

# Comparative Analysis of Genetic Diversity Among *Bacillus Thuringiensis* and *Bacillus Sphaericus* and Their Fusants Using Molecular Markers

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**Abstract:** Ten genetically stable fusants strains were obtained as a result of protoplast fusion technique between *Bacillus sphaericus* (Bs) and *Bacillus thuringiensis* (Bt), six of them produced from, Bs GHAI Bt 66 Ia experiment (fusants no. 1, 2, 16, 26, 31 and 34) and four fusants produced from Bs GHAI Bt 1977 experiment (fusants no. 1, 2, 17 and 25). Two molecular marker systems, four ISSR primers (Inter-Simple Sequence Repeat) and seven SCoT primers (Start Codon Targeted Polymorphism) were employed for detection of genetic variation between the parents and their fusants. Four ISSR primers and seven SCoT detected 55 and 97 amplicons, among which 92.696% and 81.948% bands were polymorphic respectively. The genetic similarity values among the species were calculated based on ISSR and SCoT profiles which ranged from 0.50 to 0.83 and 0.59 to 0.90 respectively. Based on the marker analysis, the three parents and the ten fusants were clustered into three major groups for ISSR primer and SCoT primers. Cluster analysis were performed to construct a dendrogram of the *Bacillus* Strains and their fusants and also reveals high genetic variation among the parents and their fusants. It could be concluded that each type of the two molecular marker approaches of DNA analysis could identify the difference between the parents, and all of their fusants under investigation have probably originated from closely related ancestors and possess high degree of genetic similarity.

**Keywords:** *Bacillus Thuringiensis*, *Bacillus Sphaericus*, Genetic Diversity, ISSR and SCoT Marker

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## 1. Introduction

Molecular markers are mainly used in molecular biology and biotechnology to recognize a specific sequence of DNA in a pool of unknown DNA. It can be defined as a variation that could be detected. A genetic marker may be a long DNA sequence, or a short one [1]. They can recognize variation at the DNA level e.g., nucleotide changes, deletion, duplication, inversion and or insertion [2].

SCoT is a simple, new and gene targeted DNA marker depend on the short-conserved region in genes [3]. Primers for SCoT marker study were planned from the conserved region surrounding the translation initiation codon ATG [4].

SCoT markers are considered as more precise than RAPD and ISSR, and it is proposed that primer length and annealing temperature are not the only factors responsible for reproducibility [5]. This procedure includes formation of single primers from the short-conserved region adjoining the ATG start codon without knowing any information about the genomic sequence [3]. The value of this primer in analysis of genetic variation has been informed in a group of plant species [4].

Inter-Simple Sequence Repeats (ISSRs) are sites in the genome lined by microsatellite sequences. PCR amplification of these regions by using a single primer produce multiple amplification products that could be used as a dominant multi

locus marker system in knowing the genetic variation in different organisms. ISSR markers are easy to use, with low cost, and with little demand in its method comparing to other dominant markers, making it a perfect genetic marker for beginners and for organisms with lacking genetic information [6].

The ISSR are semi arbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite. Each band corresponds to a DNA sequence demarcated by two inverted microsatellites [7].

In genetic variation studies, a good genetic marker must have high genetic variability with the ability to generate multi locus data from the studied genome [8]. The production of ISSR markers, tack advantage of microsatellite sequences that are highly variable and universally distributed across the genome. So ISSR is a good genetic marker for various researches, on genetic variation or diversity [9], DNA fingerprinting [10], and phylogenetic [11].

The current study was conducted to determine the genetic similarity among the three parents *Bt*(66 Ia), *Bt* (I977) and *Bs* (GHAI) and their fusants using different marker systems and to compare the efficiency between SCoT and ISSRs for diversity analysis.

## 2. Material and Methods

### 2.1. Bacterial Parental Strains

*Bacillus thuringiensis* *Bt* 66 Ia (*Bt*); a UV resistant mutant of a local *Bt* strain isolated from dead snails collected from Giza Governorate, Egypt [12], *Bt* I 977 and *Bacillus sphaericus* (*Bs*) GHAI obtained from Microbial Genetics Department, National Research Centre, Cairo, Egypt.

### 2.2. Fusants Strains

Ten fusants stains; six of them produced from *Bs* GHAI: *Bt* 66 Ia experiment (fusants no. 1, 2, 16, 26, 31 and 34) [13] and four fusants produced from *Bs* GHAI *Bt* I 977experiment (fusants no. 1, 2, 17 and 25) [14]. All fusants strains induced 100 percent mortalities against *S. littoralis* larvae after seven days of feeding at all concentrations and to *C. pipiens* larvae after one day of feeding at all concentrations.

### 2.3. DNA Extraction

Total genomic DNA of all the studied strains was obtained by DNeasy 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 5ng/μl. [15].

### 2.4. Inter Simple Sequence Repeat (ISSR) Technique

ISSR-PCR reactions were conducted using four anchored primers, which were synthesized by Eurofins, Germany. The primer names and primer sequences are shown in (Table 1). The reaction conditions were optimized and the following reagents were mixed in a final volume of 25 μl: 1 X of green

*GoTaq* Flexi buffer; 1.5mM of MgCl<sub>2</sub>; 200 μM of dNTPs (Promega); 25pucM of primer; 1 U of *GoTaq* Flexi DNA Polymerase (Promega); 25ng of template DNA and up to 25 μl double distilled water. Amplification was carried out in a T100-Bio-Rad Gradient Thermal cycler. The following programmer was used to amplify the DNA: 94°C/5min (1 cycle); [94°C/45 sec, 45°C/50 sec, 72°C/1.5 min] (40 cycles); 72°C/7 min (1 cycle) and 4°C (infinite). A volume of 10μl of the ISSR-PCR product was resolved using 1.5% agarose gel electrophoresis containing ethidium bromide. A 100 bp DNA marker (Fermentas) was used as a DNA molecular weight standard. Results were visualized on a UV transilluminator and photographed by Molecular Imager® Gel Doc™ System with Image Lab™ Software, Bio-Rad. Only the clearest and strongest ISSR bands were scored manually as present (1) or absent (0) to be used for further analysis.

Table 1. Primer name and primer sequence used in the ISSR analysis.

Primer name	Primer Sequence
ISSR-3	5'-ACACACACACACACACYT-3'
ISSR-4	5'-ACACACACACACACACYG-3'
ISSR-8	5'-AGACAGACAGACAGACGC-3'
ISSR-18	5'-GTG TGT GTG TGT GTG TC-3'

### 2.5. SCoT (Start Codon Targeted) Technique

Seven SCoT primers were used in this study (Table 2). The reaction conditions were optimized and the following reagents were mixed in a final volume of 25 μl containing 2 μL of template DNA (25 ng/μL); 2.5 μL primer at 10 pucM; 0.5 μL dNTPs at 10mM; 0.15 μL Taq DNA polymerase at 5 U/μL; 5 μL 5X PCR buffer; and 14.75 μL double distilled water. SCoT-PCR amplification was carried out in a T100-Bio-Rad Gradient Thermal cycler programmed as follows: initial denaturation was carried out at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1.5 min, and final extension at 72°C for 7 min. The amplification products were separated in 1.5% agarose gels containing 0.5 μg/mL of ethidium bromide through electrophoresis in 1X TBE buffer solution at 5 V/cm and photographed by Molecular Imager® Gel Doc™ System with Image Lab™ Software, Bio-Rad.

Table 2. Primer name and primer sequence used in the SCoT analysis.

Primer Name	Primer Sequence
SCoT-17	CCATGGCTACCACTACCC
SCoT-25	ACGACATGGCGACCGCGA
SCoT-26	ACGACATGGCGACCACTGT
SCoT-27	ACCATGGCTACCACCGTC
SCoT-35	AACCATGGCTACCACCAC
SCoT-36	CACCATGGCTACCACCAT
SCoT-47	ACCATGGCTACCACCGCG

## 3. Results and Discussion

### ISSR- and SCoT PCR Analysis

In this study, two marker systems; ISSR and SCoT, were successfully used to differentiate between *Bt* and *Bs* parents

and their ten fusants stains; six of them produced from *Bs* GHAI:: *Bt* 66 1a experiment (fusants no. 1, 2, 16, 26, 31 and 34) [13] and four fusants produced from *Bs* GHAI:: *Bt* I 977 experiment (fusants no.1, 2, 17 and 25) [14]. A total of 11 primers were used and 55 bands for ISSR and 97 bands for SCoT were produced, with a mean of 13.750 and 13.857 bands per primer, respectively, among which 92.696 % and 81.948% were polymorphic for ISSR and SCoT, respectively. ISSR and SCoT markers were calculated in order to characterize the capacity of each primer to detect polymorphic loci among the parents and their fusants.

ISSR is a suitable molecular typing technique for the study of genetic diversity of a wide variety of organisms. Screening to obtain an efficient set of primers is very important when using ISSR typing [16, 15].

Using Eleven primers; four for ISSR, and seven for SCoT, which were selected previously based on the number of bands that they produced and the polymorphism of these bands.

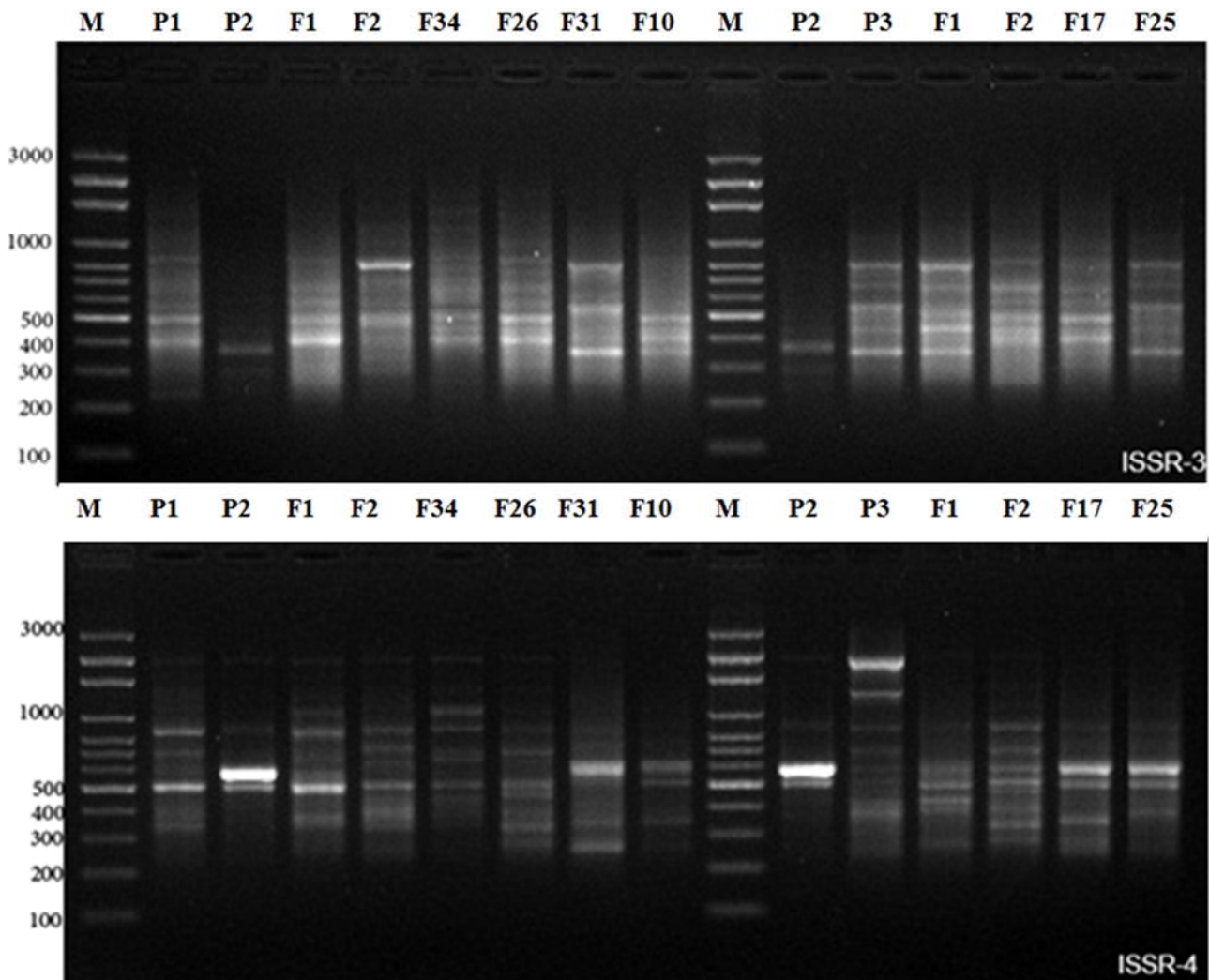
#### ISSR-Polymorphism

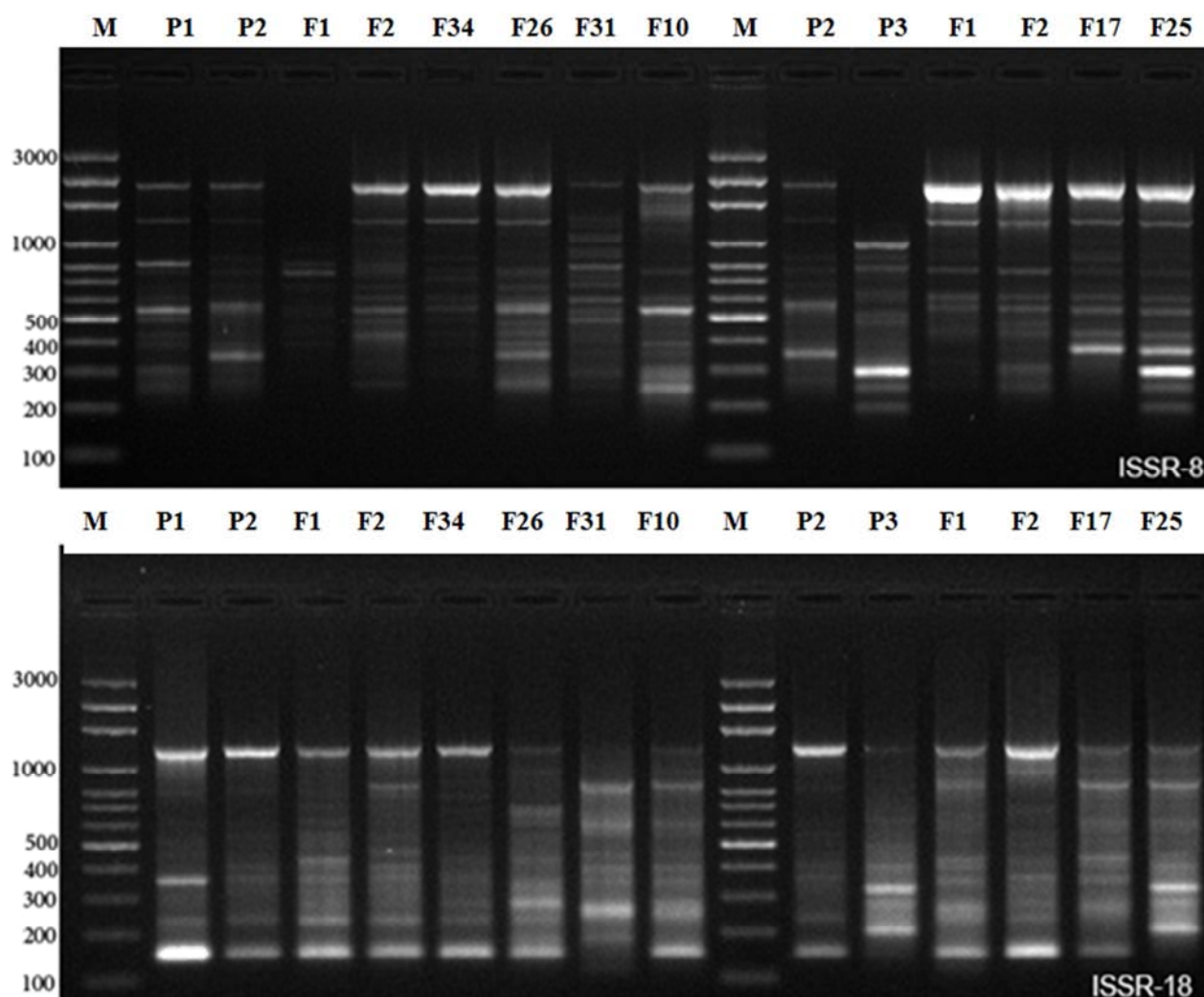
The most polymorphic primers were ISSR-8 which produced 17 bands, followed by primer ISSR-18, primer ISSR-4 and primer ISSR-3 which produced 15, 12 and 11

bands, respectively (Table 3).

(Figure 1) shows PCR products of the four ISSR primers. Among the four-decamer oligonucleotide primers, primers varied greatly in their ability to resolve variability among *Bt* 66 1a:: *Bs* GHAI and their fusants. Some primers produced several bands, while others produced a few bands.

ISSR-primers produced polymorphism ranged from 83.3 to 100%. Primer ISSR-3 produced the highest percentage of polymorphism (100%) and did not produce any monomorphic band, while primer ISSR-4 produced the lowest one(83.33) with two monomorphic bands. Mean of band frequency of each primer ranged from 0.380 % to 0.500 %. ISSR-8, appeared high polymorphic bands within the isolates in our study, while the ISSR-4 primer gave the highest mean of band frequency (0.500%) with 83.33% polymorphism efficiency. Our results showed that there is a high genetic variation among the parents and their fusants, that is in agreement with the results from previous studies carried out on *Aspergillus flavus*-strains [17]. Polymorphism of each primer was calculated as a percentage of polymorphic bands to the total number of bands produced by the designated primer [18].





**Figure 1.** PCR products with ISSR marker profiles. Lanes 2&3; P1(Bt 66 1a); P2( Bs GHAI) Lanes 4-9 are their fusants (1, 2, 16, 26, 31 and 34); Lane 11-12; P2( Bs GHAI); P3( Bt I 977) and Lanes 13-16 are their fusants (1, 2, 17 and 25); Lane (1&10) M GeneRuler100bp plus DNA ladder.

**Table 3.** Statistical analysis of ISSR primers used in this study and the amplification results.

Primer Name	ISSR Primers						
	Primer Sequence 5'-3'	No Of Scored Bands (NSB)	No Of Poly-Morphic bands (NPB)	Polymorphism (PPB)%	No Of Monomorphic Bands	No Of Unique Bands	Mean of Band Frequency
ISSR-3	5'-ACACACACACACACACYT-3'	11	11	100.000%	0	0	0.479%
ISSR-4	5'-ACACACACACACACACYG-3'	12	10	83.333%	2	1	0.500%
ISSR-8	5'-AGACAGACAGACAGACGC-3'	17	16	94.118%	1	2	0.380%
ISSR-18	5'-GTG TGT GTG TGT GTG TC-3'	15	14	93.333%	1	1	0.444%
Total		55	51		4	4	1.803%
Average		13.75	12.75	92.696%	1	1	0.45%

A total of 55 bands were generated from four ISSR primers (Table 3), and all of them were polymorphic bands. The total bands per primer ranged from 11 (ISSR-3) to 17 (ISSR-8). The size of the amplified fragments had ranged from 184 bp to 1,731 bp (Figure1).

There are many studies indicated that ISSR – PCR is depended on the sensitive reaction condition. Optimization of PCR condition is necessary to get the highest specificity and product yield [17, 19]. Optimization of PCR condition, including temperature, number of cycle, reagents and other parameters are very necessary to get successful ISSR-PCR reaction [20]. Variation in the number of bands amplified by different primers influenced by variable factors such as

primer structure and the annealing sites in the genome [21, 22], so in the present study, we used four different primer structures for reveal the genetic diversity among the strains and we found high rate of variation between them, but the intensity of the bands was not taken into account because of the DNA concentration was not controlling exactly at 50 ng for each sample, which means that the number of copies of DNA template was not the same for all sample that may

affected the intensity of the resulted bands. In this study, the unique bands and the polymorphic bands were generated. Unique band means that the presence of band in a specific variety and absence of it with the same size in all other varieties [23, 24, 25] they suggested that the application of unique markers in DNA typing and variety identification.

#### SCoT Polymorphism

The marker system Start Codon Targeted Polymorphism that is based on the short conserved region in genes surrounding the ATG translation start (or initiation) codon that has been well characterized in previous studies [26, 27]. DNA markers are produced by polymerase chain reaction (PCR) using single primers that are designed from the short conserved region flanking the ATG start codon that is conserved for all genes. Therefore, in principle, this technique is similar to RAPD or ISSR or single primer amplification reaction because a single primer is used as the forward and the reverse primer [19, 28]. Due to the basis of SCoT primer design, we expect SCoT markers to be distributed within gene regions that contain genes on both plus and minus DNA strands. It is also possible that pseudogenes and (genes within) transposable elements may be used as primer binding sites by SCoT polymorphism technique. An important factor is the distance in base pairs between primer binding sites of the template. Therefore, a relatively long extension time of the thermal cycle is important and we suggested at least 1-min. SCoT primers in this study were designed based on a consensus sequence for the flanking region around the ATG start codon derived from

the previous studies [26, 29]. Most primers differed from each other by at least one nucleotide with an emphasis on variations at the 3' end, which has been shown too critical for primer-template specificity [30, 31].

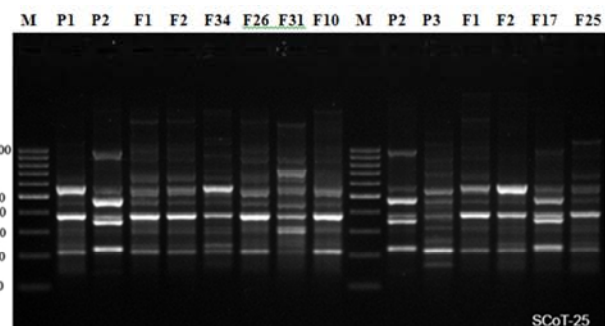
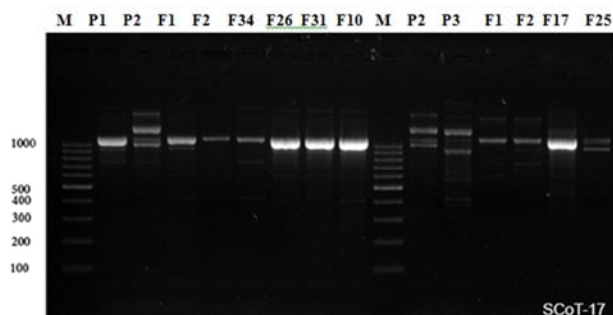
Primers sequences, code, number of scored bands (NSB), number of polymorphic bands (NPB) and percent of polymorphic bands (PPB)for SCoT primers are shown in (Table 4).

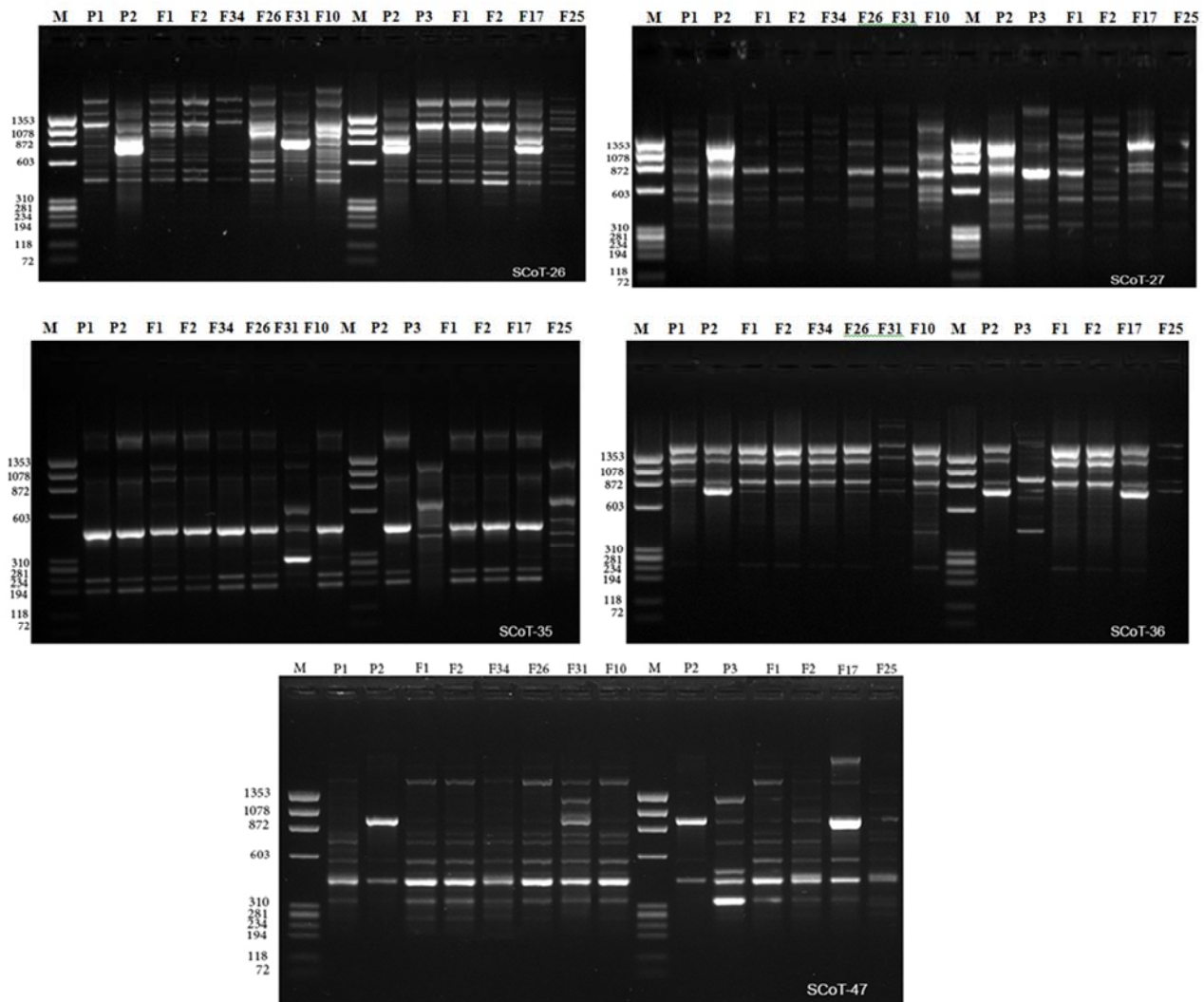
Polymorphism was observed for all primers used in this study. In addition, the total number of scored bands (97) and polymorphism was observed for 80 of them. SCoT-26 primer with 19 bands had the highest number of bands and SCoT-36 primer with 9 bands had the lowest number of bands. Bands pattern of all SCoT primers are shown in Figure.2. Average of polymorphism percent was 81.948%, the lowest percent of polymorphism (66.667%) produced by SCoT-36 primer and the highest percent of polymorphism 91.667% produced by SCOT-17 and SCT-47 primers. The average of mean of bands frequency for all primers was 0.481% and the highest value produced by SCoT-36 (0.622 %) while the lowest one produce by SCoT-17 (0.278%) (Table 4).

(Table 4) also, shows that the three primers; SCoT-25, SCoT-26 and SCoT-27 produced 16, 19 and 16 scored bands, respectively. Out of the 97 scoured bands of PCR products, 80 were polymorphic bands and 12 were unique bands. These are comparable to the number of markers typically generated by RAPD and ISSR techniques in rice [32, 33, 34] and also in Agrobacterium [35].

**Table 4.** Statistical analysis of SCoT primers used in this study and the amplification results.

SCoT Primers						
Primer Name	Primer Sequence 5'-3'	No Of Scored Bands (NSB)	No Of Poly-morphic Bands (NPB)	Polymorphism (PPB)%	No Of Unique Bands	Mean of Band Frequency
SCoT-17	CCATGGCTACCACTACCC	12	11	91.667%	3	0.278%
SCoT-25	ACGACATGGCGACCGCGA	16	12	75.000%	2	0.500%
SCoT-26	ACGACATGGCGACCACTG	19	16	84.211%	2	0.516%
SCoT-27	ACCATGGCTACCACCGTC	16	14	87.500%	2	0.450%
SCoT-35	AACCATGGCTACCACCAC	13	10	76.923%	1	0.518%
SCoT-36	CACCATGGCTACCACCAC	9	6	66.667%	0	0.622%
SCoT-47	ACCATGGCTACCACCGCG	12	11	91.667%	2	0.483%
Total		97	80		12	3.367%
Average		13.8571	11.428	81.948	1.714	0.481%





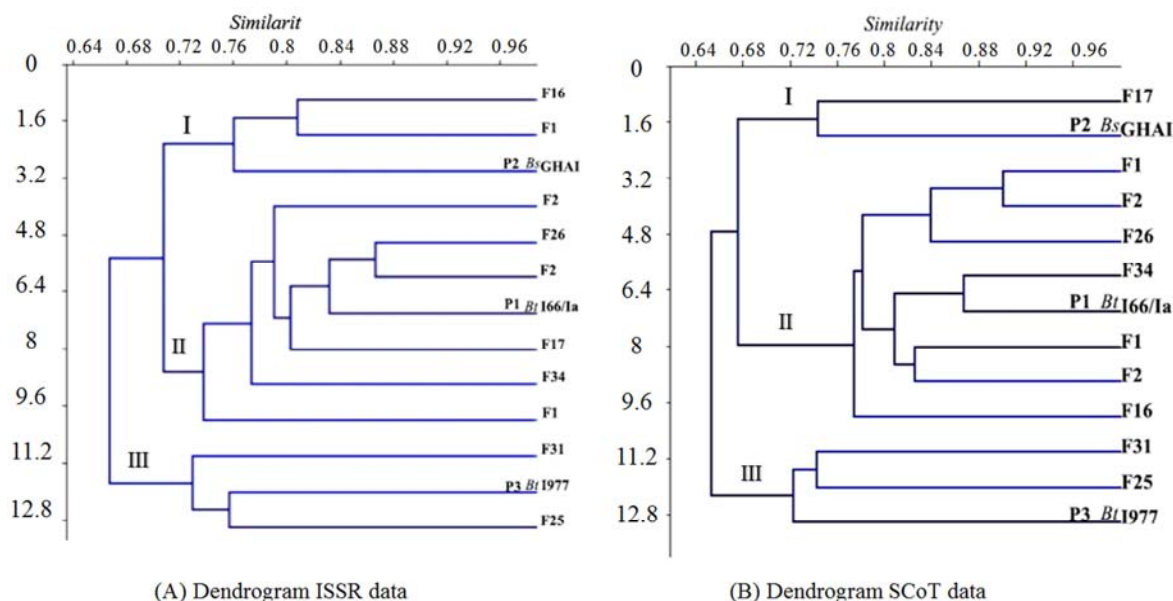
**Figure 2.** PCR products with SCoT marker profiles. Lanes 2&3; P1(*Bt* 66 1a); P2( *Bs* GHAI) Lanes 4-9 are their fusants (1, 2, 16, 26, 31 and 34); Lanes 11-12; P2( *Bs* GHAI); P3( *Bt* I 977) and Lanes 13-16 are their fusants (1, 2, 17 and 25); Lanes (1&10) M GeneRuler100bp plus DNA ladder.

#### Cluster analysis with SPSS: Hierarchical Cluster Analysis

Cluster analysis works upwards to place every variable (strain) into a single cluster. The main part of the output from SPSS is the Dendrogram that represents the phylogenetic relationships among the variable (strains) under study. The Dendrogram for the ISSR and SCoT primers patterns of all strains using average linkage (between groups) is presented in (Figure 3).

In the present study, cluster analysis was done to estimate the level of polymorphism between the parental strains and their fusants. The genetic distances (GDs) among the three parents and their fusants ranged from 0.50 to 0.82 and 0.64 to 0.90 based on ISSR and Scot profiles, respectively. The dendrogram resulted from the banding patterns of ISSR and SCoT markers (Table 3&4 and Figure 3). A dendrogram was constructed using a distance matrix by using the UPGMA method (Figure 3) depending on genetic distances from ISSR marker analysis showed that three major groups were observed. The fusants F16 and F1 were highly closely related

and put in the first group (I) with similarity ranged from 0.66 to 0.73, whereas F2, F2', F26, P1, F17, F34, F1' and F31 were placed within the second group (II): (similarity ranged from 0.65 to 0.82). However, P3 and F25, which were put in the third group: (similarity range 0.63 to 0.65). On the other hand, the cluster analysis using UPGMA depending on genetic distances from SCoT marker analysis showed that the three parents and their ten fusants could be divided into three main groups (Figure 3-B). The first group (I) consisted of F17 and P2; (*Bs* GHAI). The second group (II) involved 2 sub-groups (similarity ranged from 0.78 to 0.90) consisted of F1, F2, F1', F2', F26, F34, P1(*Bt* I66/ Ia) and F16. The third group divided into 2 subgroups; F31 and F25; which closely related to P3;(*Bt* I977) (similarity ranged from 0.72 to 0.75). These results were in-agreement with those observed by [15] who reported that microsatellite markers were highly polymorphic and so useful for genetic analysis of *Beauveria* sp.



**Figure 3.** Dendrogram illustrated genetic fingerprint and relationships between *Bt* and *Bs* strains and their fusants developed from ISSR (A) and SCoT (B) data.

P1: *Bt* 66 1a; P2: *Bs* GHAI and their fusants (F1, F2, F16, F26, F31 and F34); No: 1-6.

P2: *Bs* GHAI; P3: *Bt* 1977 and their fusants (F1', F2', F17 and F25); No: 7-10

## 4. Conclusion

In conclusion, this work has revealed that Comparative Analysis of Genetic Diversity Among *Bacillus thuringiensis* and *Bacillus sphaericus* and Their Fusants diversity exist with further experimentations, the ISSR and SCoT profile generated for each strains can be effectively used as a supporting marker for taxonomic identification and diversity.

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