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USE OF RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)-POLYMERASE CHAIN REACTION (PCR) ANALYSIS TO FINGERPRINT SOME *RHIZOBIUM LEGUMINOSARUM* BIOVAR *VICEAE* ISOLATES

[11]

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ABSTRACT

A trial was performed to use the molecular markers to differentiate between ten isolates of *Rhizobium leguminosarum* biovar *viceae* varied in their abilities to phage susceptibility (one isolate sensitive to lytic phage, four isolates sensitive to temperate phage, four isolates lysogenic and one isolate resistant to phage). The nucleic acids of these isolates were extracted, purified and separately used as templates for PCR analysis using ten short oligonucleotides (OP-A01, OP-A05, OP-A16, OP-A18, OP-B02, OP-B03, OP-B04, OP-B06, OP-B08 and OP-B10) of arbitrary nucleotide sequences. The amplified DNA polymorphisms were electrophoresed on 1.2% agarose gel in TAE buffer. The molecular relationship between the ten applied isolates was determined. Results of random amplified polymorphic of DNA (RAPD)- polymerase chain reaction (PCR) showed that, polymorphic (not common for all isolates) as well as monomorphic (common for all isolates) fragments were obtained for each isolate. The number of amplified fragments as expected was differed with some different oligonucleotides. In some cases, the number and size of amplified fragments differed from one isolate to another for the same oligonucleotide. In addition, unique band(s), whatever present or absent, were characterized for some isolates. Statistical analysis of RAPD-PCR polymorphisms and the phylogenetic tree revealed a degree of similarities ranged from 90.4 to 99.1 %. Therefore, the results of this research paid an attention to encourage the use of RAPD-PCR technique as a new sensitive and reliable molecular tool for fingerprinting the *Rhizobium* strains.

Key words: *Rhizobium. leguminosarum* biovar *viceae*, RAPD-PCR, DNA fingerprinting, Molecular markers, Phage susceptibility

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INTRODUCTION

The leguminous crops play a significant and fundamental role in agriculture production. The Nile valley has adequate levels of native populations of *Rhizobium leguminosarum* permitting the development of a good symbiotic nitrogen fixation system (**Hamdi, 1982**). **Teaumroong and Boonkerd (1998)** reported that rhizobia have been extensively used in agricultural systems for enhancing the ability of legumes to fix atmospheric nitrogen. **Keyser and Cregan (1987)** showed that competition from indigenous *Rrhizobium* strains usually limits the performance of the inoculant strains. It is therefore important to distinguish inoculant strains from other indigenous rhizobia and to identify the strain responsible for nodulation.

In the 1980s, the stability of markers that were used for the identification of rhizobial field isolates was crucial importance for successful interpretation of results from inoculation and competition experiments. The identification methods commonly used were phage typing (**Bromfield *et al* 1986**), intrinsic antibiotics resistance "IAR" (**Kremer and Peterson, 1982 and El-Hassan *et al* 1986**), protein profiles and serological properties (**Bohlool and Schmidt, 1980**; **Martensson and Gustafsson, 1985** and **Demezas and Bottomley, 1986**). Either separately or in combination some of the previous method and plasmid profiles were also used for evaluating the diversity of indigenous rhizobia in soil (**Brockman and Bezdicek, 1989**).

De Bruijn (1992) developed new approaches to evaluate the diversity of indigeneous rhizobia using the polymerase chain reaction (PCR). **Dovkishinevsky *et al*** (**1996**) determined the cultural and physiological properties, serology, plasmid profiles and infective traits for 23 strains of rhizobia from various *Hedysarum* species. They reported that no particular plasmid profile pattern was observed in relation to the host or geographical region of the strains. They also used the random amplified polymorphic DNA (RAPD) fingerprinting method with five *H. coronarium* and three *H. alpinum* strains. **Perret and Broughton (1998)** used the targeted PCR fingerprinting (TPF) technique for rapid identification of *Rhizobium* strains as well as descriminating between *Rhizobium* species NGR234 and *R. fredii* USDA257 using PCR primers specific for the *nifH* and *recA* genes.

In this study, a trial to use the molecular markers *via* RAPD-PCR analysis to fingerprint ten *R. leguminosarum* biovar *viceae* varied in their abilities to phage susceptibility was carried out.

MATERIAL AND METHODS

Source of *Rhizobium* isolates

Ten isolates of *R. leguminosarum* biovar *viceae* (S2, S23, d89, A107, S112, S24, I41, D96, D97 and 2435) varied in their phage susceptibility (Table, 1) were obtained from the Department of Agricultural Botany, Faculty of Agriculture, Zagazig University, Zagazig, Egypt.

Samples preparation

The isolates of *Rhizobium* were grown in *Rhizobium* minimal medium (RMM, **Broughton *et al* 1986**). Fifty ml portions of the medium were dispensed into 250-ml conical flasks. Five ml of the inoculum of each of the ten *Rhizobium* isolates

Table 1. Source and phage susceptibility of *R. leguminosarum* biovar *viceae* isolates **(Radwan, 2000)**

Rhizobium isolate	Name	Source	Phage susceptibility
1	S2	Sharkia	Sensitive to lytic phage
2	S23	Sharkia	Sensitive to temperate phage
3	d89	Dakahlia	Sensitive to temperate phage
4	A107	Alexandria	Sensitive to temperate phage
5	S112	Sharkia	Sensitive to temperate phage
6	S24	Sharkia	Lysogenic isolate
7	I41	Ismalia	Lysogenic isolate
8	D96	Damietta	Lysogenic isolate
9	D97	Damietta	Lysogenic isolate
10	2435	USAD-USA	Resistant strain (standard)

were added to each flask, then incubated at 28°C±2 for 3-4 days on a rotary shaker (120 rpm). Two flasks were used for each isolate and a flask was left without inoculation to serve as control.

DNA extraction of *Rhizobium* isolates

The total nucleic acid extracts were prepared based on the method recomended by **Marmur (1961) and Mohamed *et al* (2001)** with some modifications. Five ml of each culture were transferred into a fresh centrifuge tube and equal volume of phenol: chloroform (v:v) mixture was added. The mixture was handly shaked gently for two times followed by centrifugation at 13000 rpm at room temperature (RT). To the upper phase an equal volume of chloroform was added and again handly shaked gently for 15 sec. The mixture was then centrifuged for 15 min at 13000 rpm at RT. The upper layer was transferred to a new tube and double volumes of cold absolute ethanol and tenth volume of 3M sodium acetate (pH 5.2) was added followed by incubation at -20°C overnight. The pellets of nucleic acids were collected by centrifugation at 14000 rpm for 20 min at 4°C followed by washing with 70 % ethanol, brief drying and resuspended in 500 µl of TE buffer **(Sambrook *et al* 1989)**.

DNA purification

To the nucleic acids suspension, the ribonuclease (free DNase) was added with a final concentration of 1 µg/ml followed by digestion for 30 min at 37°C in a water bath. The protein molecules were removed by adding the proteinase K with a final concentration of 50 µg/ml followed by incubation for 1 hr in a water bath. The DNA was then re-extracted with phenol/chloroform and precipitated as mentioned above **(Mohamed *et al* 2001)**.

Purity and concentration of DNA

The purity and concentration of DNA extracts of the ten *Rhizobium* isolates were determined as recommended by **Brown (1990)** using the ultraviolet absorbence spectrophotometer (Model Du-40 spectrophotometer Beckman). With a pure sample of DNA the $A_{260/280}$ is 1.8-2, the DNA concentration was then adjusted to 100 ng/µl.

Primers used for RAPD-PCR

Ten decamer oligonucleotide primers; OP-A01, OP-A05, OP-A16, OP-A18, OP-B02, OP-B03, OP-B04, OP-B06, OP-B08 and OP-B10 from OPERON Technologies, Alameda, CA., kits A and B, were used for RAPD-PCR. The nucleotide sequences of the applied primers are presented in Table (2).

RAPD-PCR

RAPD-PCR was carried out based on the procedure described by **Mohamed *et al* (2001).** The amplification reaction was conducted on a volume of 50 µl. Each reaction mixture contained 100 ng genomic DNA (as a template), 50 pmole primer, 2 units of *Taq* DNA polymerase (Promega Crop., Madison, WI, USA), 5 µl of 10X-PCR buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs (dATP, dCTP, dGTP and dTTP) and deionized distilled water (dd.H_2O). The PCR amplification was performed in a Perkin-Elmer GeneAmp PCR System 2400 for 35 cycles after initial denaturation for 5 min at 94°C. Each cycle consisted of denaturation at 94°C for 1 min, annealing temperature at 37°C for 2 min and extension at 72°C for 2 min. The final primer extension cycle was extended to 7 min.

DNA electrophoresis

The amplified products were resolved by electrophoresis in a 1.2 % agarose gel in TBE buffer at 100 volts for 1 hr **(Sambrook *et al* 1989)**. PCR products were visualized by staining gel with ethidium bromide (0.5 µg/ml) and photographed under UV light using a Polaroid camera.

RAPD analysis

Amplified products were visually examined and scored as present (1) or absence (0). Bands of the same mobility were scored as identical. The similarity coefficient (F) between isolates was defined by the formula of **Nei and Li (1979).** A dendrogram was derived from the distance by unweighted paired-group method, arithmetic mean (UPGMA) contained in the computer program package NTSYS 1.5 **(Rohlf, 1990).**

RESULTS AND DISCUSSION

Williams *et al* (1990) were the first who 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). These polymorphisms, simply detected as DNA segments which amplified from one parent but not the other inherited in a Mendelian fashion and can be used to construct genetic maps in a variety of species. Those results suggested that these polymorphisms be called RAPD markers.

Table 2. The nucleotide sequences of the primers used for RAPD-PCR analysis

No.	Primer names	Sequences (5'-----3')
1	OP-A01	CAG GCC CTT C
2	OP-A05	AGG GGT CTT G
3	OP-A16	AGC CAG CGA A
4	OP-A18	AGG TGA CCG T
5	OP-B02	TGA TCC CTG G
6	OP-B03	CAT CCC CCT G
7	OP-B04	GGA CTG GAG T
8	OP-B06	TGC TCT GCC C
9	OP-B08	GTC CAC ACG G
10	OP-B10	CTG CTG GGA C

In this study ten isolates of *R. leguminosarum* biovar *viceae* varied in their susceptibility to phage were used. Data of RAPD-PCR presented in Tables (3), (4), (5), (6), (7), (8), (9), (10), (11) and (12) and Figures (1), (2), (3), (4) and (5) clearly indicated difference and relationship between the ten applied *R. leguminasarum* biovar *viceae* isolates based on RAPD-PCR fingerprinting analysis. The present data therefore confirms those of belonging to the same species.

The absence of amplified bands was observed in the negative controls revealed that the reaction mixtures were free from any strange DNA contamination. For each isolate, number of amplified fragments differed with different primers, as expected (Table, 13) was found. As the number of produced fragments for these isolates under investigation was ranged from 51 to 58 out of 62 fragments produced using the ten applied primers (Table 13). On the other direction, the number and size of amplified fragments differed from one isolate to another for the same primer. Moreover, the isolates were characterized by unique band(s) with the same primers used (Table, 14). In other word, 7, 7, 4, 3, 3, 2, 3, 4, 7 and 4 out of 15 unique fragments were obtained with the ten *Rhizobium* isolates, respectively. However, some bands were common for all isolates (Monomorphic bands). Statistical analysis of RAPD-PCR polymorphisms revealed a degree of similarities with ratio from 90.4 to 99.1 % between the *Rhizobium* isolates (Table, 15).

In addition, data of DNA fingerprinting based on RAPD-PCR represented in Table (15) and Figure (6) confirmed the geographical distribution of the *Rhizobium* isolates under investigation. As the phylogenetic tree in Figure (6) showed that isolates 5, 6 and 7 obtained from Sharkia and Ismalia soils represented a cluster with similarity ranged from 97.2 to 99.1%. Isolates 1, 2 and 3 obtained

Table 3. Scoring the amplified fragments produced using OP-A01 primer based on RAPD-PCR of DNA extracted from ten *R. leguminosarum* biovar *viceae* isolates (1-10, Refer to Table 1)

Fragment	M_r (bp)	*R. leguminosarum* biovar *viceae* isolates									
		1	2	3	4	5	6	7	8	9	10
F1	2500	1	1	1	1	1	1	1	1	1	1

Table 4. Scoring the amplified fragments produced using OP-A05 primer based on RAPD-PCR of DNA extracted from ten *R. leguminosarum* biovar *viceae* isolates (1-10, Refer to Table 1)

Fragment	M_r (bp)	*R. leguminosarum* biovar *viceae* isolates									
		1	2	3	4	5	6	7	8	9	10
F2	4885	1	1	1	1	1	1	1	1	1	1
F3	2222	0	0	0	1	0	0	0	0	0	0
F4	1912	1	1	1	1	1	1	1	1	1	1
F5	1449	1	1	1	1	1	1	1	1	1	1
F6	1235	1	1	1	1	1	1	1	1	1	1
F7	1169	0	0	0	0	0	0	0	1	1	1
F8	0953	1	1	1	1	1	1	1	1	1	1
F9	0320	0	0	0	0	0	0	0	1	1	1

Table 5. Scoring the amplified fragments produced using OP-A16 primer based on RAPD-PCR of DNA extracted from ten *R. leguminosarum* biovar *viceae* isolates (1-10, Refer to Table 1).

Fragment	M_r (bp)	*R. leguminosarum* biovar *viceae* isolates									
		1	2	3	4	5	6	7	8	9	10
F10	2728	1	1	1	1	1	1	1	1	1	1
F11	1927	1	1	1	1	1	1	1	1	1	1
F12	1460	1	1	1	1	1	1	1	1	1	1
F13	1223	1	1	1	1	1	1	1	1	1	1
F14	1103	1	1	1	1	1	1	1	1	1	1
F15	0951	1	1	1	1	1	1	1	1	1	1
F16	0539	1	1	1	1	1	1	1	1	1	1

Table 6. Scoring the amplified fragments produced using OP-A18 primer based on RAPD-PCR of DNA extracted from ten *R. leguminosarum* biovar *viceae* isolates (1-10, Refer to Table 1)

Fragment	M_r (bp)	*R. leguminosarum* biovar *viceae* isolates									
		1	2	3	4	5	6	7	8	9	10
F17	2042	1	1	1	1	1	1	1	0	1	1
F18	1645	1	1	1	1	1	1	1	1	1	1
F19	1417	1	1	1	1	1	1	1	1	1	1
F20	0999	1	1	1	1	1	1	1	1	1	1

Table 7. Scoring the amplified fragments produced using OP-B02 primer based on RAPD-PCR of DNA extracted from ten *R. leguminosarum* biovar *viceae* isolates (1-10, Refer to Table 1)

Fragment	M_r (bp)	*R. leguminosarum* biovar *viceae* isolates									
		1	2	3	4	5	6	7	8	9	10
F21	4600	1	1	0	0	0	0	0	0	0	0
F22	4500	0	0	0	0	0	0	0	0	1	0
F23	2188	1	1	1	1	1	1	1	1	0	1
F24	2154	0	0	0	0	0	0	0	0	1	0
F25	1482	1	1	1	1	1	1	1	1	1	1
F26	0650	1	1	1	1	1	1	1	1	1	1

Table 8. Scoring the amplified fragments produced using OP-B03 primer based on RAPD-PCR of DNA extracted from ten *R. leguminosarum* biovar *viceae* isolates (1-10, Refer to Table 1)

Fragment	M_r (bp)	*R. leguminosarum* biovar *viceae* isolates									
		1	2	3	4	5	6	7	8	9	10
F27	3700	1	1	0	0	0	0	0	0	1	0
F28	2629	1	1	1	1	1	1	1	1	1	1

Table 9. Scoring the amplified fragments produced using OP-B04 primer based on RAPD-PCR of DNA extracted from ten *R. leguminosarum* biovar *viceae* isolates (1-10, Refer to Table 1)

Fragment	M_r (bp)	*R. leguminosarum* biovar *viceae* isolates									
		1	2	3	4	5	6	7	8	9	10
F29	4000	0	0	0	1	0	1	1	1	1	1
F30	279	1	1	1	1	1	1	1	1	1	1
F31	2265	1	1	1	1	1	1	1	1	1	1
F32	1946	1	1	1	1	1	1	1	1	1	1
F33	1742	1	1	1	1	1	1	1	1	1	1
F34	1498	1	1	1	1	1	1	1	1	1	1
F35	1327	1	1	1	1	1	1	1	1	1	1
F36	1134	1	1	1	1	1	1	1	1	1	1
F37	0950	1	1	1	1	1	1	1	1	1	1
F38	0852	1	1	1	1	1	1	1	1	1	1
F39	0774	1	1	1	1	1	1	1	1	1	1

Table 10. Scoring the amplified fragments produced using OP-B06 primer based on RAPD-PCR of DNA extracted from ten *R. leguminosarum* biovar *viceae* isolates (1-10, Refer to Table 1)

Fragment	M_r (bp)	*R. leguminosarum* biovar *viceae* isolates									
		1	2	3	4	5	6	7	8	9	10
F40	2618	0	0	1	1	1	1	1	1	1	1
F41	2176	1	1	1	1	1	1	1	1	1	1
F42	1787	1	1	1	1	1	1	1	1	1	1
F43	1580	1	1	1	1	1	1	1	1	1	1
F44	1356	1	1	1	1	1	1	1	1	1	1
F45	1057	1	0	1	1	1	1	1	0	0	1
F46	0920	0	1	0	0	0	0	0	1	1	0
F47	0849	0	1	0	0	0	0	0	0	1	0
F48	0796	1	1	1	1	1	1	1	1	1	1

Table 11. Scoring the amplified fragments produced using OP-B08 primer based on RAPD-PCR of DNA extracted from ten *R. leguminosarum* biovar *viceae* isolates (1-10, Refer to Table 1)

Fragment	M_r (bp)	*R. leguminosarum* biovar *viceae* isolates									
		1	2	3	4	5	6	7	8	9	10
F49	3293	1	1	1	1	1	1	1	1	1	1
F50	2788	1	1	1	1	1	1	1	1	1	1
F51	1926	1	1	1	1	1	1	1	1	1	1
F52	1702	1	1	1	1	1	1	1	1	1	1
F53	1518	1	1	1	1	1	1	1	1	1	1
F54	1315	1	1	1	1	1	1	1	1	1	1
F55	1000	0	1	0	1	1	1	1	1	1	1
F56	0698	1	1	1	1	1	1	1	1	1	1

Table 12. Scoring the amplified fragments produced using OP-B10 primer based on RAPD-PCR of DNA extracted from ten *R. leguminosarum* biovar *viceae* isolates (1-10, Refer to Table 1)

Fragment	M_r (bp)	*R. leguminosarum* biovar *viceae* isolates									
		1	2	3	4	5	6	7	8	9	10
F57	4531	1	1	1	1	1	1	1	1	1	1
F58	2025	1	1	1	1	1	1	1	1	1	1
F59	1556	1	1	1	1	1	1	1	1	1	1
F60	1267	1	1	1	1	1	1	1	1	1	1
F61	0933	1	1	1	1	1	1	1	1	1	1
F62	0820	1	1	1	1	1	1	1	1	1	1

Table 13. Number of amplified fragments of ten *R. leguminosarum* biovar *viceae* isolates (1-10, Refer to Table 1) based on RAPD-PCR analysis using ten random primers

Primers	TNAF	*R. leguminosarum* biovar *viceae* isolates									
		1	2	3	4	5	6	7	8	9	10
OP-A01	1	1	1	1	1	1	1	1	1	1	1
OP-A05	8	5	5	5	6	5	5	5	7	7	7
OP-A16	7	7	7	7	7	7	7	7	7	7	7
OP-A18	4	4	4	4	4	4	4	3	4	4	4
OP-B02	6	4	4	3	3	3	3	3	3	4	3
OP-B03	2	2	2	1	1	1	1	1	1	2	1
OP-B04	11	10	10	10	11	10	11	11	11	11	11
OP-B06	9	6	7	7	7	7	7	7	7	8	7
OP-B08	8	7	8	7	8	8	8	8	8	8	8
OP-B10	6	6	6	6	6	6	6	6	6	6	6
Total	62	52	54	51	54	52	53	52	57	58	55

TNAF: Total number of amplified fragments.

Table 14. Number of polymorphic and unique fragments of ten *R. leguminosarum* biovar *viceae* isolates (1-10, Refer to Table 1) based on RAPD-PCR analysis using ten random primers

Primers	TNAF (TUF)	M_r (bp) of Unique band	Present (+) or absent (-) of unique markers *R. leguminosarum* biovar *viceae* isolates									
			1	2	3	4	5	6	7	8	9	10
OP-A01	1 (0)	*	*	*	*	*	*	*	*	*	*	*
OP-A05	8 (3)	2222	-	-	-	+	-	-	-	-	-	-
		1169	-	-	-	-	-	-	-	+	+	+
		0320	-	-	-	-	-	-	-	+	+	+
OP-A16	7 (0)	*	*	*	*	*	*	*	*	*	*	*
OP-A18	4 (1)	2042	-	-	-	-	-	-	+	-	-	-
OP-B02	6 (4)	4600	+	+	-	-	-	-	-	-	-	-
		4500	-	-	-	-	-	-	-	-	+	-
		2188	+	+	+	+	+	+	+	+	-	+
		2154	-	-	-	-	-	-	-	-	+	-
OP-B03	2 (1)	3700	+	+	-	-	-	-	-	-	+	-
OP-B04	11 (1)	4000	+	+	+	-	+	-	-	-	-	-
OP-B06	9 (4)	2618	+	+	-	-	-	-	-	-	-	-
		1057	+	-	+	+	+	+	+	-	-	+
		0920	-	+	-	-	-	-	-	+	+	-
		0849	-	+	-	-	-	-	-	-	+	-
OP-B08	8 (1)	1000	+	-	+	-	-	-	-	-	-	-
OP-B10	6	*	*	*	*	*	*	*	*	*	*	*
Total	62	15	7	7	4	3	3	2	3	4	7	4

*: No unique bands were amplified. +: Present. -: Absent.

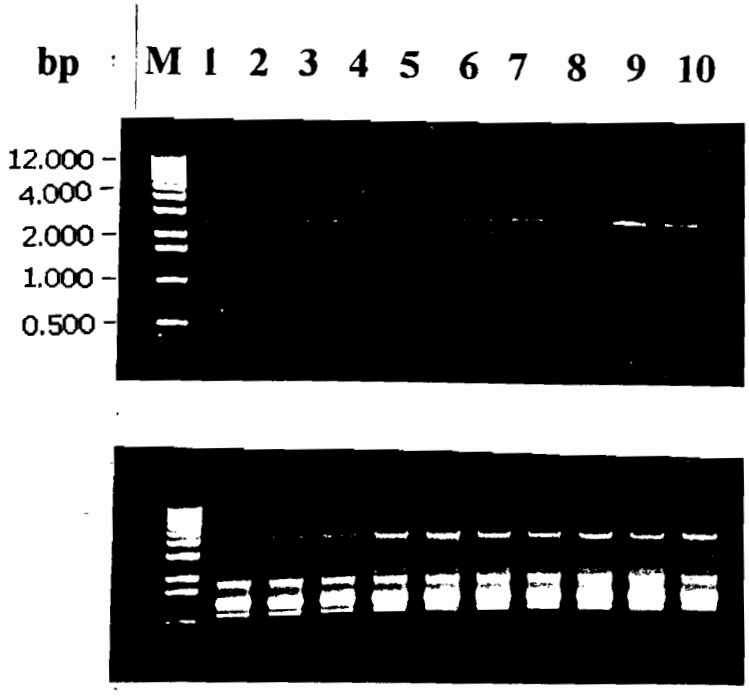

Figure 1. 1% agarose gel in TEB buffer stained with ethidium bromide showing RAPD-PCR polymorphism of DNA of ten *R. leguminosarum* biovar *viceae* isolates (1-10, Refer to Table, Lanes, 1-10, respectively) using OP-A01 (Upper) and OP-OA05 (Lower). M: DNA marker (1 Kb ladder, 500, 1000, 2000, 4000 and 12000 bp)

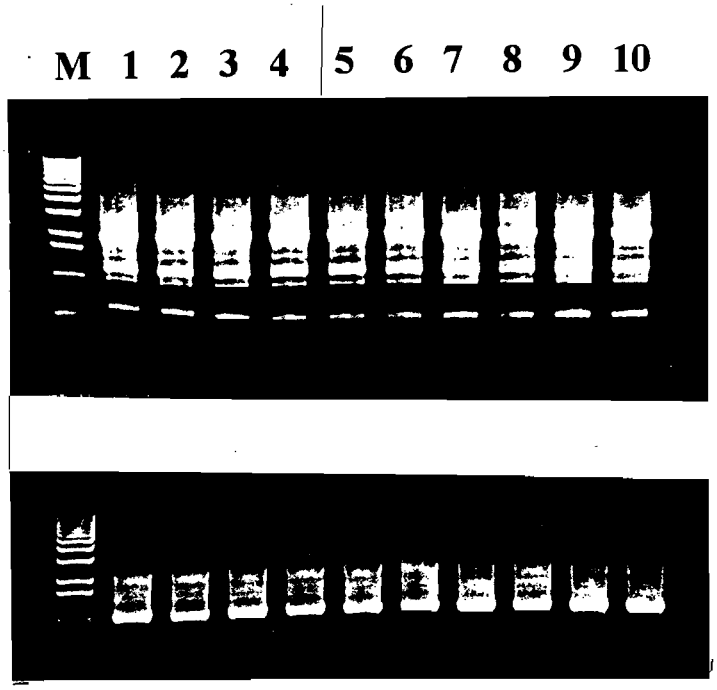

Figure 2. 1% agarose gel in TBE buffer stained with ethidium bromide showing RAPD-PCR polymorphism of DNA of ten *R. leguminosarum* biovar *viceae* isolates (1-10, Refer to Table 1, Lanes, 1-10, respectively) using OP-A16 (Upper) and OP-OA18 (Lower). M: DNA marker (1 Kb ladder, 500, 1000, 2000, 4000 and 12000 bp).

Figure 3. 1% agarose gel in TBE buffer stained with ethidium bromide showing RAPD-PCR polymorphism of DNA of ten *R. leguminosarum* biovar *viceae* isolates (1-10, Refer to Table 1, Lanes, 1-10, respectively) using OP-B02 (Upper) and OP-B03 (Lower). M: DNA marker (1 Kb ladder, 500, 1000, 2000, 4000 and 12000 bp).

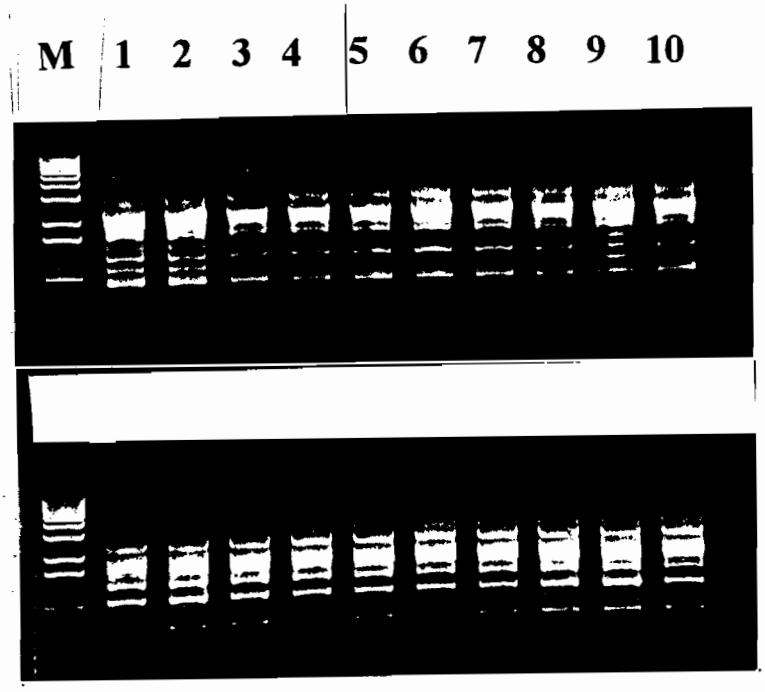

Figure 4. 1% agarose gel in TBE buffer stained with ethidium bromide showing RAPD-PCR polymorphism of DNA of ten *R. leguminosarum* biovar *viceae* isolates (1-10, Refer to Table 1, Lanes, 1-10, respectively) using OP-B04 (Upper) and OP-B06 (Lower). M: DNA marker (1 Kb ladder, 500, 1000, 2000, 4000, 12000 bp).

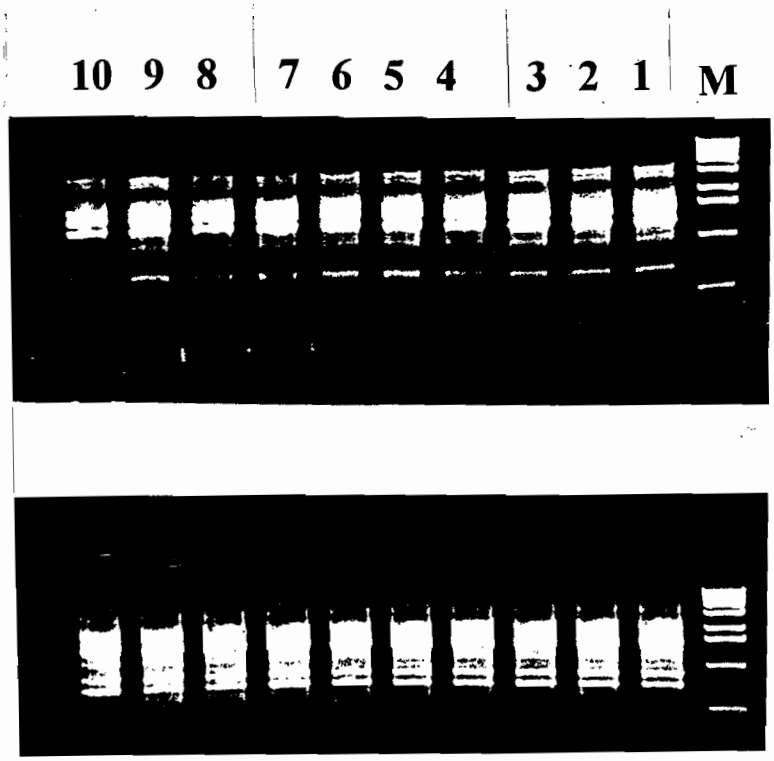

Figure 5. 1% agarose gel in TBE buffer stained with ethidium bromide showing RAPD-PCR polymorphism of DNA of ten *R. leguminosarum* biovar *viceae* isolates (1-10, Refer to Table 1, Lanes, 1-10, respectively) using OP-B08 (Upper) and OP-OB10 (Lower). M: DNA marker (1 Kb ladder, 500, 1000, 2000, 4000 and 12000 bp).

Table 15. Similarity between ten *R. leguminosarum* biovar *viceae* isolates (1-10, Refer to Table 1) based on RAPD-PCR analysis using ten random primers

Isolates	Location	Similarity between ten *R. leguminosarum* biovar *viceae* isolates									
		1	2	3	4	5	6	7	8	9	10
1	Sharkia	100	98.1	98.0	94.3	95.3	94.2	94.3	92.2	94.4	93.3
2	Sharkia		100	96.2	92.6	93.6	92.5	92.6	90.4	96.4	93.5
3	Dakahlia			100	94.2	93.3	94.1	92.3	92.0	92.5	93.2
4	Alexandria				100	95.4	94.3	94.4	94.2	92.7	93.5
5	Sharkia					100	97.2	99.1	95.2	97.3	94.4
6	Sharkia						100	98.1	96.1	94.4	93.3
7	Ismalia							100	96.2	96.4	95.3
8	Damietta								100	94.3	93.2
9	Damietta									100	93.6
10	USAD-USA										100

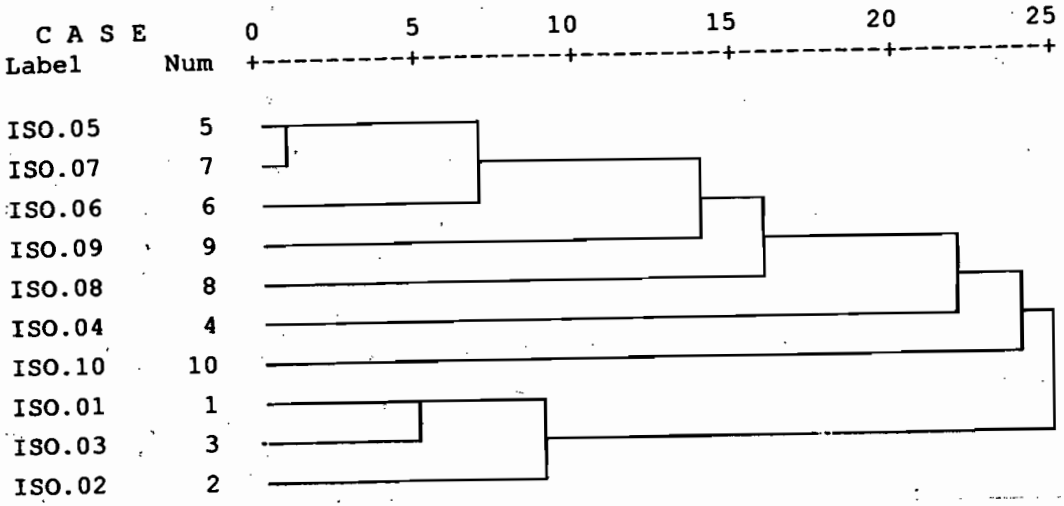

Figure 6. Dendrogram showing molecular relationship between ten *R. leguminosarum* biovar *viceae* isolates (1-10, Refer to Table 1, 1-10, respectively) based on RAPD-PCR analysis.

from Sharkia and Dakahlia represented a cluster with 96.2-98.1% homology. While isolates 8 and 9 that obtained from Damietta soil were close to each other. Finally isolates 4 and 10, which obtained from Alexandria and USAD-USA, respectively, created a separate group for each other. This study also recommends the use of RAPD-PCR technique as a new molecular tool for detection of bacteria as the technique is fast, technically easy and requires little materials. Most importantly, no previous nucleotide sequence information is needed for the construction of primers. Application of the RAPD assay is a good technique in finding genetic variations where it had not previously been recognized, and is confirming differences that have been detected by other methods of characterization (**Williams *et al* 1990**). Furthermore, many markers can readily be identified for a variety of taxonomic levels.

The use of RAPDs for the typing of microorganisms has documented by several investigators (**Fouly *et al* 1994; Huff *et al* 1994; Amoah *et al* 1995; Fouly and Wilkinson, 1999; Mohamed *et al* 2001; El-Helali, 2001 and Mahfouz and Mohamed, 2002**). The RAPD assays were successfully used in identifying conserved regions of actinomycete genomes using various arbitrary primers as well as pUC18/19 'reverse' sequencing primers (**Mehling *et al* 1995**).

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REFERNCES

Amoah, B.K.; N. Rezanoor; P. Nicholson and M.V. MacDonald (1995). Variation in the *Fusarium* section Liseola: Pathogenicity and genetic studies of isolates of *Fusarium moniliforme* Sheldon from different hosts in Ghana. ***Plant Pathology 44: 563-572.***

Bohlool, J.L. and E.L. Schmidt (1980). The immunoflurescence approach in microbial ecology. ***Adv. Micro. Ecol. 4: 203-241.***

Brockman, F.J. and D.F. Bezdicek (1989). Diversity within serogroups of *Rhizobium leguminosarum* biovar *viceae* in the Palouse region of eastern Washington as indicated by plasmid profiles, intrinsic antibiotic resistance and topography. ***Appl. Environ. Microbiol. 55*(1): *109-115.***

Bromfield, E.S.P.; I.B. Sinha and M.S. Wolynetz (1986). Influence of location, host cultivar and inoculation on the composition of naturalized populations of *Rhizobium meliloti* in *Medicago sativa* nodules. ***Appl. Environ. Microbiol. 51*(5): *1077-1084.***

Broughton, W.J.; C.H. Wong; A. Lewin; U. Samrey; H. Myint; Z.A.H. Meyer; D.N. Dowling and R. Simon (1986). Identification of *Rhizobium* plasmid sequences involve in recognition of *Psophocarpus*, *Vigna*, and other legumes. ***J. Cell Biol. 102: 1173-1182.***

Brown, T.A. (1990). Purification of DNA from living cells. In: ***Gene Cloning: An Introduction, pp. 27-42,*** (Brown,T.A., 2nd Ed.), Chapman and Hall, St. Edmundsbury Press Ltd., London.

De Bruijn, F.J. (1992). Use of repetitive (Repetitive extragenic palindromic and enterobacterial repetitive intergeneric

consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. ***Appl. Environ. Microbiol. 58(7): 2180-2187.***

Demezas, D.H. and P.J. Bottomley (1986). Interstrain competition between representatives of indigenous serotypes of *Rhizobium trifolii*. ***Appl. Environ. Microbiol. 52(5): 1020-1025.***

Dovkishinevsky, B.; S. Dipankar and Y. Guary (1996). Diversity of rhizobia isolated from various *Hedysarum* species. ***Plant and Soil 186: 21-28.***

El-Hassan, G.A.; B.S. Hernandez and D.D. Focht (1986). Comparison of hup trait and intrinsic antibiotic resistance for assessing rhizobial competitiveness axenically in soil. ***Appl. Environ. Microbiol. 51(3): 546-551.***

El-Helali, M.F.M. (2001). ***Studies on Some Biotic and Environmental Factors Affecting the Activity of Rhizobium leguminosarum Biovar viceae, p.229.*** Ph.D Thesis in Botany (Microbiology), Botany Department, Faculty of Science, Zagazig University, Egypt.

Fouly, H.M. and H.T. Wilkinson (1999). Use of RAPD markers as a diagnostic tool in the identification of *Gaeumannomyces graminis* isolates that cause patch diseases of grassess. ***Arab Journal of Biotechnology 2: 143-160.***

Fouly, H.M.; L.L. Domier; H.T. Wilkinson and W.L. Pedersen (1994). Use of RAPD and ribosomal RNA specific primers in PCR to characterize isolates of *Gaeumannomyces* species. ***Phytopathology 84:Abs.No 630, p. 1145.***

Hamdi, Y.A. (1982). Application of nitrogen-fixing systems in soil management. ***FAO Soil Bull. 49: 11-40.***

Huff, D.R; T.E. Bunting and K.A. Plumley (1994). Use of random amplified polymorphic DNA markers for the detection of genetic variation in *Magnoporthe poae*. ***Phytopathology 84: 1312-1316.***

Keyser, H.H. and P.B. Cregan (1987). Nodulation and competition for nodulation of selected soybean genotypes among *Bradyrhizobium japonicum* serogroup 123 isolates. ***Appl. Environ. Microbiol. 53(11): 2631-2635.***

Kremer, R.J. and H.L. Peterson (1982). Nodulation efficiency of legume inoculation as determinated by intrinsic antibiotic resistance. ***Appl. Environ. Microbiol. 43: 636-642.***

Mahfouz, H.T. and Sonya H. Mohamed (2002). Physiological, antagonistic and fingerprinting studies on some halotolerant *Streptomyces* strains. ***Arab Journal of Biotechnology 5: 103-120.***

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. ***Journal of Molecular Biology 3:208-218.***

Martensson, A.M. and J.G. Gustafsson (1985). Competition between *Rhizobium trifolii* strains for nodulation, during growth in a fermentor, and in soil-based inoculants, studied by ELISA. ***J. Gen. Microbiol. 131: 3070-3082.***

Mehling, A.; U.F. Wehmerier and W. Piepersberg (1995). Application of random amplified polymorphic DNA (RAPD) assays in identifying conserved regions of actinomycete genomes. ***FEMS-Microbiol-Lett. 128 :119-125***

Mohamed H. Sonya; H.I. Abdel-Fattah; Sh.M. Selim and M.S. Sharaf (2001). Identification and molecular studies on some halotolerant actinomycetes isolated from Sinai sandy soil. ***Arab Journal of Biotechnology 4: 179-196.***

Nei, M. and W.H. Li (1979). Mathematical model for studying genetic varia-

tion in terms of restriction endonuclease. *Proceedings of the National Academy of Sciences of the United States of America 76: 5269-5273.*

Perret. X. and W.J. Broughton (1998). Rapid identification of *Rhizobium* strains by targeted PCR fingerprinting. ***Plant and Soil 204: 21-34.***

Radwan, M.A. (2000). ***Influence of Rhizobiophage on Competition Between Root-nodule Bacteria, p.227***. Ph.D Thesis in Agric. Microbiol., Agric. Botany Dept., Faculty of Agric., Zagazig Univ., Zagazig, Egypt.

Rohlf, F.J. (1990). NTSYS-pc, ***Numerical Taxonomy and Multivariate Analysis System, Version 1.60, pp.10-35***. Exeter Software Publisher, Seteutet, New York.

Sambrook, J.; E.F. Fritsch and T. Maniatis, (1989). Gel electophoresis of DNA. In: ***Molecular Cloning : A Laboratory Mannual, Part 6, pp. 1-15, 2nd Ed.*** Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Teaumroong, N. and N. Boonkerd (1998). Detection of *Bradyrhizobium* spp. and *B. japonicum* in Thailand by primer-based technology and direct DNA extraction. ***Plant and Soil 204: 127-134.***

Williams, J.G.K.; A.R. Kubelik; K.J. Livak; J.A. Rafolski and S.V. Tingey (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. ***Nucleic Acids Research 18: 6531-6535.***

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استخدام تكنيك الـ RAPD-PCR في عمل بصمة وراثية لبعض عزلات من الريزوبيا ليجيومنزارم بيوفار فياسي

[١١]

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تم أجراء محاولة لاستخدام الدلائل الجزيئية للتفرقة بين عشرة عزلات من بكتريا الريزوبيا ليجيومنزارم بيوفار فياسي مختلفة في قدرتها للإصابة بالفاج (عزلة حساسة للفاج المحلل وأربعة عزلات حساسة للفاج الهادئ وأربعة عزلات ليسوجينية وعزلة مقاومة للفاج). وقد استخدم الحامض النووي المستخلص من هذه

العزلات بصورة منفصلة كبادئ للتحليل بواسطة الـ PCR وذلك باستخدام عشرة بادئات قصيرة التتابع وعشوائية الارتباط وهي OP-A01, OP-A05, OP-A16, OP-A18, OP-B02, OP-B03, OP-B04, OP-B06, OP-B08 and OP-B10 . كما تم إجراء الكتروفوريسيس علي اجاروز جل تركيزه ١,٢% مجهز في محلول منظم TAE. وقد قدرت العلاقة الجزيئية بين العشرة عزلات وقد أوضحت النتائج أن الأجزاء من النوع البوليمورفيك والمونومورفيك قد تم الحصول عليها لكل عزلة وان عدد الأجزاء المتحصل عليها قد اختلف مع استخدام البادئات وهذا متوقع من الجهة الوراثية. وفي بعض الحالات نجد أن عدد الأجزاء المتحصل عليها وأحجامها قد اختلف من عزلة لأخرى . وبالإضافة إلى ذلك فان بعض الأجزاء المميزة قد تم الحصول عليها لبعض العزلات سواء كانت موجودة او غائبة. كما اظهر التحليل الإحصائي للبوليمرفيزم وشجرة التقارب الوراثي نسبة تشابه تتراوح ما بين ٩١,٩ إلى ٩٩,١ % وبناء علي ذلك فان نتائج البحث تلفت الانتباه وتشجع استخدام تكنيك الـ RAPD-PCR كوسيلة جزيئيه جديدة وحساسة لعمل البصمة الوراثية لسلالات الريزوبيا .

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