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Molecular Characterization of Bacillus sp. with Inter- Simple Sequence Repeat (ISSR) Markers

By

A. I. H. Sayed(*); H. F. A. EL-Shaer(**); M. M. S. Asker(*);

R.E. EL-Kholy(*)

Abstract :

Interspecific crosses between some mutants by EMS belonging to both two species (*B. subtilis* NRRL B3411 and *B. licheniformis* 11945 BT3) gave some hybrids produced 1.5 folds α-amylase enzyme comparing with parental strains. Inter-simple sequence repeat (ISSR) markers were used to investigate genetic diversity among of isolates of *Bacillus* and Genetic variations of thirteen *Bacillus* species were evaluated using random amplified polymorphic DNA. The amplified products varied in size from 150- 2537 bp. The SDS-PAGE protein banding pattern of these hybrids was performed and compared with protein banding pattern of their parental strains. Furthermore determination of the α-amylase produced by the thirteen selected strains.

(*) Botany Dept. (Genetics unit), Fac. of Agric. Al-Azhar University, Cairo, EGYPT

(**) Microbial Biotechnology Department, National Research Centre, Giza, EGYPT

INTRODUCTION

α-amylase (*EC 3.2.1.1*), an extracellular enzyme degrades α-1,4-glucosidic linkages of starch and related substrates in an endo-fashion producing oligosaccharides including maltose, glucose and alpha limit dextrins. The enzyme is used particularly in starch liquefaction, brewing, textile, pharmaceuticals, paper, detergents, drugs, toxic wastes removal and oil drilling (Ajayi & Fagade, 2003). The enzyme production is largely dependent on the type of strain, medium composition, cell growth, initial pH and thermostability (Qirang & Zhao, 1994; Haq *et al*., 2005&2010). Many researchers using the interspecific hybridization for improving the production of microorganisms and also for understanding. The enzyme or vitamins production. (Ali *et al.,* 1977; Sayed *et al.,* 1982 & EL-Shaer, 2007). The inter-simple sequence repeat (ISSR) is a new kind of molecular marker that amplifies intermicrosatellite sequences at multiple loci throughout the genome by a single primer 16–18 bp long composed of a repeated sequence anchored at the 3k or 5k end by 2–4 arbitrary nucleotides (Zietkiewicz *et al.,*1994). Each amplified band corresponds to a unique DNA sequence delimited by two inverted micro-satellites, leading to multilocus and highly polymorphic patterns in which fragments are often polymorphic between different individuals (Nagaoka & Ogihara, 1997). ISSRs are dominant inheritance markers and can generate large numbers of highly informative and reproducible alleles. Up to now, no reports are available for using ISSR markers

to study *Bacillus sp.* The objectives of the present study are: to test the efficiency of ISSR markers with the goal of using these polymorphisms as genetic markers; (2) to investigate the genotypic variability within *Bacillus sp* by analyzing ISSRs of thirteen *Bacillus* isolated from different insect hosts and geographical origins; and to calculate and to compare genetic diversities, genetic similarities and genetic distances within and among isolates. SDS-PAGE whole cell protein pattern analysis had been emerged as a powerful tool for bacterial identification having the advantages of being fairly fast and easy and, when performed under highly standardized conditions, it offers a better taxonomic resolution at species or subspecies level (Perez *et al.,* 2000). The SDS-PAGE protein profiles analysis had been successfully used to characterize various species of cyanobacteria (Liu *et al.,* 2006).The molecular characterization of *Bacillus* sp. with inter simple sequence repeat markers.

MATERIALS and METHODS

Organism and culture maintenance

The bacterial culture *B.subtilis* NRRL B3411 and *B.licheniformis* 11945 BT3 were obtained from the Microbiological resources centre (Cairo Mircen) of IIB. and mutants from (Sayed *et al.,* 2016). It was maintained on nutrient agar medium containing nutrient broth 8.0 g and agar 20.0 (g/l) with pH 7.0. The slants were incubated at 37°C for maximum growth and stored at 4°C in a cold cabinet.

Production Media

Soluble starch 10g, Peptone 5g, KH_2PO_4 1g, Yeast extract 5g, NaCl 5g, $MgSO_4$ 0.2g, $CaCO_2$ 0.2g, pH 7.0 (Haq *et al.,* 2010).

Luria broth Medium(LB): Davis et al., 1980):

Tryptone 10g, Yeast extract 5g, NaCl 5g Agar was added as solidified agent 2% (w/v).

Minimal medium (Faires et al., 1999):

K_2HPO_4 70mM, KH_2PO_4 30 mM, $(NH_4)_2SO_4$ 25 mM, $MgSO_4$ 0.5 mM, $MnSO_4$ 10 mM, Ferric ammonium citrate 22 mg**,** Glucose 1g, agar was added as solidified agent 2% (w/v).

Enzyme assay

α- amylase was estimated according to the method of Rick and Stegbauer (1974). One milliliter of enzyme extract was added to a test tube containing 1.0 ml of 1.0 % soluble starch solution, pH 7.0. The mixture was incubated at 40ºC for 10 min. After the incubation, 3.0 ml of DNS reagent was added to each of the tubes. The tubes were placed in boiling water for 5 min and cooled to room temperature. The contents of tubes were diluted up to 10 ml with distilled water. The optical density (OD) of reaction mixture was determined at 570 nm using a spectrophotometer. One unit of enzyme activity is equivalent to that amount of enzyme, which in 1 min librates1mg reducing sugars from 1 % soluble starch.

DNA Extraction

DNA was extracted from the thirteen *Bacillus* isolates using DNeasy Tissue Mini Kit (QIAGEN). The concentration of DNA was then determined based on a comparison of the DNA samples with standard lambda DNA on 1% (w/v) agarose gel, after which it was adjusted to 5 ng/µl (Sambrook, *et al*; 1989).

ISSR-PCR Amplification

Five ISSR primers were used in this study. PCR amplification was done using a Perkin Elmer (Gene Amp PCR system 9600 –USA). Amplification was performed for 35 cycles with denaturation at 94oC for 1 min, annealing at 45oC for 1 min. and extension at 72oC for 2 minutes. Initial denaturation was done at 94oC for 5 min and a final extension step of 5 min. at 72oC was also included. The reaction mixture (25 µl) contained 20 ng of DNA template, 10 mM Tris-HCl pH 9.0; 50 mM KCl; 0.1% Triton X-100; 1.5 mM MgCl2; 0.1 mM dNTP; 2 mM primer; 0.5 unit of Taq DNA polymerase. Master mixes of each reaction were overlaid with 10 µl of mineral oil to prevent evaporation. Amplification products were separated on 1.2% agarose gel in 1X TAE (Tris base, acetic acid and EDTA) buffer (Sibao, *et al*; 2005).

Table 1: Code, sequence and nucleotide length of primers used in the ISSR analysis.

Primer code	Primer sequence 5' to 3'	Nucleotide length
ISSR6	CGCGATAGATAGATAGATA "CGC$(GATA)_4$ "	19-mers
ISSR8	AGACAGACAGACAGACGC "$(AGAC)_4$GC"	18-mers
ISSR9	GATAGATAGATAGATAGC "$(GATA)_4$GC"	18-mers
ISSR10	GACAGACAGACAGACAAT " $(GACA)_4$AT"	18-mers
ISSR13	AGAGAGAGAGAGAGAG " (AG)8YT"	18-mers
ISSR14	CTCCTCCTCCTCCTCTT " (CTC)5TT "	17-mers
ISSR-HB09	GTGTGTGTGTG " (GT)6GC "	14- mers
ISSR-HB11	GTGTGTGTGTGTCC " (GT)6CC"	17- mers
ISSR-HB12	CACCACCACGC "(CAC)3GC"	11-mers
ISSR-HB13	GAGGAGGAGGC "(GAG)3GC"	11-mers

Gel electrophoresis

Amplification products of ISSR were separated on 1.5% agarose gels in 1XTAE buffer with DNA ladder (1Kb) and 100bp DNA Ladder RTU and detected by staining with ethidium bromide (Sambrook *et al.,* 1989).

SDS-PAGE electrophoresis technique

will be done by the method of Laemmli (1970) and modified by Sambrook *et al*. (1989).

SDS-PAGE

The total soluble protein profiles of *B. bassiana* isolates and its mutants subjected to the above mentioned were analyzed by SDS-PAGE. Protein samples were treated with treatment buffer (0.125M Tris pH 6.8, 20% glycerol, 2% SDS and 14.4mM β- Mercaptoethanol) for 10 minutes in a boiling water bath at 100°C. The samples were cooled to ambient temperature and 50µl of protein samples were loaded on Tris glycine gels (5% stacking and 15% resolving). Electrophoresis was performed on Biotech vertical gel electrophoresis unit.

Intraspecific crosses

Two mutants from *B.licheniformis* and five mutants from *B.subtilis* were chossen for intraspecific crosses, α-amylase enzyme production were messured in all parental and intra. or interspecific hybrids in order to understand the genetic behaviour for this enzyme (Mayur, 1965).

Results and Dissection

I- Genetic analysis for Interspecific

The genetic analysis for interspecific crosses were used as around spore analyses at MM medium to obtained the progenies requires and its gene or genes number in each cross. Results obtained were presented in table (1). Tow Interspecific crosses were placed on MM as fallows;

1- Crosses between *B. subtilis* (A18) X *B. licheniformis* (B2)

A18 mutant were required for (Ornithine, Folic acid), while B2 was required for (Adenine – Ornithine - Folic acid), the genetic analysis of progenies were R1 (0) : R2 (0) : R1R2 (1) and W.T (2),which gave ratio (0) P.D : (3) N.P.D, this results is clearly to give 0:3 ratio which in indicated that the two requires is functionally in one or more linked genes.

2- Crosses between *B.subtilis* (A8)X *B. licheniformis* (B1)

A8 mutant were required for (Adenie), while B2 was required for (Cysteine), were crossed and plated on MM, all progenies were tasted for genotypes, results were presented in **Table (1).** Showed that we obtained only W.T colonies (11 colonies), while the R1,R2 or R1R2 colonies were not obtained. This results gave the ratio (0) P.D: (11) N.PD . and also indicate clearly that the two requires were functionally not in unlinked genes, but in one gene or more closed linked genes belonging to one linkage group.

Table (2): Interspecific Crosses in *Bacillus licheniformis* 11945 BT3 and Interspecific Crosses in *Bacillus subtilis* NRRL B3411 its F1 production.

No.	Crosses		No. F1 production				Expected Ratio of P.D:N.PD	No. of genes
	R1	R2	R1	R2	R1.R2	W.T		
VI	A18 (Orn) (Folicacid)	B2 (Ade) (Orn) (Folic acid)	0	0	1	2	0:3	One or more linked genes
V	A8 (Ade)	B1 (Cys)	0	0	0	11	0:11	2 gene

All the previews results in Table (1) were agreed with those reported by Ayala, (1976) Ali *et al.,* (1977) , Sayed *et al.,* (1982) & EL-Shaer, (2007) and disagree with reviewed by Mayur, (1965).

3- Interspecific hybrid α- amylase enzyme production

Tables (3) presented the amylase production (Unit) in each *B. subtilis, B. licheniformis* and interspecific hybrids and its progenies. This table showed that the cross [between mutant no.A18 and mutant no.B2] and the cross [between mutant no.A8 and mutant no. B1], we can notice from these previous crosses that each of them was between higher amylase production mutant (B2, A18 and A8) and one of the lowest amylase production mutant (B1).

Table (3): α-amylase production of (A18XB2) and (A8XB1) interspecific crosses growing on nutrient starch agar medium for 3 days on 30°C.

Strains		Enzyme activity (U)	Upper		Genotype
			100% (A)	100% (B)	
Parent	*Bacillus subtilis* (A0)	200.59	100	100	W.T
	B. licheniformis (B0)	153.25	100	100	W.T
Mutant					
	(A18)	323.64	161.34	211.18	Ade- Orn- Folic acid
	(B2)	294.08	146.61	191.90	CYS
					Hybrids
	(51)	132.00	65.81	86.13	W.T
	(52)	128.00	63.81	83.52	CYS- Ade- Orn- Folic acid
	(53)	340.00	169.50	221.86	W.T
Mutant					
	(A8)	355.00	176.98	231.65	Ade
	(B1)	119.52	59.58	77.99	Ade- Orn- Folic acid
Hybrids					
	(61)	154.00	76.77	100.49	W.T
	(62)	192.00	95.72	125.29	W.T
	(63)	188.00	93.72	122.68	W.T
	(64)	544.00	271.20	354.98	W.T
	(65)	300.00	149.56	195.76	W.T
	(66)	176.00	87.74	114.85	W.T
	(67)	128.00	63.81	83.52	W.T
	(68)	356.00	177.48	232.30	W.T
	(69)	188.00	93.72	122.68	W.T
	(70)	176.00	87.74	114.85	W.T
	(71)	252.00	125.63	164.44	W.T

II-Protein Profiles of *Bacillus* strains

Sodium dedocyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell protein extract of the thirteen *Bacillus* strains **(Table 2).** Data obtained were presented in **Table (3)** and illustrated in **Fig (1).** Data in **Table (3)** showed a high heterogeneous profile (Fig.1). The main difference in protein pattern was related to some high accumulated polypeptides with different molecular weight present in almost *Bacillus* cells, the molecular weight for these bands ranged from 9 to 95 KDa. SDS-PAGE protein banding patterns of the thirteen *Bacillus* strains were showed the maximum number of bands in this pattern was 14 bands for B2 strain while the minimum numbers of bands were 6 bands for *B. licheniformis* (B0) and A8 strains SDS-PAGE aanalysis of these banding patterns suggested that four common bands were present in all of the thirteen *Bacillus* strains with molecular weight; 12,15,10 and 9 KDa. Meanwhile, one specific band was found in VI 61 strain (marker band) at molecular weight 43 KDa. On the other hand, there were few observable differences in the protein banding pattern among all tested strains. Each of the parental strains *B.subtilis* (A0) and B1 has 13 bands, while the parental strain A18 have 12 bands the hybrid V 51, V 52, V 53, VI 61, VI 64, VI 67 and VI 68 has 11, 11, 13, 7, 9, 9 and 10 bands, respectively. These differences could be attributed to the differences in the new genetic recombination that occur in every diploid fusion before regeneration.

Table (4): protein profiles for the thirteen *Bacillus* strains by using SDS-PAGE techniguc:

Band	Marker KDa	A0	B0	B2	A18	B1	A8	V 51	V 52	V 53	VI 61	VI 64	VI 67	VI 68
1	95	-	+	+	+	+	-	-	-	-	-	-	-	-
2	83	-	+	+	+	-	-	-	-	+	+	+	+	+
3	72	+	-	+	-	+	-	-	-	+	-	-	+	+
4	61	+	-	+	-	+	+	+	+	+	+	+	+	+
5	56	+	-	+	+	+	+	+	+	+	-	-	-	+
6	51	+	-	-	+	-	-	-	+	-	-	+	+	+
7	46	+	-	-	+	-	-	-	+	+	-	+	-	-
8	43	-	-	-	-	-	-	-	-	-	*+	-	-	-
9	42	+	-	+	+	+	-	+	-	+	-	+	-	-
10	39	+	-	+	-	+	-	+	+	+	-	-	-	-
11	36	+	-	+	-	+	-	-	-	+	-	-	-	-
12	29	+	+	+	+	+	+	+	+	+	+	+	+	+
13	26	+	-	+	-	+	-	+	-	-	-	-	-	-
14	25	-	-	-	-	-	-	-	+	-	-	-	+	+
15	23	-	-	+	+	+	-	+	-	-	-	-	-	-
16	15	+	+	+	+	+	+	+	+	+	+	+	+	+
17	12	-	-	-	+	-	-	+	+	+	-	-	-	-
18	10	+	+	+	+	+	+	+	+	+	+	+	+	+
19	9	+	+	+	+	+	+	+	+	+	+	+	+	+
Total		13	6	14	12	13	6	11	11	13	7	9	9	10

Nates: Band (+) presence and (−) absence

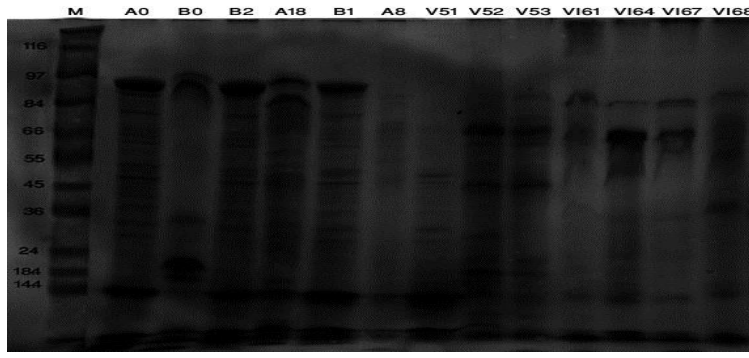

Figure (1): Electrophoretic profile (SDS-PAGE) of whole protein extract of 13 bacteria Numbers 1 to 13 indicate bacterial A0 to VI 68, respectively. M = Protein Molecular weight markers, PageRuler™ Unstained Protein Ladder (Fermentas).

The previous Sodium dedocyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis is generally used to compare protein profiles of *Bacillus* strains, For example, Attallah *et al*. (2014) used SDS-PAGE analysis in order to determine the differences among the *Bacillus* isolates collected from different area in Egypt. results are in good agreement with previous researches, it is known that protein profiles of whole-cell and extracellular protein are good enough to distinguish most of bacterial genera at species level (Berber *et al.,* 2003).

III-Polymorphisms as detected by DNA markers based on ISSR analysis

To find more moor different among 13 strains, they were exposed to the PCR- based marker of monomorphic fragments and percentage of polymorphism obtained pare ach primer are shown in table (5). These primers were; ISSR 8, ISSR 10, ISSR 13, ISSR HB-9, ISSR HB-11, ISSR HB-13 and ISSR 9. Both the number and MW of the amplified products varied considerably with the different primers. The results of ISSR-PCR of the studied *Bacillus* strains and presented in table (5) showed clearly that the seven primers were used for amplifying DNA fragments produced 89 bands. An average of 12.71 bands /primer were amplified which ranging from approximately 150 - 2537 bp. The polymorphic bands without unique band across all were gave 58 bands with an average of 8.28 bands /primer.

Table (5): List of primers, their sequence, numbers and size of the amplified fragments (bands) generated by ISSR primers in bacillus.

Primers	MW Range (bp)		Bands total no.	Mono-morphic		Polymorphic			
	mix	min		No	%	Unique	Poly-morphic	Total	%
ISSR 8	1800	150	20	8	40	0	12	12	60
ISSR 9	2537	321	15	0	0	2	13	15	100
ISSR 10	2000	280	10	2	20	1	7	8	80
ISSR 13	1500	200	11	4	36	0	7	7	64
ISSR HB-9	2500	390	8	1	12	1	6	7	88
ISSR HB-11	2500	250	11	6	55	1	4	5	45
ISSR HB-13	2200	220	14	4	29	1	9	10	71
Total			89	25	192	6	58	64	508
Average	2537	150	12.71	3.57	27.43	0.86	8.28	9.14	72.57

A total of polymorphic bands were 64 bands polymorphic with unique bands across all with an average of 9.14 bands / primer. Six unique bands with average of 0.86 bands /primer were identified of them. The polymorphism bands percentage was 72.57 %. ISSR 8 amplified the highest number of polymorphism bands (20 Bands). However three primers which are ISSR 9, ISSR HB-9 and ISSR 10 showed the highest polymorphic differences (100, 88 & 80 %, respectively) among strains which are useful in *Bacillus* DNA identification, while ISSR HB-11 exhibited the lowest polymorphic difference (45%). Although in this work the ISSR markers showed high percentage of polymorphism and it could be useful for DNA and genomic fingerprinting. It was be acceptable with (Iruela *et al*., 2002).

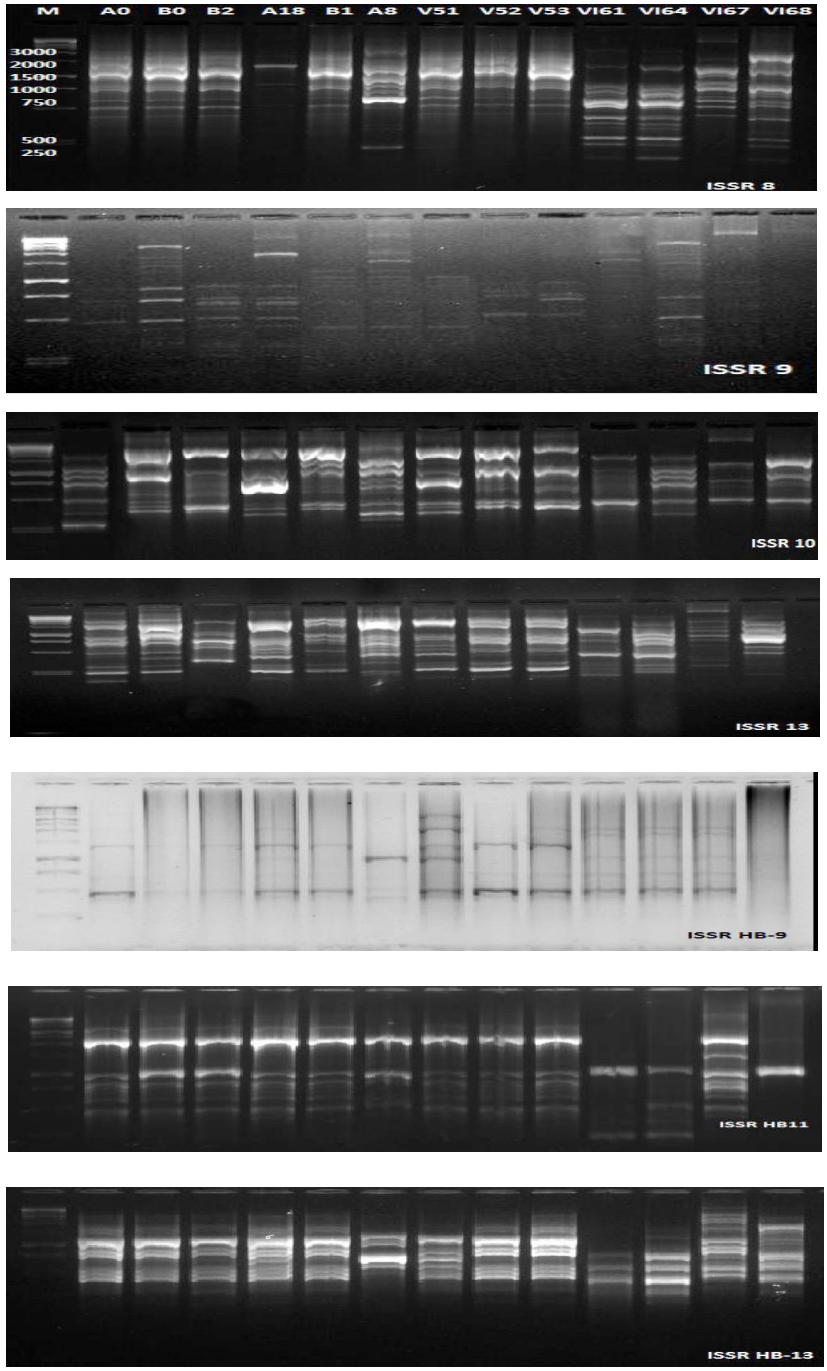

Fig. (2): Genetic comparison with 13 different strains of bacillus by 7 ISSR markers.

***A*-Cluster analysis and similarity indices of 13 strains**

To examine the genetic relationships among 13 strains; A0, B0, B2, A18, B1, A8, V 51, V 52, V 53, VI 61, VI 64, VI 67 and VI 68. Based on ISSR-PCR results, the scored data were analyzed using the Dice coefficient to compute the similarity matrices. These similarity matrices were used to generate a dendrogram using the UPGMA method. The dendrogram tree and similarity indices among the 13 strains utilizing ISSR-PCR. The UPGMA cluster analysis was carried out to represent graphically the genetic distances among the 13 strain. As shown in **(Figure 3),** a phylogenetic tree illustrated that all the 13 strains had two distinct groups G1 and G2. The first group (G1) includes one cluster with two strains; VI 61 and VI 64 with close distance between them. High genetic similarity ratio between the two strains about 85% with the same parent (A8XB1) interspecific crosses **(Table 3)**.

On the other hand, the second group (G2) includes 11 strains; A0, B0, B2, A18, B1, A8, V 51, V 52, V 53, VI 67 and VI 68 with different distance between them in nine pool-clusters; a, b, c, d, e, f, g, h and i and one strain only VI 67. It's had one sup-group (cluster a) include two division; b and d sub- group b include strain VI 68 and sup-group c include strain A8 and V 51 with close distance between them 80%, while sup-group d divided into two group e and i, which include B0 and B2 with distance 91%. Sup-group e dived into group f and one strain only A 18, while sup-group f dived into group g and one

strain only B1. The end sup-group g dived into group h and one strain only A0, while sup-group h have two strains V 52 and V 53 with close distance between them 93% with the same parent (A18XB2) interspecific crosses in Table (3). This result was in agreement with those obtained by (Iruela et al., 2002).

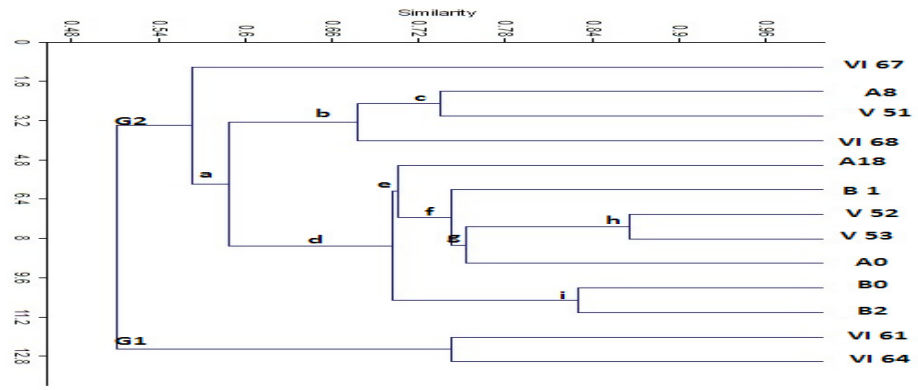

Fig 3: Dendrogram generated using Un-weighted Pair Group Method with arithmetic average (UPGMA) analysis showing relationships among 13 different bacillus strains using ISSR.

The ISSR Similarity matrix

Table (5) elucidate similarity matrix among the 13 strains of *Bacillus* under study. the genetic similarities derived from the data of the ISSR marker analysis varied from 0.62 *Bacillus subtilis* NRRL B3411 (A0) WT and strain VI 64 to 0.91 between *Bacillus licheniformis* 11945 BT3 (B0) WT and strain B2.

Table: (6): Similarity matrix among 13 strains of *Bacillus* under study Among the 13 *bacillus*, strains, V 52 showed maximum genetic similarity with strain V 53 (0.93), based on the ISSR marker analysis.

	A0	**B0**	**B2**	**A18**	**B1**	**A8**	**V51**	**V52**	**V53**	**VI61**	**VI64**	**VI67**	**VI68**
A0	100												
B0	84	100											
B2	80	91	100										
A18	83	83	82	100									
B1	85	86	80	81	100								
A8	76	72	67	75	79	100							
V51	78	77	75	80	80	85	100						
V52	85	81	81	80	85	72	80	100					
V53	87	85	82	86	85	69	78	93	100				
VI61	63	64	60	70	67	73	73	68	69	100			
VI64	62	67	65	72	63	72	70	63	67	85	100		
VI67	73	66	65	73	77	76	69	72	73	65	67	100	
VI68	69	75	70	67	76	83	79	71	68	74	73	73	100

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