Physiological, antagonistic and fingerprinting studies on some halotolerant *Streptomyces* strains

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Hesham T. Mahfouz* and Sonya H. Mohamed**
* Agricultural Genetic Engineering Research Institute (AGERI), ARC, Giza, P.O. Box 12619, Egypt
** Department of Agricultural Microbiology, Institute of Soil, Water and Environment Research, ARC, Giza, P.O. Box 12619, Egypt.

ABSTRACT

Five selected halotolerant Streptomyces strains revealed variable degrees of tolerance to different concentrations of Sencor or Basta herbicides in the growth medium. Results of enzymatic activities of these strains showed their ability to hydrolyze casein and starch, and their failure to degrade pectin in the growth medium. The Streptomyces strains belonging to the violet series were able to utilize the cellulose as a carbon source, while others belonging to the red series could not. The gelatin in the growth medium was only utilized by S. muavecolor Si-9. On the other direction, the highest antagonistic activities were obtained by the red series strains (S. melanogenes Si-11 followed by S. lateritius Si-6 and S. tuirus Si-4), as they antagonized 11, 7 and 7 out of 11 tested organisms, respectively. These organisms represent bacteria, fungi and yeast. Only S. melanogenes Si-11 and S. lateritius Si-6 showed high antiviral activities against tobacco mosaic tobamovirus (TMV), as the local lesions on the inoculated leaves of Datura metel appeared when the filtrates were diluted up to1:80 and 1:40, respectively. Therefore, these strains could be used for production of antimicrobial substance(s). For RAPD-PCR analysis, a total of 327 DNA bands generated by 16 random 10-mer primers were detected in which 311 (about 95%) were considered as useful markers for the five Streptomyces strains used in the present study. A very few number of bands were common (monomorphic) for all strains. As high as 192 out of the 327 (59%) RAPD-PCR markers were strain-specific. The similarity indices among the five Streptomyces strains utilizing RAPD-PCR markers were detected. The strongest relationship was scored between Streptomyces strains Si-11 and Si-4 (similarity of 70.3%), while Streptomyces strains Si-1 and Si-9 were shown to be the most genetically distant strains (similarity index of 29.0%). From the dendrogram tree, the Streptomyces sp. *Si-1 strain ensured to be a novel species with a similarity index of 33.0% when compared with the other four strains. The Streptomyces muavecolor* Si-9 *strain was also shown to be genetically distant when compared with the other three strains Si-6, Si-4 and Si-11. This study also suggests the use of RAPD-PCR technology as a new taxonomical tool for actinomycetes beside the classical proposed keys used in Bergey s Manual.*

Key words: *Streptomyces, halotolerant, antiviral, herbicides, RAPD-PCR, TMV, fingerprinting, antibacterial, antifungal activities.*

INTRODUCTION

Actinomycetes are widely distributed in soils, where they have an important role in the degradation of the lignocellulose of plant cell walls (Lacey, 1973; Puniya *et al.*, 1995). In Egypt, El-Nasser and El-Shafei (1994) tested sixty different strains of *Streptomyces* for their enzymatic hydrolysis of cellulose and related materials. The most active strains were *S. mutabilis, S. chromofuscus and S. cyanoviridis*. Mohamed *et al.* (2000) isolated halotolerant actinomycete isolates from sandy soil from different locations in Egypt. The four highly halotolerant isolates having the ability to grow on 15-18% NaCl were identified as *S. violans* Da-3, *S. alboflavus* Is-10, *S. bobili* PS-12 and *S. hawaiiensis* Si-8. These species were able to utilize starch and casein for growth.

Actinomycetes are universally distributed worldwide and considered to be the most important source of antibiotics (Paul and Banerjee, 1983, De and Gupta, 1991, Zaki *et al.*, 1993; El-Abyad *et al.*, 1996a). A new antifungal antibiotic having some phytotoxic effects produced by *S. galbus* was isolated from a soil sample of West Bengal by Paul and Banerjee (1983) and was active against a wide variety of fungi and bacteria. In Egypt, Mohamed *et al.* (2000) studied the antagonistic activity of four *Streptomyces* species against 12 microorganisms representing bacteria, fungi and yeast. Surprisingly, *S. hawaiiensis* Si-8 isolated from Sinai showed a strong activity against all tested microorganisms in the presence of 3% NaCl in starch nitrate broth medium. An antiviral activity due to some *Streptomyces* species was also reported by Pridham and Tresner (1974).

The biological decontamination of pesticide (acaricide, fungicide, herbicide, insecticide, miticide and rodenticide) wastes has become an increasingly important area of research. It would be desirable if a microbial method of degradation was available for the various pesticidal compounds (Kaufman, 1974). Sencor [4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1, 2, 4-traizin-5(4H)-one] (Jarczyk, 1983) is a selective herbicide for the control of weeds in potatoes, soybeans, sugarcane, tomatoes, alfalfa (Lucerne) and asparagus. Basta [L-2-amino-4-hydroxymethylphosphinol-butyric acid, called phosphinothricin (PPT) or glufosinate] (Tachibana, 1987) is a recommended herbicide for controlling weeds in mango, grapes and citrus in Egypt. The importance of microorganisms in the degradation and detoxication of pesticides was established, and biodegradation, chiefly by microorganisms (eg., actinomycetes) was recognized as a major mean of destroying these chemicals in soil (Kaufman *et al.*, 1963, Kaufman, 1974, Krause *et al.*, 1985, Shelton *et al.*, 1996; Mohamed, 1998).

Over the last decade, the polymerase chain reaction (PCR) technology has been developed as a novel genetic assay based on selective DNA amplification (Saiki *et al.*, 1988; Krawets, 1989; Innis *et al.*, 1990). The prerequisite of DNA sequence information has reduced the application of this technology. Williams *et al.* (1990) described a new DNA polymorphism assay based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence called random amplified polymorphic DNA (RAPD). Mohamed (1998) used RAPD-PCR to differentiate between five *Streptomyces* species with dissimilarities ranging from 41.4 to 57.4%.

The aim of the present work is to study some physiological and antibiosis activities of some *Streptomyces* strains obtained from Sinai sandy soil in Egypt. The study included the antibacterial, antifungal and antiviral activities of the applied strains. In addition, the DNA fingerprinting based on RAPD-PCR technique were used to obtain unique patterns that could be used as markers for the characterization of *Streptomyces* strains under investigation and to elucidate the phylogenetic relationships among them.

MATERIALS AND METHODS

Source of streptomycetes

Five halotolerant streptomycete strains, i.e., *Streptomyces* species Si-1 (a new isolate), *S. tuirus* Si-4, *S. lateritius* Si-6, *S. muavecolor* Si-9 and *S. melanogenes* Si-11 isolated from Sinai sandy soil in Egypt were provided by Dept. Agric. Microbiol., Institute of Soil, Water and Environment Research, ARC, Giza, Egypt. These strains have the ability to grow on 9 and/or 12% salt in the growth medium.

Selected herbicides

The Sencor herbicide was kindly provided by Bayer Company, Cairo, Egypt. The Basta (glufosinate) herbicide was kindly provided by AGERI, ARC, Giza.

Herbicide tolerance of streptomycetes

The five selected *Streptomyces* strains were tested for their abilities to grow in the presence of Sencor and Basta herbicides in starch nitrate agar medium (SNAM) (Waksman and Lechevalier, 1961) *in vitro*. For each herbicide, three concentrations were applied, i.e., equivalents to the recommended dose (RD) (0.75 g/l for Sencor and 2 g/l for Basta), and 5 and 10 folds of the RD (3.75 and 7.5 g/l for Sencor, and 10 and 20 g/l for Basta). Inoculated plates were incubated at 28°C±2 for 15 days. Plates containing herbicide free SNAM were used to serve as control. The growth of *Streptomyces* strains withstanding the toxicity of the herbicide was determined and recorded as no growth (-), weak (+), moderate (++) and abundant (+++) growth.

Enzymatic activities

The ability of the five selected *Streptomyces* strains to utilize casein, cellulose (carboxy methylcellulose), gelatin, pectin and starch in SNAM as described by Mohamed *et al.* (2000) were determined. The normal SNAM was used as a control. The results were determined according to the method given by Skerman (1967).

Antimicrobial activities

The antimicrobial activities of the selected *Streptomyces* species against eleven microorganisms were investigated on the basis of the method described by Zaki *et al.* (1993). The microorganisms used included fungi, i.e., *Aspergillus niger*, *Alternaria solani*, *Botrytis faba*, *Helminthosporium gramenium*-133, *Fusarium oxysporum F.sp.* lycopersci-123, *Rhizoctonia solani* and *Rhizopus nigricans*; yeast, i.e., *Candida tropicalis* CAIM-2, and bacteria, i.e., *Erwinia carotovora*, *E. coli* - 1319 and *Pseudomonas* sp. These test organisms were kindly provided by Cairo MIRCEN and Dr. Ahmed Mousa, Dept. Plant Pathology, Faculty of Agric., Ain Shams University. The antibiosis activity of the *Streptomyces* strains under investigation was carried out as described by Mohamed *et al.* (2000). The antimicrobial activity was determined by measuring the inhibition zones (in mm) using the diffusion method as recommended by British Pharmacopoeia (1968).

Antiviral activity

The antagonism activity of the five selected *Streptomyces* strains was determined

against the tobacco mosaic tobamovirus (TMV). In this experiment, standard inoculum for each applied strain was prepared by scraping the heavy spores from the surface of the growth of starch nitrate slant in the presence of 5 ml sterilized distilled water ($d.H_2O$). An aliquot of 2 ml of this standard inoculum (containing 1.5-ml spores ml^{-1}) was transferred aseptically to 50 ml starch nitrate broth medium (Waksman and Lechevalier, 1961) in 250 ml conical flask. Inoculated flasks were incubated at 28° C ± 2 for 6 days on a rotary shaker (160-rpm min^{-1}). Thereafter, growth was centrifuged at 14000 rpm at 4°C for 5 min. The pellets were discarded and four dilutions, i.e., 1/10, 1/20, 1/40 and 1/80 were prepared for each *Streptomyces* supernatant. Then, one gram of TMV-infected leaves of *Nicotiana tabacum* cv. Samsun exhibiting systemic infection was triturated in 2 ml of the prepared dilution as well as undiluted supernatant. For each treatment, five leaves of *Datura metel* plant were inoculated by the conventional leaf rub method with a cotton swab, using carborandum (600 mesh) as an abrasive. The inoculated plants were maintained in an insect proof glasshouse for local lesion (L.L) developing.

RAPD-PCR analysis

The DNA extracts from the five *Streptomyces* strains were prepared and purified following a modified protocol of CTAB (2% CTAB in 0.7 M NaCl). Sixteen random decamer oligonucleotide primers from, kits A, B, D, E, O and Z synthesized at AGERI, ARC, Giza, Egypt according to the nomenclature of Operon Technologies (Alameda, CA) were applied. Names and sequences of these 16 primers are listed as the following:

No.	Primer name	Sequence (5` → 3`)
1	A13	CAGCACCCAC
2	A20	GTTGCGATCC
3	B02	TGATCCCTGG
4	B19	ACCCCCGAAG
5	D07	TTGGCACGGG
6	D08	GTGTGCCCCA
7	D20	ACCCGGTCAC
8	E01	CCCAAGGTCC
9	E02	GGTGCGGGAA
10	E06	AAGACCCCTC
11	E11	GAGTCTCAGG
12	E20	AACGGTGACC
13	O10	TCAGAGCGCC
14	O11	GACAGGAGGT
15	Z01	TCTGTGCCAC
16	Z18	AGGGTCTGTG

The reaction conditions were optimized and mixtures (50 µl total volume) were composed of dNTPs (0.2 mM), $MgCl_2$ (1.5 mM), 1X buffer, primer (0.2 µM), DNA (100 ng), *Taq* DNA polymerase (2 units). Amplification was carried out in a PCR Express (HYPAID) thermocycler programmed for 37 cycles as follows : 94°C/4 min. (1 cycle); 94°C/1 min., 34°C/50 sec., 72°C/2 min. (infinitive). The min. (1 cycle); then 4° C (infinitive). The electrophoresis of RAPD-PCR fragments amplified from the DNA extracts of the five selected *Streptomyces* strains was carried out at 100 V for 45 min. and gels (1.5%) were photographed under UV light using a Polaroid camera.

Marker nomenclature

Each RAPD-PCR marker was named by the primer used and DNA fragment size in base bair (bp). For example, A13-3627 refers to a marker with a band size of 3627 bp amplified against primer A13.

RAPD-PCR computer analysis

RAPD-PCR patterns were scanned and scored both visually and using Gel Doc Computer System. Variations among strains were evaluated from similarity indices for the proportion of shared bands, i.e., two times the number of common bands (shared fragments) divided by the total number of bands for each pair of strains against all markers. Therefore, DICE computer package was used to calculate the similarity indices and plot the dendrogram among strains (Yang and Quiros, 1993).

RESULTS AND DISCUSSION

Herbicide-tolerance of Streptomycetes

Actinomycetes have an important role in the degradation of pesticides and cleaning soil and water of contaminated residues. In Egypt, Sencor is a broad-spectrum herbicide especially notable for its powerful action against both grass and broad-leaf weeds. Shelton *et al.* (1996) reported that *Streptomyces* (strain PS 1/5) was able to metabolize atrazine (metribuzin, Sencor) contaminated soil as a nitrogen source within 28 days.

In the present investigation, all strains were tested for their tolerance to Sencor and Basta herbicides at RD, and five and ten folds RD for each. Results in Table (1) showed that the *Streptomyces* strains varied in their tolerance to the two herbicides used and/or concentrations applied. Basta at 10 RD suppressed the growth of *S. tuirus* Si-4 and *S. muavecolor* Si-9, while the remaining strains showed weak (*S. lateritius* Si-6 and *S. melanogenes* Si-11) to moderate (*Streptomyces* sp. Si-1) growth. Only *Streptomyces* sp. Si-1 strain showed abundant growth at 5 RD of Basta, while the other four species gave a weak growth. Data in Table (1) also revealed that the growth of the applied isolates was more abundant in the presence of Sencor than in Basta treatment. This may be due to the high toxicity of Basta to Streptomycete strains than Sencor. Mohamed (1998) obtained similar results, in which she reported that 70 % of the tested actinomycete isolates can tolerate Sencor at RD, while only 38 % could withstand the toxicity of Basta at its RD. With increasing Sencor and Basta concentrations to ten folds of the RD, the inhibitive effect was severe and more pronounced in the case of Basta herbicide.

Table (1): Tolerance levels of some Streptomyces strains to different concentrations of the two herbicides Sencor and Basta ***on SNAM.***

Streptomyces Strains	Growth in the presence of herbicides							
	Sencor				Basta			
	Cont.	RD	5 RD	10 RD	Cont.	RD	5 RD	10 RD
S. sp. Si-1	+++	+++	+++	+++	+++	+++	+++	++
S. muavecolor Si-9	+++	+++	++	+	+++	++	+	-
S. tuirus Si-4	+++	+++	++	+	+++	++	+	-
S. lateritius Si-6	+++	+++	++	++	+++	+++	+	+
S. melanogenes Si-11	+++	+++	++	++	+++	+++	+	+

RD = Recommended dose (0.75 g/l and 2g/l for Sencor and Basta, respectively). + = Weak growth. ++ = Moderate growth. +++ = Abundant growth. - = No growth. Cont.= Control, herbicide free SNAM.

Prokopenko (1986) studied the effect of 0.5-1 kg Sencor (metribuzin)/ha on the soil microflora (including bacteria and actinomycetes) in Primorsk and Siberia. He noted that, by 60 days after incorporation, Sencor stimulated the growth of bacteria and actinomycetes but not that of spore-forming bacteria.

Enzymatic activities

Cellulase research was mainly focused on fungi. Species of *Streptomyces,* as an actinomycetes genus, appeared in the early studies on cellulolytic microorganisms (Enger and Sleeper, 1965). Li and Gao (1996) isolated a new bacterium, *Streptomyces* sp. LX from soil, which was aerobic Gram-positive and could decompose crystalline cellulose completely.

Data in Table (2) show that cellulose was utilized by the *Streptomyces* strains belonging to the violet series, i.e., *Streptomyces* sp. Si-1 and *S. muavecolor* Si-9, while others belonging to the red series could not utilize cellulose. The gelatin in the growth medium was only hydrolyzed by *S. muavecolor* Si-9. All the *Streptomyces* strains were able to utilize casein and starch as a sole source of nitrogen and carbon, respectively. All the tested strains were not able to degrade pectin. In Egypt, Mohamed *et al.* (2000) reported that four halotolerant *Streptomyces* species belonging to yellow, white and violet series showed variation in their activities to degrade cellulose and gelatin. They also reported that these strains utilized starch and casein for growth, while failed to utilize pectin.

Table (2): Enzymatic activities of some Streptomyces strains grown on SNAM.

Streptomyces strains	Source of nitrogen		Source of carbon		
	Casein	Gelatin	Cellulose	Pectin	Starch
S. sp Si-1	+	-	+	-	+
S. muavecolor Si-9	+	+	+	-	+
S. tuirus Si-4	+	-	-	-	+
S. lateritius Si-6	+	-	-	-	+
S. melanogenes Si-11	+	-	-	-	+

+ = Growth. - = No growth.

Antimicrobial activity

Hornby (1990) has reported biological control of soil-borne diseases by bacterial antagonists. The rhizosphere streptomycetes can protect plant roots by inhibiting the growth of fungal pathogens based on their ability to produce antifungal antibiotics *in vitro*, but the production of antibiotics in natural soil is lacking (Williams, 1982). De and Gupta (1991) isolated soil actinomycetes with antifungal activity against *F. oxysporum* and *R. solani.*

Data in Table (3) show that the highest antagonistic activities were obtained by the red series strains, i.e., *S. melanogenes* Si-11 followed by *S. lateritius* Si-6 and *S. tuirus* Si-4, as they antagonized 11, 7 and 7 out of 11 organisms used, respectively. *S. melanogenes* Si-11 could be recommended as a source for the production of antimicrobial substance(s) against a variety of test micro organisms belonging to Gram positive, gram negative and yeast as well as fungi. The production of lytic enzymes (chitinolytic enzymes) by *S. melanogenes* Si-11 could be a possible mode of action against the tested fungi, in particular, *F. oxysporum, R. solani* and *A. solani*, the causal agents of Fusarium wilt, seedling

damping-off and early blight diseases, respectively. Sneh (1981), Lortio *et al.* (1993), Nuero *et al.* (1993) and DeCal *et al.* (1995) reported similar observations.

Vikineswary *et al.* (1997) reported that the streptomycete isolate S6 isolated from a tropical mangrove ecosystem showed strong antifungal activity against *F. monoliforme*, *Aspergillus niger* and *Candida ablicans*. In Egypt, El- Abyad *et al.* (1996a) reported that 9 out of 36 isolates of actinomycetes species isolated from fertile soils showed antagonistic activities against the French bean-wilt fungus *F. oxysporum* f. sp. Phaseoli, and 19 isolates antagonized the bacterial-wilt pathogen of banana *pseudomona solanacearum in vitro*. The most active isolates against the pathogens were identified as *S. corchrusii* and *S. spiroverticillatus*. El-Abyad *et al.* (1996b) reported that on SNAM, 14 strains of *Streptomyces* were active against *F. oxysporum* f. sp. lycopersici and 18 strains against *A. solani*.

Table (3): Antagonistic activities of some Streptomyces strains grown on starch nitrate broth medium for 6 days at 28°C±2 under shaking conditions against different microorganisms.

	Streptomyces strains				
	Violet series		Red series		
Tested organisms	Si-1	Si-9	Si-4	Si-6	Si-11
Bacteria					
Erwinia carotovora	1.1*	0.0	0.0	0.0	1.1
E. coli-1319	1.1	1.0	1.0	1.0	1.4
Pseudomonas sp.`	1.0	1.0	1.0	1.0	1.1
Fungi					
Aspergillus niger	1.1	1.0	0.0	0.0	1.2
A. solani	0.0	0.0	1.0	1.0	1.2
Botrytis faba	0.0	0.0	0.0	0.0	1.1
H. gramenium-133	0.0	0.0	1.0	0.0	1.2
F. oxysporum F.sp. lycopersci-123	1.0	1.0	1.0	1.0	1.2
R. solani	0.0	0.0	1.1	1.1	1.1
Rhizopus nigricans	0.0	1.0	0.0	1.0	1.2
Yeast					
C. tropicalis CAIM-2	1.1	1.0	1.0	1.0	1.5
Total no. inhibited organisms	6	6	7	7	11

* = Zone of inhibition (mm). Note: LB agar medium (Sambrook *et al.*, 1989) and potato glucose agar medium (Waksman and Lechevalier, 1961) were used for growing the bacteria and fungi and yeast, respectively.

Antiviral activity

Results in Table (4) revealed that *S. melanogenes* Si-11 and *S. lateritius* Si-6 showed high antiviral activities against TMV, as the local lesions on the inoculated leaves of *D. metel* appeared when the filtrates were diluted up to 1:80 and 1:40, respectively. This could be due to the low concentration of the antiviral substance(s) in the diluted filtrates. The present results clearly recommend that an antiviral substance(s) could be extracted,

identified and produced by these *Streptomyces* strains.

Pridham and Tresner (1974) reported that *Streptomyces lavendulae*, *S. luridus*, *S. violaceus* (Rossi Doria), *S. violaceus* subsp. confinus, *S. violaceus* subsp. vicinus and *S. violarus* have an antiviral activity beside their antibacterial and antifungal activities.

Table (4): Antiviral activity of some Streptomyces strains grown on starch nitrate broth medium for 6 days under shaking conditions against TMV as judged by number of L.L on D. metel plant.

Streptomyces isolates	Dilution filtrates				
	Crude	1:10	1:20	1:40	1:80
S. sp. Si-1	00*	15	25	45	60
S. muavecolor Si-9	00	10	20	35	55
S. tuirus Si-4	00	15	25	40	55
S. lateritius Si-6	00	00	00	30	45
S. melanogenes Si-11	00	00	00	00	35
Positive control**	375	290	210	135	75
Healthy	00	00	00	00	00

* = No. L.L produced by TMV on five leaves of *D. metel* plant inoculated with infectious sap prepared using filtrate(s). ** = Leaves of *D. metel* plant inoculated with 1 ml infectious sap prepared using d.H_2O.

Identification of RAPD-PCR molecular markers in *Streptomyces* strains

The analysis of RAPD-PCR provides a novel and effective dimension for distinguishing among organisms according to the banding patterns of PCR products, which provides a new means of obtaining genetic markers (Williams *et al.*, 1990; Welsh and McClelland, 1990). Many publications have been developed concerning the use of this technology in the identification of bacteria, protozoa, nematodes, insects, trematodes and fungi (Rollinson and Stothard, 1994).

Two types of polymorphisms were considered in the present study for RAPD-PCR analysis. They are presence of bands in one or more strains and differences in band intensities (quantitatively different phenotypes). Most markers were scored for presence phenotype, but few of them were scored for band intensity. For computer analysis to detect the phenogram involving the five strains, intensive bands were considered as present (+), while weak bands were considered as absent (-). Data revealed that no amplified bands were observed in any of the negative controls, which indicates that the reaction mixtures were free from any DNA contaminants.

A total of 327 DNA bands were detected in which 311 (about 95%) were useful as RAPD-PCR markers generated by the 16 random primers for the five *Streptomyces* strains used in the present study (Figure 1 and Tables 5 and 6). The size of the amplified fragments ranged from 334 bp for primer D08 to 7337 bp for D07 (Table 5). The least number of RAPD-PCR markers was detected for primer A20 (8 markers out of 10 amplified bands), while the largest number of RAPD-PCR markers was detected for primer O11 (27 markers out of 27 bands). However, a very few number of bands were common (monomorphic) for all strains ranging from 3

Table (5): Survey of RAPD-PCR markers in five Streptomyces strains (1=Si-11, 2=Si-4, 3=Si-1, 4=Si-6, 5=Si-9) using 16 random primers, where (+) means presence and (-) means absence.

Marker	1	2	3	4	5	Marker	1	2	3	4	5	Marker	1	2	3	4	5	Marker	1	2	3	4	5
A13-3627	-	-	+	-	-	D08-2304	+	+	-	+	+	E02-628	+	+	-	-	-	O10-1169	-	-	+	+	+
3276	+	+	-	+	+	2074	-	-	+	-	-	608	-	-	+	-	-	1000	+	+	-	-	-
2667	+	+	+	+	+	1915	+	+	+	+	+	596	-	-	-	-	+	931	-	-	+	+	-
2315	-	-	-	-	+	1778	-	-	+	+	-	590	-	-	-	-	+	914	-	-	-	-	+
2181	-	-	-	+	-	1605	+	+	-	+	+	E06-4269	-	-	+	-	-	813	+	+	+	+	+
1997	+	+	+	+	+	1391	+	+	+	+	-	2760	-	-	-	-	+	728	+	+	+	+	+
1712	-	-	+	+	+	1307	+	+	+	+	+	2133	-	-	-	-	+	676	+	+	+	+	-
1557	-	-	+	+	-	1192	-	-	-	-	+	1758	-	-	+	-	-	633	-	-	-	-	+
1389	-	-	+	-	+	1124	+	+	-	+	+	1557	-	-	+	-	-	619	+	+	-	+	-
1193	+	+	+	+	-	816	+	+	+	+	-	1423	-	-	-	+	-	598	+	+	+	+	-
1050	-	-	-	-	+	763	-	-	+	+	-	1228	-	-	-	+	-	550	-	-	-	+	-
995	-	-	+	-	-	721	+	+	-	-	+	1130	+	+	+	-	-	O11-6676	-	-	-	-	+
860	+	+	-	+	-	651	-	-	+	-	-	1007	+	-	-	-	-	5375	-	-	-	-	+
790	-	-	+	+	-	594	-	-	+	-	-	938	-	+	+	-	-	4479	-	-	+	-	-
730	+	+	+	-	+	553	-	-	-	+	-	884	-	-	-	+	-	4098	+	+	-	-	+
583	-	-	-	-	+	514	+	+	-	+	-	845	+	-	-	-	-	3543	-	-	+	-	-
419	+	+	-	+	-	474	-	-	+	-	-	774	-	-	-	+	-	3312	-	-	-	-	+
364	-	-	+	-	-	415	+	+	-	-	-	752	+	+	+	-	+	2731	+	+	-	-	-
A20-3699	-	-	-	-	+	334	-	-	-	-	+	727	-	-	-	+	-	2467	-	-	+	-	-
2612	-	-	+	-	-	D20-3086	-	+	-	-	+	697	+	-	-	-	-	2187	-	-	+	-	-
2055	+	+	+	-	+	2554	-	+	-	-	+	678	-	-	-	+	-	2046	-	-	-	-	+
1528	-	-	+	-	+	2344	-	-	+	-	-	667	-	+	-	-	-	1696	+	+	-	+	-
1297	-	-	+	-	+	2018	-	+	-	+	-	640	+	-	-	-	-	1515	-	-	+	-	+
1147	-	-	-	-	+	1670	-	-	+	-	+	609	-	+	+	-	-	1348	-	+	-	+	-
1009	+	+	+	+	+	1581	+	+	-	-	-	589	+	-	-	-	-	1208	-	+	+	+	-
862	+	+	-	+	+	1525	-	-	-	-	-	572	-	+	-	+	-	1088	-	+	+	+	+
830	-	-	+	-	-	1460	-	-	+	-	-	E11-4815	-	-	+	-	-	1019	-	+	-	-	-
694	+	+	-	+	-	1365	-	-	-	-	+	2103	-	-	+	-	-	918	+	+	-	-	+
B02-1275	-	-	-	+	-	1240	-	-	-	+	+	1616	-	-	-	+	-	878	+	+	-	+	-
1171	-	-	+	-	-	1176	-	+	-	-	-	1480	-	+	-	-	+	835	-	-	-	-	+
1058	-	+	-	-	-	1103	+	-	+	-	-	1289	-	-	+	-	-	798	+	+	-	+	-
912	+	-	+	-	-	993	-	-	+	-	-	1125	-	-	-	-	+	780	-	+	-	-	-
821	-	+	+	-	-	935	-	-	+	-	-	1092	-	+	+	-	-	762	+	-	-	+	-
729	-	-	-	-	+	875	+	+	-	+	+	988	+	-	-	+	+	734	-	-	+	-	-
697	-	-	-	+	-	846	-	-	+	-	-	918	-	+	-	-	-	728	+	+	-	+	+
679	-	-	+	-	-	770	+	-	+	-	+	869	+	-	+	+	-	716	-	-	-	-	+
667	+	-	-	-	-	748	-	+	-	+	-	791	+	-	+	-	-	688	-	-	+	-	-
640	+	-	-	+	+	730	+	-	-	-	-	722	+	+	+	-	-	678	-	-	-	+	-
628	-	+	-	-	-	695	+	+	-	+	-	689	-	-	-	+	-	Z01-2780	-	-	-	-	+
612	-	-	-	+	-	686	-	-	+	-	-	667	+	+	-	-	-	2294	-	-	-	-	+
605	-	-	-	-	+	675	-	-	-	-	+	636	-	-	+	+	-	1741	-	-	+	+	-
592	-	-	+	-	-	650	-	-	+	-	-	611	-	-	-	+	-	1548	-	-	+	-	-
572	-	-	-	+	+	611	-	-	-	-	+	600	+	+	-	-	-	1426	-	-	-	+	-
566	-	-	-	-	-	594	-	+	-	+	-	582	-	-	-	+	-	1374	-	+	-	-	-
555	+	-	-	+	-	E01-3698	-	-	-	-	+	575	-	-	+	-	+	1226	-	+	-	-	+
543	-	-	-	-	+	2612	-	-	-	-	+	554	+	+	-	-	-	1158	-	-	+	+	-
B19-3193	+	-	-	-	-	2114	-	-	-	-	+	537	-	-	+	+	-	1057	+	-	-	-	-
2631	-	-	+	-	-	1737	+	+	+	+	+	543	+	-	-	-	-	1020	-	-	-	+	-
1756	-	-	-	-	+	1354	+	+	+	+	+	529	-	+	-	-	-	935	+	+	-	-	+
1465	-	-	-	-	+	1073	+	+	+	+	+	523	+	+	-	-	-	850	-	-	+	-	-
1323	+	+	+	+	-	928	+	+	-	+	+	E20-4025	-	-	+	-	-	802	+	+	-	+	-
1026	+	-	-	-	-	848	+	+	-	+	+	3383	+	+	-	+	+	755	-	+	-	-	+
995	-	-	+	-	-	759	+	+	-	+	+	2748	-	-	+	-	+	725	-	-	+	-	-
899	-	+	-	+	-	715	+	+	-	-	+	2330	-	-	-	+	+	702	-	-	-	+	-
783	+	-	-	-	-	683	+	-	-	-	-	2177	+	+	+	+	-	684	-	+	+	-	-
750	+	-	+	-	+	669	-	+	+	+	+	1806	-	-	+	-	-	635	-	+	-	-	-
706	-	+	+	+	-	621	+	+	-	+	+	1630	+	+	-	+	+	604	-	-	-	+	-
680	+	-	-	-	-	597	-	-	-	+	+	1455	+	+	-	+	+	623	+	+	-	-	-
665	-	-	-	-	+	584	+	+	-	-	-	1342	-	-	+	-	+	Z18-4620	-	-	+	-	-
D07-7337	-	-	-	-	+	549	-	+	-	-	+	1200	+	+	-	-	-	3214	-	-	-	-	+
4620	-	-	-	-	+	550	+	+	-	+	-	1094	-	-	+	-	+	2664	+	+	+	+	+
3578	-	-	+	-	-	525	-	-	-	-	+	981	-	-	-	-	+	2374	-	-	+	-	-
2906	-	+	-	+	+	E02-3922	-	-	+	-	-	936	+	+	+	+	+	2235	-	-	-	-	+
2343	+	+	-	-	+	3553	-	-	-	+	+	858	+	+	-	+	-	1961	-	-	+	-	-
2051	+	+	-	+	-	3026	+	+	+	+	-	811	-	-	+	-	-	1723	+	-	-	-	+
1565	+	+	-	+	+	2362	+	+	+	+	-	749	+	+	-	+	-	1556	+	-	+	-	-
1363	+	+	+	+	-	1866	+	+	-	+	+	735	-	-	+	-	+	1389	-	-	+	+	-
1179	-	-	-	+	+	1530	+	+	-	+	+	684	+	+	+	+	+	1279	-	+	+	-	-
944	+	+	-	+	-	1402	-	-	+	-	+	656	+	+	+	+	-	1233	-	-	-	-	+
902	-	-	+	-	-	1270	-	-	-	+	+	637	+	-	-	-	+	1094	-	+	-	-	+
860	-	+	-	+	-	1223	-	-	+	-	-	628	-	-	-	-	+	1047	+	-	+	-	-
821	-	-	+	-	+	1141	-	-	-	-	+	611	-	+	+	-	-	943	-	+	-	-	-
754	-	-	-	-	+	1086	+	+	+	+	-	597	-	+	-	-	-	921	-	-	+	-	+
715	+	+	+	+	-	927	-	-	-	-	+	595	+	-	-	-	-	900	+	-	-	-	-
662	-	-	+	-	+	805	+	+	+	+	-	O10-3126	-	-	+	-	-	865	-	+	-	+	-
633	+	+	-	+	-	769	+	-	-	-	+	2851	-	-	-	-	+	843	-	-	-	-	+
618	+	-	-	-	-	712	-	-	+	+	-	2156	-	-	-	-	+	798	-	+	-	-	-
590	-	+	-	-	+	700	-	-	-	+	-	1873	-	+	+	+	-	771	+	-	-	-	-
578	-	-	+	-	-	702	+	+	-	-	-	1554	-	-	+	-	-	733	-	+	-	-	-
D08-3290	-	-	+	-	-	673	+	-	+	-	-	1524	+	+	-	+	+	718	+	-	-	-	-
2850	-	-	+	-	-	654	+	-	-	+	-	1325	-	-	+	-	-	699	-	-	+	-	-
2486	+	+	-	+	-	638	-	-	+	+	-	1293	-	+	-	-	+						

bands for primers A13, E01 and O10, 2 bands for primers A20, D08 and E20; while one band for primer Z18. There were few unstable (unrepeatable) bands, which were suggested to result from the formation of artificial heteroduplexes between multiple amplified fragments (Wenger and Nielsen, 1991), or from non-specific amplification. He *et al.* (1992) described that these artifacts were minimized on the gradient gel, where the latter controls the consistency of PCR products by denaturing artificial heteroduplexes. However, these artifacts were excluded from the data collected for RAPD-PCR analysis. Due to the high number of polymorphic bands, we found that the use of denaturing gel electrophoresis was not necessary. In conclusion, the primers used in this study provided high level of resolution. All of them were sensitive enough to differentiate among the five *Streptomyces* strains (Figure 1).

Strain-specific markers

The specific markers for *Streptomyces* strains generated from RAPD-PCR analysis are shown in Tables (6 and 7). As high as 192 out of the 327 (59%) RAPD-PCR markers were found to be useful as strain-specific markers. The largest number of RAPD-PCR specific markers was scored for the new *Streptomyces* sp. Si-1 (72 markers), while the lowest (14 markers) was scored for strain Si-4. A number of 157 specific markers were scored for the presence of a unique band for a given strain (positive marker), while 35 were scored for the absence of a common band (negative marker). Thirty-two of them were scored for either the new strain of *Streptomyces* Si-1 or Si-4, while 2 and 1 only were scored for strains Si-11 and Si-6, respectively. In the meantime, the largest number of RAPD-PCR strain-specific markers was generated by primer E06 (18 markers) followed by primer O11 (17 markers). On the other hand, the least number of RAPD-PCR specific markers were generated by primer A20 (5 markers) followed by A13 (9 markers).

In conclusion, all primers used in the present study allowed for enough distinction among the five *Streptomyces* strains. Overall comparison among strains across the 16 primers revealed the power of RAPD-PCR analysis in distinguishing among closely related *Streptomyces* strains. These strain-specific markers can be used in subsequent experiments to detect molecular markers for polymorphic genes among these and other *Streptomyces* strains. In other words, the present study calls for the search for more RAPD-PCR markers to initiate the genome map of *Streptomyces* sp. that involves molecular genetic markers linked to several genes of interest obtained from contrasting genotypes.

Phylogenetic relationships

The dendrogram tree and the similarity indices among the five *Streptomyces* strains utilizing RAPD-PCR markers (Figure 2 and Table 8, respectively) were detected by DICE computer package. The analysis was based on the number of markers that were different between any given pair of strains. The strongest relationship was scored between *Streptomyces* strains Si-11 and Si-4 (similarity of 70.3%), while *Streptomyces* strains Si-1 and Si-9 were shown to be the most genetically distant strains (similarity index of 29.0%) when compared with the other 3 strains (Figure 2 and Table 8). In addition, pairs of strains Si-4/Si-6 (similarity index of 59.8%) followed by strains Si-11/Si-6 (similarity index of 55.9%) can be considered as moderately related pairs of strains using RAPD-PCR analysis. From the dendrogram tree, the *Streptomyces* sp. Si-1 strain appeared to be a novel species with a similarity index of 33.0% when compared with the other four

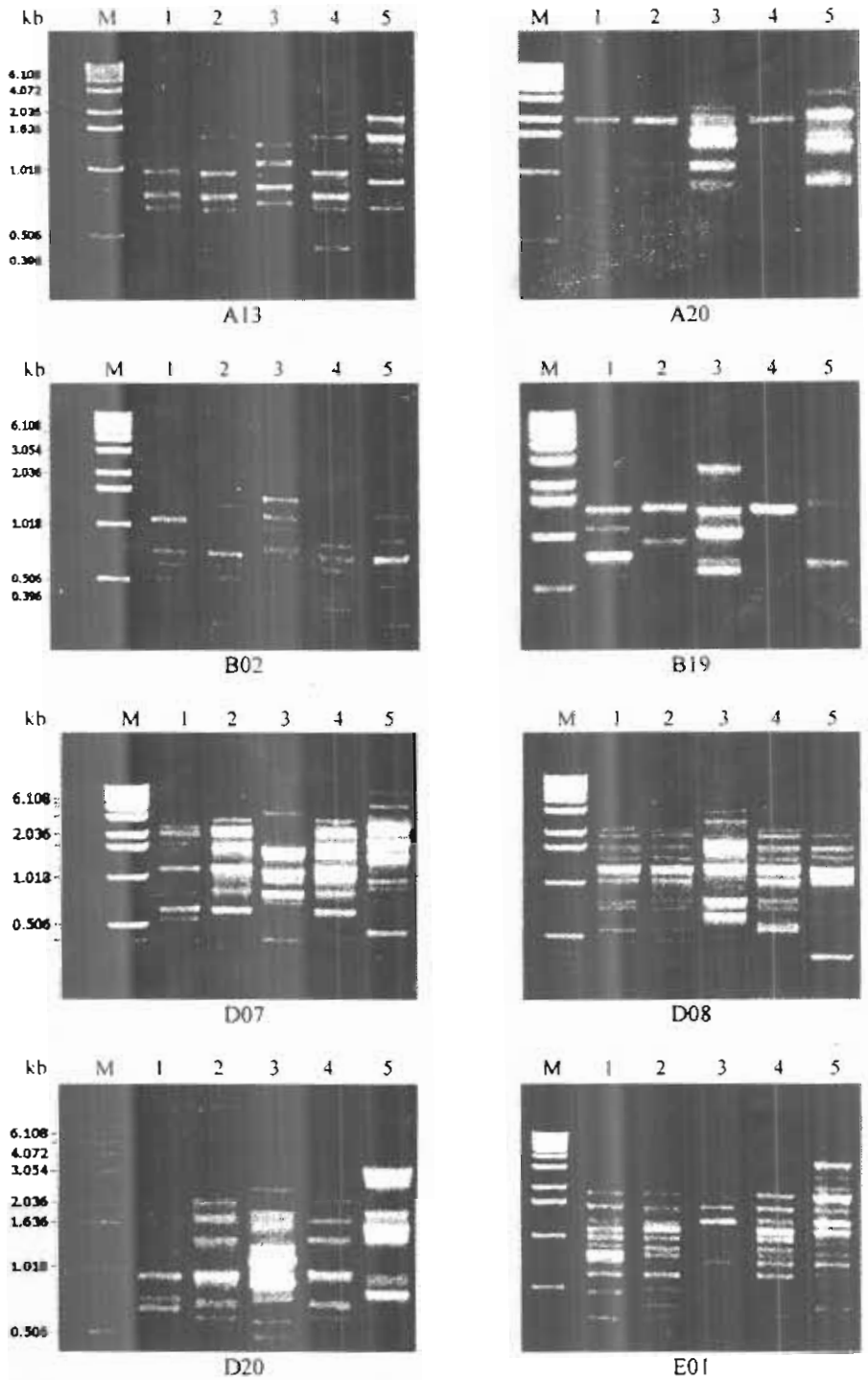

Fig. (1): ***Agarose gel (1.5%) in TAE buffer stained with ethidium bromide showing RAPD-PCR polymorphism of DNA of Streptomyces strains (Lanes 1-5, Streptomyces sp. Si-11, S. muavecolor Si-4, S. tuirus Si-1, S. melanogenes Si-6, S. lateritius Si-9, respectively) using A13, A20, B02, B19, D07, D08, D20, E01, E02, E06, E11, E20, O10, O11, Z01 and Z18 random primers. M refers to 1-kb DNA ladder.***

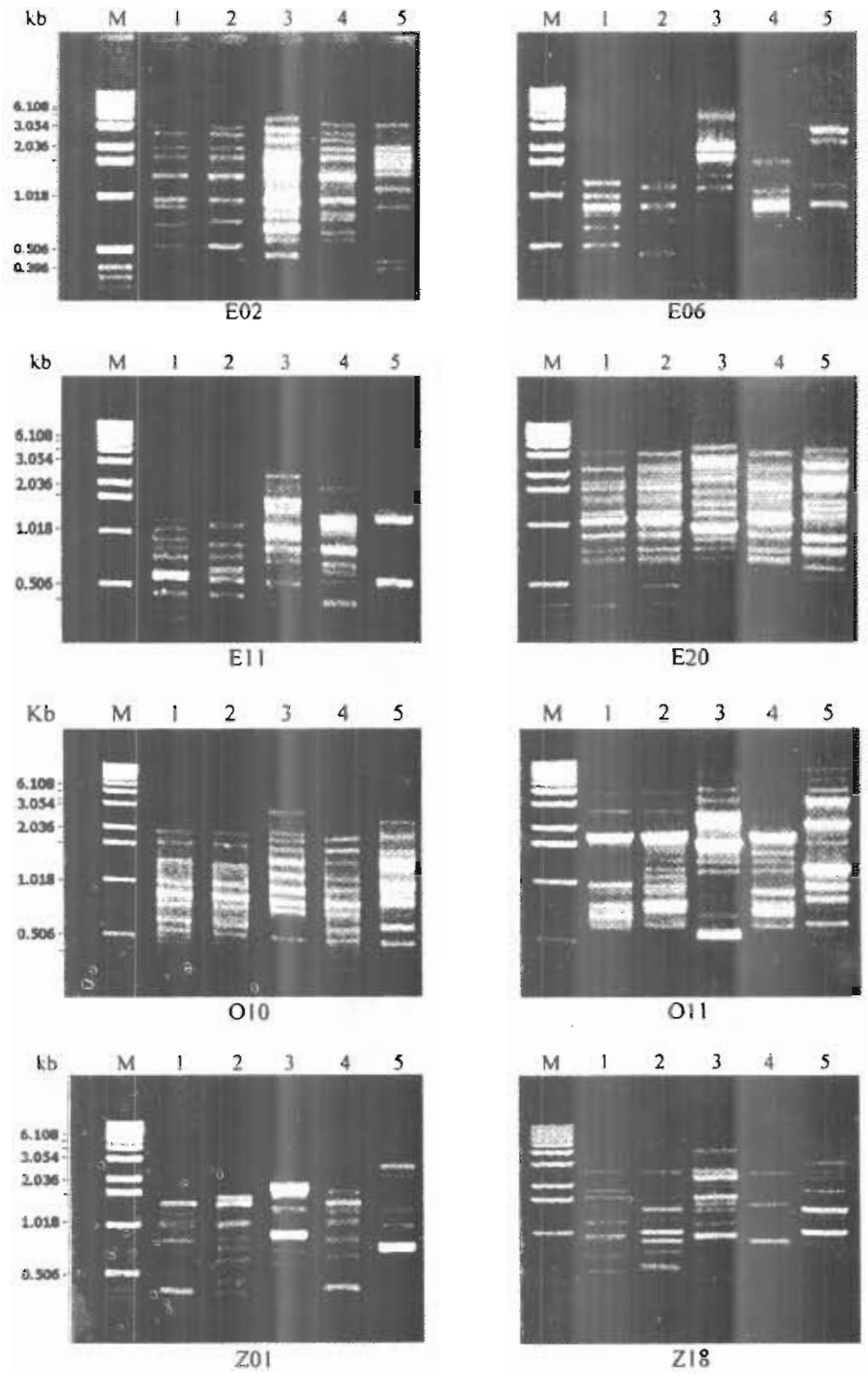

Fig. (1): Continued

strains (Figure 2). The *Streptomyces muavecolor* Si-9 strain was also shown to be genetically distant when compared with the other three strains Si-6, Si-4 and Si-11. The latter three strains can be considered as closely related *Streptomyces* strains with a similarity index of 62.0% across the three strains.

Table (6): Number of amplified fragments and specific markers of five Streptomyces strains based on RAPD-PCR analysis.

			Violet series strains				Red series strains						
			Si-1		Si-9		Si-4		Si-6		Si-11		
Primer	TAF	PB	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	TSM
A13	18	15	11	4	9	4	7	0	11	1	7	0	9
A20	10	8	6	3	7	2	4	0	4	0	4	0	5
B02	18	18	6	3	6	3	5	2	6	3	5	0	11
B19	13	13	5	2	4	4	3	0	3	0	6	4	10
D07	20	20	7	4	10	5	10	0	9	0	8	1	10
D08	22	20	12	9	8	4	11	0	12	1	11	0	14
D20	25	25	10	8	9	3	9	1	7	1	6	1	14
E01	18	15	4	4	15	4	12	0	10	0	11	2	10
E02	24	24	11	5	10	8	8	0	12	1	11	0	14
E06	22	22	7	3	4	2	6	1	7	7	7	5	18
E11	24	24	10	3	4	1	10	2	8	4	9	1	11
E20	24	22	12	6	13	4	12	1	10	0	12	1	12
O10	19	16	10	4	10	6	9	0	10	1	9	0	11
O11	27	27	9	7	11	6	12	2	9	1	8	1	17
Z01	20	20	6	3	5	2	8	2	7	4	5	1	12
Z18	23	22	10	4	8	4	7	3	3	0	7	3	14
Total	327	311	136	72	133	62	133	14	128	24	126	20	192

TAF = Total amplified fragment, PB = Polymorphic bands, AF = Amplified fragment, SM = Specific marker, including either the presence or absence of a band in specific strain, TSM = Total no. specific markers across strains.

In addition, compiling the data available for the differences in mycelial color as a morphological characteristic resulted in the detection of 30 RAPD-PCR markers for both colors, red (*S. melangenes* Si-11, *S. tuirus* Si-4 and *S. lateritius* Si-6) and violet (*Streptomyces* sp. Si-1 and *S. muavecolor* Si-9). More studies are needed to be done to ensure the use of one or more of these RAPD-PCR markers to detect color of different strains.

Utilizing RAPDs as molecular markers in *Streptomyces* gained a wide acceptance in the latest publications (Mohamed *et al.*, 2001). The present study also recommends the use of RAPDs as a new taxonomical tool for actinomycetes besides the classical proposed keys used in Bergey's Manual. The molecular analyses used in the present study provide a simple mean for verification of phylogenetic relationships without the need of additional sources of information that might be subjected to experimental error over time. More importantly, the level of polymorphism detected by using RAPDs will provide molecular biologists with environment-independent markers. Furthermore, many markers can readily be identified for a variety of taxonomical levels and to target several characteristics of the microorganism.

Table (7): Strain-specific markers in five Streptomyces strains resulting from RAPD-PCR analysis.

Streptomyces strain	Marker		Total
	Positive	Negative	
S.melanogenes Si-11	B19-3193, B19-1026, B19-783, B19-680, D07-618, D20-730, E01-683, E06-1007, E06-845, E06-697, E06-640, EO6-589, E11-543, E20-595, ZO1-1057, Z18-900, Z18-771, Z18-718.	E01-669, O11-1088.	20
S. tuirus Si-4	B02-1058, B02-628, D20-1176, E06-667, E11-918, E11-529, E20-597, O11-1019, O11-780, Z01-1374, Z01-635, Z18-943, Z18-798, Z18-733.		14
Streptomyces sp. Si-1	A13-3627, A13-995, A13-364, A20-2612, A20-830, B02-1171, B02-679, B02-566, B19-2631, B19-995, D07-3578, D07-902, D07-578, D08-3290, D08-2850, D08-2074, D08-651, D08-594, D08-474, D20-2344, D20-1460, D20-993, D20-935, D20-846, D20-686, D20-650, E02-3922, E02-1223, E02-608, E06-4269, E06-1758, E06-1557, E11-4815, E11-2103, E11-1289, E20-4025, E20-1806, E20-811, O10-3126, O10-1554, O10-1325, O11-4479, O11-3543, O11-2467, O11-2187, O11-734, O11-688, Z01-850, Z01-1548, Z01-725, Z18-4620, Z18-2374, Z18-1961, Z18-699.	A13-3276, A20-862, D07-1565, D08-2304, D08-1605, D08-1124, D20-875, E01-928, E01-848, E01-759, E01-621, E02-1866, E02-1530, E20-3383, E20-1630, E20-1455, O10-1524, O11-728.	72
S. lateritius Si-6	A13-2181, B02-1275, B02-697, B02-612, D08-553, D20-1525, E02-700, E06-1423, E06-1228, E06-884, E06-774, E06-727, E06-678, E11-1616, E11-689, E11-611, E11-582, O10-550, O11-678, Z01-1426, Z01-1020, Z01-702, Z01-604.	E06-752.	24
S. muavecolor Si-9	A13-2315, A13-1050, A13-583, A20-3699, A20-1147, B02-729, B02-605, B02-543, B19-1756, B19-1465, B19-665, D07-7337, D07-4620, D07-754, D08-1192, D08-334, D20-1365, D20-675, D20-611, E01-3698, E01-2612, E01-2114, E01-525, E02-1141, E02-927, E02-596, E02-590, E06-2760, E06-2133, E11-1125, E20-981, E20-628, O10-2851, O10-2156, O10-914, O10-633, O11-6676, O11-5375, O11-3312, O11-2046, O11-835, O11-716, Z01-2780, Z01-2294, Z18-3214, Z18-2235, Z18-1233, Z18-843.	A13-1193, B19-1323, D07-1363, D07-715, D08-1391, D08-816, E02-3026, E02-2362, E02-1086, E02-805, E20-2177, E20-656, O10-1873, O10-676.	62
Total	157	35	192

Table (8): Similarity indices (%) calculated by DICE computer package among five Streptomyces strains based on RAPD-PCR analysis.

Streptomyces strain	Si-11	Si-4	Si-1	Si-6	Si-9
S. melanogenes Si-11	100.0				
S. tuirus Si-4	70.3	100.0			
S. sp. Si-1	32.8	34.2	100.0		
S. lateritius Si-6	55.9	59.8	37.1	100.0	
S. muavecolor Si-9	39.4	41.4	29.0	36.8	100.0

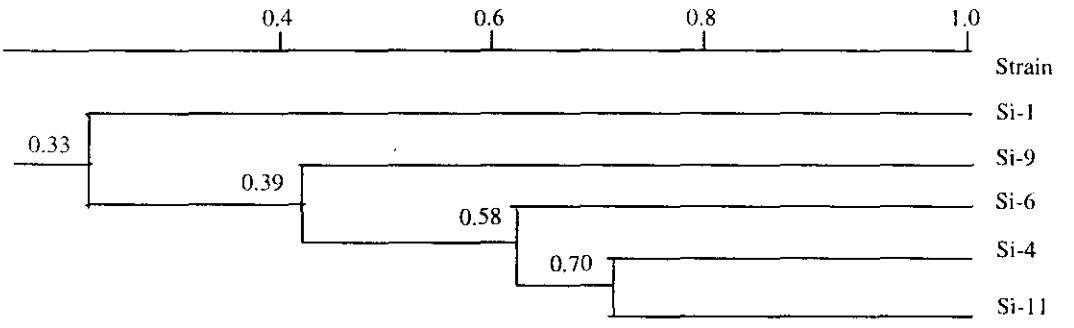

Fig. (2): Phenogram demonstrating the relationships among five Streptomyces strains based on a compiled data set.

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الملخص العربي

بعض الدراسات الفسيولوجية والتضادية والبصمة الوراثية لبعض الاستربتوميسيتات المتحملة للملوحة

هشام طه محفوظ* وسونيه حموده محمد**

* معهد بحوث الهندسة الوراثية الزراعية، مركز البحوث الزراعية، الجيزة، ص.ب.١٢٦١٩، مصر

*** قسم الميكروبيولوجيا الزراعية، معهد بحوث الأراضي والمياه والبيئة، مركز البحوث الزراعية، الجيزة، ص.ب.١٢٦١٩ ، مصر*

في هذا العمل تم استخدام خمس سلالات من الاستربتوميسيتات المتحملة للملوحة. وقد ثبت ان هذه السلالات تختلف في قدرتها علي تحمل تركيزات مختلفة من مبيدي الحشائش السينكور والباستا في بيئة النمو. كما أوضحت نتائج النشاط الانزيمي لهذه السلالات قدرتها علي تحليل كل من الكازين والنشا، بينما فشلت في تكسير البكتين في بيئة النمو. و قد أظهرت السلالات بنفسجية اللون القدرة علي استخدام السليلوز كمصدر للكربون بينما لم تستطع السلالات حمراء اللون. أما الجيلاتين كمصدر للبروتين في بيئة النمو فقد استخدم فقط بواسطة السلالة *S. muavecolor* Si-9 . ومن جهة اخرى فقد أظهرت السلالات حمراء اللون S. *melanogenes* Si-11, *S. lateritius* Si-6, *S. tuirus* Si-4 أعلي نشاط تضادى لـ ١١ كائن مختبرمن البكتريا و الفطريات والخميرة حيث أظهروا قدرة تضاديه لـ ٧،٧،١١ كائن على التوالى. وفي اختبار القدرة التضاديه لهذه السلالات لفيروس موزيك الدخان أظهرت السلالتان *S.melanogenes* Si-11, *S. lateritius* Si-6 أعلي نشاط تضادي للفيروس، حيث ان النقط الميتة الموضعية قد ظهرت علي أوراق نبات الداتورة ميتل بعد ان تم تخفيف الراشح لكلا السلالتين إلى ١:٨٠ و ١:٤٠. وبناء علي هذا فان هاتين السلالتين يمكن استخدامهما لانتاج مواد تضاديه. ومن خلال دراسة البصمه الوراثية للسلالات الخمس من الستربتوميسيتات بإستخدام تحليل RAPD-PCR فقد تم تحديد ٣١١ حزمه دنا (DNA) من مجمل عدد ٣٢٧ حزمه (تمثل حوالى ٩٥%) ناتجه من ١٦ بادئ عشوائى والتى يمكن إعتبارها كواسمات RAPD-PCR markers. وأوضحت النتائج ان عدد قليل من الحزم كانت شائعة (وحيدة المظهر Monomorphic) فى جميع السلالات. كما تم تحديد ١٩٢ واسمه مميزة للسلالات تمثل نسبة ٥٩% من مجمل حزم الدنا. تم تحديد درجة التشابه بين السلالات الخمسة من الستربتوميسيتات باستخدام واسمات الـ DNA وقد سجلت السلالتان *S. melanogenes* si-11, *S. uirus* si-4 أعلى درجات التشابه (٧٠,٣%) بينما سجلت السلالتان *S. muavecolor* Si-9, *Streptomyces* sp. Si-1 أقل درجات التشابه ٢٩%. وبينت شجرة القرابة Dendrogram tree أن السلالة *Streptomyces* sp. Si-1 تعتبر سلالة جديدة بالمقارنة بالأربعة سلالات الأخرى وأعطت درجة التشابه معهم مقدارها ٣٣%. ومن ناحية أخرى وجد أن السلالة *S. muavecolor* Si-9 كانت أقل تشابها بالمقارنة بالثلاثة سلالات الأخرى *S. uirus* Si,-4, *S. lateritius* Si-6, *S. melanogenes* Si-11. وقد أكدت نتائج هذه الطرق الجزيئية التعريف السابق لهذه السلالات وتقسيمهم إلى مجموعتين. كما اتضح ان السلالة *Streptomyces* sp. Si-1 تعتبر جديده لاختلافها عن باقي السلالات. ولهذا فان هذه الدراسة أيضا توصي باستخدام هذه التقنيات الحديثة كأداة جديدة لتصنيف الأكتينوميسيتات بجانب المفاتيح المقترحة والمستخدمة في كتاب برجي.