This was confirmed by the phylogenetic relationships in Figure (16) as *S. aureocirculatus*-IS-01 and *S. rochei*-IS-2 were found together in the same cluster, while, *S. caeaoi*-IS-03 was found in a separate cluster. The experimental results were in harmony with that reported by Mahfouz and Mohamed (2002), Hussein *et al.* (2006), Luo et al. (2010), Mohamed *et al.* (2012), Shori *et al.* (2012), Mohamed *et al.* (2013), Maleki *et al.* (2013), Biswas *et al.* (2017), Qattan and Khattab (2019) and Shoukry *et al.* (2019).

Table 5: Scoring of DNA polymorphisms of three bacterial strains amplified using P-01 and P-02 primers among ISSR method.

DNA Fragments	Bacterial strains		
	***S. aureocirculatus*-IS-01**	***S. rochei*-IS-02**	***S. caeaoi*-IS-03**
P-0101	0	0	1
P-0102	1	0	0
P-0103	0	0	1
P-0104	0	0	1
P-0105	1	0	1
P-0106	1	0	0
P-0107	1	0	1
P-0108	0	1	0
Total	**4**	**1**	**5**
P-0201	0	1	0
P-0202	0	1	1
P-0203	1	0	0
P-0204	0	1	1
P-0205	1	0	0
P-0206	0	1	0
P-0207	1	0	0
P-0208	1	0	1
P-0209	0	1	0
P-0210	0	1	0
Total	**4**	**6**	**3**

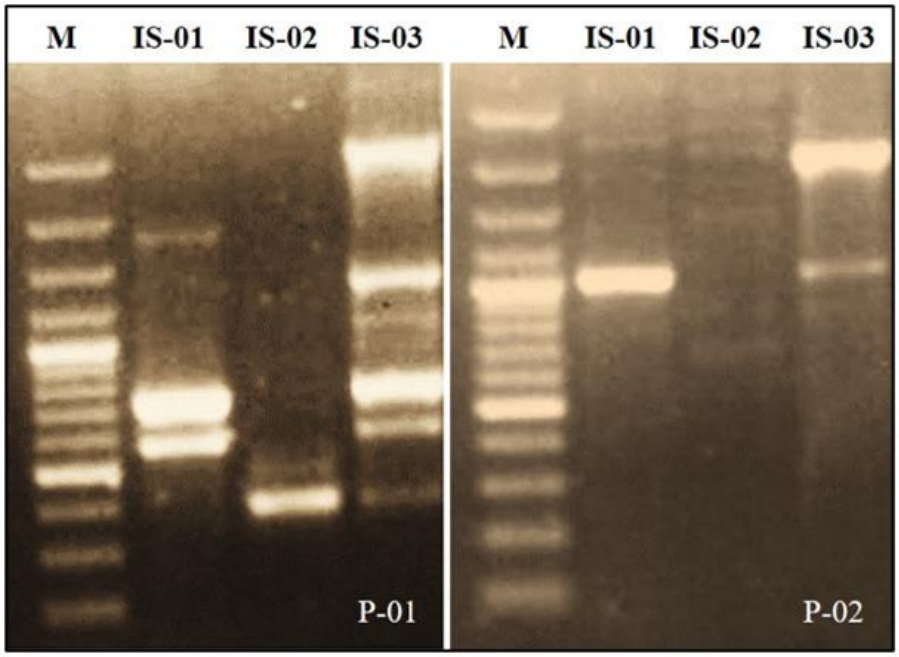

Fig. 12: Agarose gels (1.2%) stained with ethidium bromide show DNA polymorphisms of three bacterial strains amplified using P-01 and P-02 primers among ISSR method.

Table 6: Scoring of DNA polymorphisms of three bacterial strains amplified using P-03 and P-04 primers among ISSR method.

DNA Fragments	Bacterial strains		
	***S. aureocirculatus*-IS-01**	***S. rochei*-IS-02**	***S. caeaoi*-IS-03**
P-0301	1	0	0
P-0302	1	1	0
P-0303	0	1	0
P-0304	0	0	1
P-0305	1	1	1
P-0306	1	0	1
P-0307	1	0	1
P-0308	0	1	0
P-0309	0	1	1
P-0310	1	1	1
P-0311	1	1	0
P-0312	1	1	1
P-0313	0	1	0
Total	**8**	**9**	**7**
P-0401	0	1	0
P-0402	0	0	1
P-0403	1	0	1
P-0404	1	0	0
P-0405	1	0	1
P-0406	0	0	1
P-0407	0	1	0
P-0408	0	1	0
P-0409	0	1	0
P-0410	1	0	1
P-0411	1	1	0
P-0412	1	1	0
Total	**6**	**6**	**5**

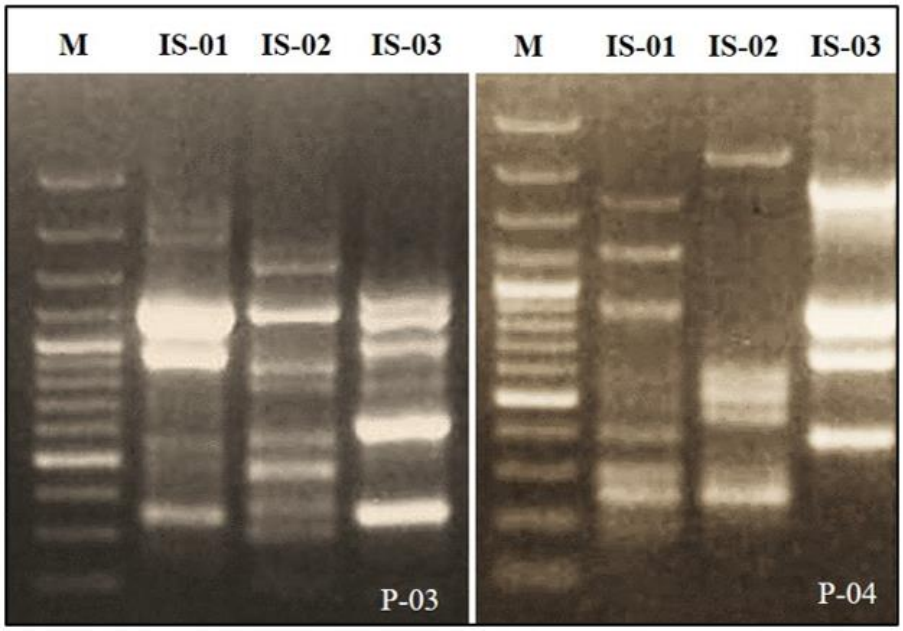

Fig.13: Agarose gels (1.2%) stained with ethidium bromide show DNA polymorphisms of three bacterial strains amplified using P-03 and P-04 primers among ISSR method.

Table 7: Scoring of DNA polymorphisms of three bacterial strains amplified using P-05 and P-06 primers among ISSR method.

DNA Fragments	Bacterial strains		
	***S. aureocirculatus*-IS-01**	***S. rochei*-IS-02**	***S. caeaoi*-IS-03**
P-0501	1	0	0
P-0502	0	1	1
P-0503	0	1	1
P-0504	1	0	0
P-0505	0	1	0
P-0506	0	1	1
P-0507	0	0	1
P-0508	0	1	0
P-0509	1	0	1
P-0510	0	0	1
P-0511	1	0	0
P-0512	1	0	0
Total	**5**	**5**	**6**
P-0601	1	0	1
P-0602	1	0	1
P-0603	1	0	1
P-0604	1	1	0
P-0605	1	1	0
P-0606	0	1	0
P-0607	0	1	0
P-0608	1	1	0
P-0609	1	0	0
P-0610	1	0	1
Total	**8**	**5**	**4**

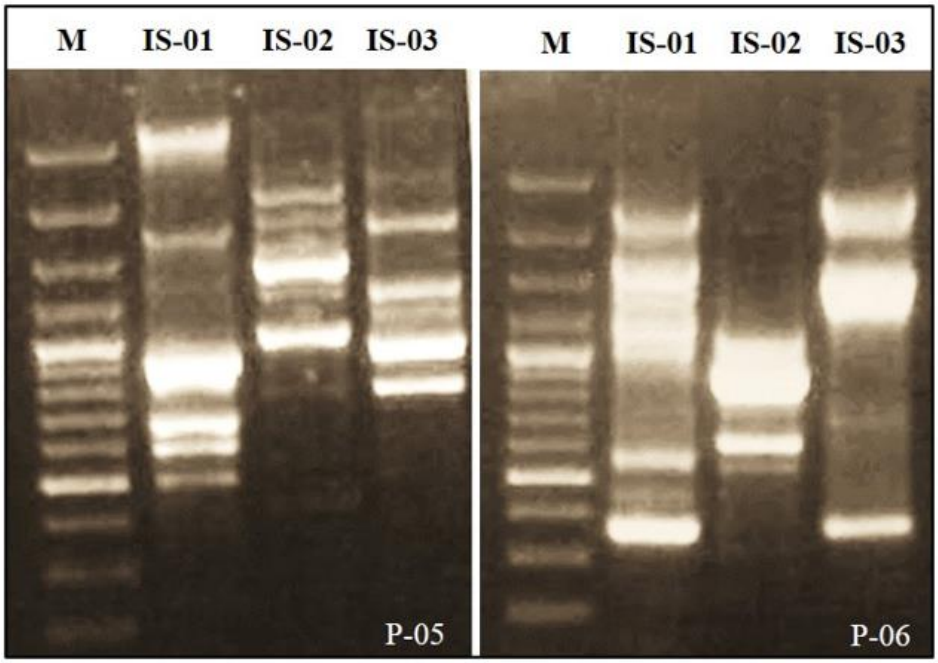

Fig.14: Agarose gels (1.2%) stained with ethidium bromide show DNA polymorphisms of three bacterial strains amplified using P-05 and P-06 primers among ISSR method.

Table 8: Scoring of DNA polymorphisms of three bacterial strains amplified using P-07 and P-08 primers among ISSR method**.**

DNA Fragments	Bacterial strains		
	***S. aureocirculatus*-IS-01**	***S. rochei*-IS-02**	***S. caeaoi*-IS-03**
P-0701	0	0	1
P-0702	1	1	1
P-0703	1	0	1
P-0704	1	0	0
P-0705	1	1	1
P-0706	0	1	1
P-0707	0	0	1
P-0708	1	1	0
P-0709	0	0	1
P-0710	1	0	0
P-0711	0	1	0
Total	**6**	**5**	**7**
P-0801	0	1	1
P-0802	1	0	0
P-0803	1	0	0
P-0804	1	1	1
P-0805	1	0	1
P-0806	1	0	1
P-0807	1	1	1
P-0808	1	1	0
P-0809	0	1	0
Total	**7**	**5**	**5**

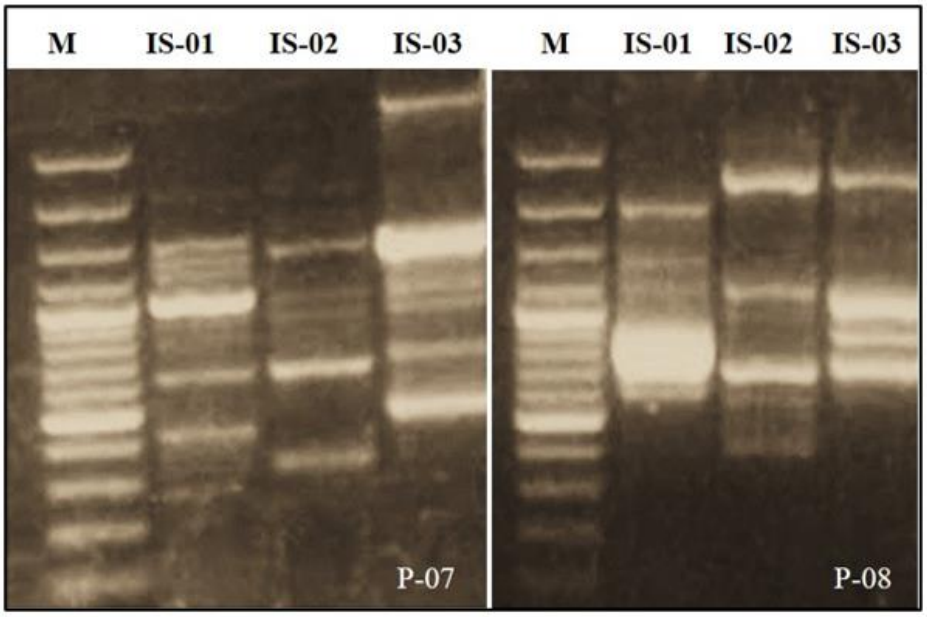

Fig. 15: Agarose gels (1.2%) stained with ethidium bromide show DNA polymorphisms of three bacterial strains amplified using P-07 and P-08 primers among ISSR method.

Table 9: Summary of scoring of DNA polymorphisms of three bacterial strains amplified using eight primers among ISSR method.

DNA Fragments	TAFs	Bacterial strains		
		***S. aureocirculatus*-IS-01**	***S. rochei*-IS-02**	***S. caeaoi*-IS-03**
P-01	08	4	1	5
P-02	10	4	6	3
P-03	13	8	9	7
P-04	12	6	6	5
P-05	12	5	5	6
P-06	10	8	5	4
P-07	11	6	5	7
P-08	09	7	5	5
Total	**85**	**48**	**42**	**42**
%	**100**	**56.47**	**49.41**	**49.41**

TAFs: Total amplified fragments.

Table 10: DNA unique DNA fragments (Markers) of three bacterial strains amplified using eight primers among ISSR method.

DNA Fragments	TAFs	MMFs	PMFs	Bacterial strains		
				***S. aureocirculatus*-IS-01**	***S. rochei*-IS-02**	***S. caeaoi*-IS-03**
P-01	08	0	8	2	1	3
P-02	10	0	10	3	4	0
P-03	13	1	12	1	3	1
P-04	12	0	12	1	4	2
P-05	12	0	12	4	2	2
P-06	10	0	10	1	2	0
P-07	11	1	10	2	1	3
P-08	09	2	7	2	1	3
Total	**85**	**4**	**81**	**16**	**18**	**14**
%	**100**	**4.71**	**95.29**	**19.75**	**22.22**	**17.28**

TAFs: Total amplified fragments. PMFs: Polymorphic fragments. MMFs: Monomorphic fragments.

Table 11: Similarities between the three bacterial strains based on analysis of DNA polymorphisms produced *via* ISSR method using eight primers.

DNA Fragments	***S. aureocirculatus*-IS-01**	***S. rochei*-IS-02**	***S. caeaoi*-IS-03**
***S. aureocirculatus*-IS-01**	100		
***S. rochei*-IS-02**	76.158	100	
***S. caeaoi*-IS-03**	66.332	73.485	100

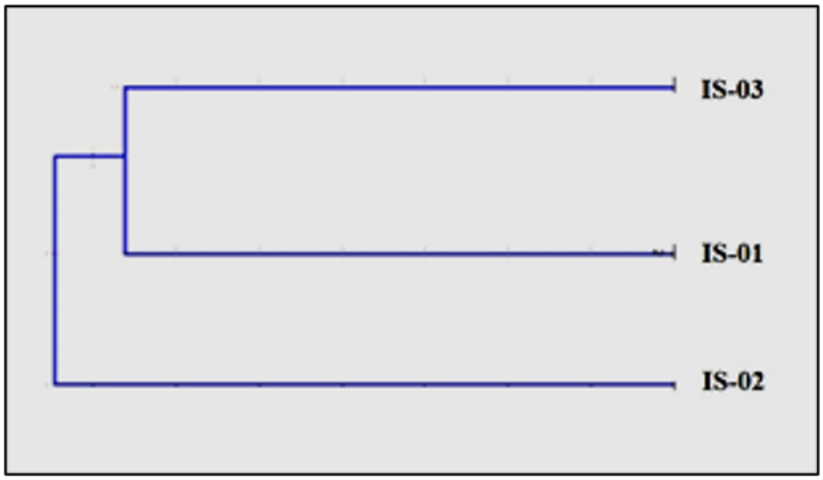

Fig. 16: Dendrogram shows the genetic relationship between the three bacterial strains based on analysis of DNA polymorphisms produced *via* ISSR method using eight primers**.**

Declarations:

Ethical Approval: It is not applicable.

Conflicts of Interest: The authors declare that they have no conflict of interest.

Authors Contributions: I hereby verify that all authors mentioned on the title page have made substantial contributions to the conception and design of the study, have thoroughly reviewed the manuscript, confirm the accuracy and authenticity of the data and its interpretation, and consent to its submission.

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