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Molecular Characterization of *Beauvaria sp.* with Inter-Simple Sequence Repeat (ISSR) and RAPD Markers

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Abstract: Inter-simple sequence repeat (ISSR) markers were used to investigate genetic diversity among of isolates of *Beauveria* and Genetic variations of nine *Beauvaria* species were evaluated using random amplified polymorphic DNA (RAPD). Amplification of genomic DNA of the nine genotypes by RAPD analysis were used and twelve primers generated 165 fragments of which 97 were polymorphic with an average of 8 bands/ primer. The amplified products varied in size from 1777 to 71 bp. five selected ISSR primers produced 57 bands across nine genotypes ten of which were polymorphic with an average of 8 bands/ primer. The size of amplified bands ranged from 264 to 2838 bp. Similarity index values ranged from value 0.531 to 0.876 (RAPD), 0.625 to 0.967 (ISSR) and 0.567 to 0.907 (RAPD and ISSR). These results indicated that both of the marker systems RAPD and ISSR, individually or combined can be effectively used in determination of genetic relationship among *Beauvaria* species. It could be concluded that, the information of genetic similarities and diversity among *Beauvaria* genotypes are necessary for identification and differentiation at molecular level methods.
Key words: Beauvaria, ISSR, RAPD, genetic diversity.

Introduction

Study conducted on the efficacy and persistence of a microbial control agent require identification of specific sequences of selective strain of *Beauveria bassiana* with highly polymorphic molecular markers. These techniques are critical for the entomopathogenic fungus *B. bassiana* because the fungus has a wide insect host range and is common in nature[1]. There are also various *B. bassiana* based mycoinsecticides currently registered or under commercial development worldwide for agricultural pests[2]. Molecular markers have been utilized to assess genetic variation among isolates of *B. bassiana* and other entomopathogenic fungi, thereby providing means to identify strains of interest, determine origin of isolates, or study population structure. One technique that has been used to differentiate strains of *B. bassiana* is polymerase chain reaction (PCR) based random amplified polymorphic DNA (RAPD). This technique utilizes short primers of arbitrary sequence that anneal to multiple target sequences producing diagnostic patterns[3,4 and 5].

RAPD analysis has also been utilized to generate unique PCR products or amplicons in filamentous fungal species or strains of interest and identification of specific sequence of *Beauveria bassiana* for the selective strains. RAPD primers are designed based on known DNA sequences of the organism in the study. This allows for the development of sensitive and diagnostic assays to amplify specific fungal DNA in selective

strains containing mixed DNA because primers anneal specifically to fungal sequences. This is in contrast to RAPD analysis that requires the establishment of single spore isolates for strain identification. In this study we utilized RAPD–PCR technique to screen for markers that would differentiate the *B. bassiana* strains of the fungus. These RAPD markers develop a sensitive diagnostic assay for the selective detection of *B. bassiana* strains or isolates[6,7 and 8].

The inter-simple sequence repeat (ISSR) is a new kind of molecular marker that amplifies inter-microsatellite 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[9]. 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 [10]. 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 *Beauveria sp.*

The objectives of the present study are: (1) to test the efficiency of ISSR markers with the goal of using these polymorphisms as genetic markers; (2) to investigate the genotypic variability within *B.sp* by analysing ISSRs of nine *Beauveria* isolated from different insect hosts and geographical origins; and (3) to calculate and to compare genetic diversities, genetic similarities and genetic distances within and among isolates.

Material and Methods

DNA extraction. Total genomic DNA extracted from all isolates of *Beauvaria* by using DNeasy Tissue Kits (Qiagene). Its integrity was checked on agarose gel electrophoresis[11].

DNA amplification

RAPD fingerprinting:

RAPD amplification was carried out with twelve primers (Invitrogen Custom Primers ICP by life technology.(Table 1) in a 25µL volume, containing (5 µL of 5x buffer, 3.0 µL of dNTPs (2.5mM) 3 µL of Mgcl2 (25 mM), 3.0 µL primer (2.5 µL), 0.3 µL of Taq polymerase (5U/ µL) and 2.0 µL of genomic DNA (50 ng/ µL). Amplification was performed in (Perkin Elmer GeneAmp PCR system 9600 –USA). programmed for an initial denaturation at 94^{o}C 5 min, 40 cycles of 1 min denaturation at 94^{o}C, 1 min annealing at 36^{o}C and 2 min extension at 72^{o}C followed by final extension for 5 min at 72^{o}C. Each PCR reaction was repeated twice in order to ensure that RAPD banding patterns were consistent and reproducible and only stable products were scored.

ISSR fingerprinting:

ISSR-PCR reaction for the nine genotypes of *Beauvaria* species was conducted using five primers (Table1). Amplification was carried out in 25 µl reaction mixture contained 2 µl of genomic DNA,1 µl of the primer, 2.5 µl of 10X Taq DNA polymerase reaction buffer, 1.5 unit of Taq DNA polymerase and 200 mM of each dNTP. Amplifications were performed in DNA amplification Thermocycler (Perkin Elmer GeneAmp PCR system 9600–USA).The apparatus is programmed to execute the following conditions, denaturation step of 5 min at 94°C, followed by 35 cycles composed of 30s at 94°C, 60s at the annealing temperature, and 60s at 72°C. A final extension of 72°C for 5 min. Amplifications were performed at least twice and only reproducible products were taken into account for further data analysis.

Gel electrophoresis: Amplification products of RAPD and ISSR were separated on 1.5% agarose gels in 1X TAE buffer with DNA ladder (1Kb) and 100bp DNA Ladder RTU and detected by staining with ethidium bromide[11]. Then, the PCR products were visualized by UV-transilluminator and photographed by gel documentation system, Bio-Rad - Gel Doc.XR+ with Image lab Software.

Pairwise comparison of genotypes based on the presence (1) or absence (0) of reproducible polymorphic DNA bands was used to generate the similarity coefficients by SPSS program version-18[12]. The similarity coefficients was used to construct a dendrogram by the unweight pair group method with arithmetical average (UPGMA).

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

Primer code	Primer sequence 5' to 3'	Nucleotide length
OPD-01	**ACCGCGAAGG**	**10-mers**
OPD-02	**GGACCCAACC**	**10-mers**
OPD-03	**GTCGCCGTCA**	**10-mers**
OPD-04	**TCTGGTGAGG**	**10-mers**
OPD-06	**ACCTGAACGG**	**10-mers**
OPD-07	**TTGGCACGGG**	**10-mers**
OPC-01	**TTCGAGCCAG**	**10-mers**
OPC-13	**AAGCCTCGTC**	**10-mers**
OPE-03	**CCAGATGCAC**	**10-mers**
OPE-06	**AAGACCCCTC**	**10-mers**
OPL-20	**TGGTGGACCA**	**10-mers**
OPQ-15	**TCGCCCAGTC**	**10-mers**
ISSR-HB09	**$(GT)_6GC$**	**14- mers**
ISSR- HB11	**$(GT)_6CC$**	**17-mers**
ISSR-HB12	**$(CAC)_3GC$**	**11mers**
ISSR-HB-13	**$(GAG)_3GC$**	**11-mers**
ISSR-HB14	**$(GTG)_3GC$**	**11-mers**

Results and Discussion

RAPD analysis:

For RAPD analysis twelve Operon series (OPC, OPD, OPE, OPL and OPQ) were tested out of which most reproducible primers were used for the fingerprinting. In the present study twelve RAPD primers were used for the fingerprinting, two each from OPC and OPE series and six from OPD series and one from OPL and OPQ (Table 1).

All the RAPD primers used in the present study produced polymorphic bands, number of bands produced by each primer varied from 1-32 bands, only in case of OPD-02, OPD-04, OPC-13 and OPQ-15 it has produced one polymorphic band. All the bands produced in the present study were scored for the analysis. Twelve primers were used in the study of RAPD markers analysis to standardization of suitable specific primers amplifying the genetic materials of *Beauveria sp.* accessions. All the primers yielded maximum amplification products with all *Beauveria sp.* but the two primers (OPD-01 and OPC-01) produce 41 and 25 band respectively. The primers amplified DNA products from each *Beauveria* generating reproducible band patterns. The remaining primers gave patterns that were identical or had differences too small to provide information on the genetic diversity. Analysis of nine accessions of *Beauveria sp.* revealed 58.7% of polymorphism. A total of 165 distinct bands, consistent with repeated amplifications, were amplified using the DNAs from the nine *Beauvaria* isolates as templates. Depending on the isolate-primer combination, the bands were scored ranging in size from 71 bp to 1777 bp among the isolates of *Beauvaria* collected (Fig. 1).

The total 165 bands were scored for the 12 RAPD primers out of which 97 bands were monomorphic. Using SPSS software Jaccard's similarity coefficient were calculated for each primer. Then based on Jaccard's similarity coefficient Dendrogram was constructed for each genotype of *Beauveria sp*. The PCR amplified band patterns of nine *Beauveria sp* were shown in Fig 1. The size of amplified bands also varied with different primers. The largest 1777 bp band was amplified by primer OPC-01, while the smallest size was amplified by primer OPD-01 and detected about 71 bp (Fig.1) Our results are in agreement with data reported for the *Beauveria* genus fungi isolates, which presented considerable genetic diversity[13] and are also in accordance with the high variability amongst *B. bassiana* populations from natural reserves already observed [14] .It seems that isolates from natural reserves present considerable genetic diversity due to multiplicity of habitats and hosts.

The genetic similarity coefficients based on RAPD markers of the nine studied *Beauvaria* isolates shown in (Table 2) illustrated that the highest similarity value was 0.876 which recorded between B8 and B9, while the lowest similarity value was 0.526 between B.1. and B.3. Cluster analysis based on dissimilarity values between species generated a dendrogram that represent the phylogenetic relationships among the nine species under study (Fig.2).A high co-phenetic correlation coefficient between the dissimilarity matrix and Neighbor-

joining clustering methods was obtained. According to our results, the species under study are distributed in three clusters. The first cluster included, B.8and B.9 with highly related value(0.876) then come B. 7 which is moderately related with B.6 (0.615), while the second cluster contained three genotypes, B.4, B.5 with similarity index value of (0.78)Then, came the third cluster comprised B.1 and B.2 which are highly related to each other record value is (0.737). These results demonstrated that, RAPD analysis through UPGMA dendrogram revealed substantial polymorphism and gave precise phylogenetic relationship among investigated *Beauvaria* species. The same results were reported and the significant variation and a large genetic diversity were calculated within and among *Beauvaria sp.* genotypes by RAPD analysis[15]. Several studies have reported using RAPD markers for the identification of the genetic relationships among species of some *Beauvaria*[16,17,18 and 19]. A result of this study suggests that the molecular diagnoses of strains of *Beauveria* differ very little among themselves.

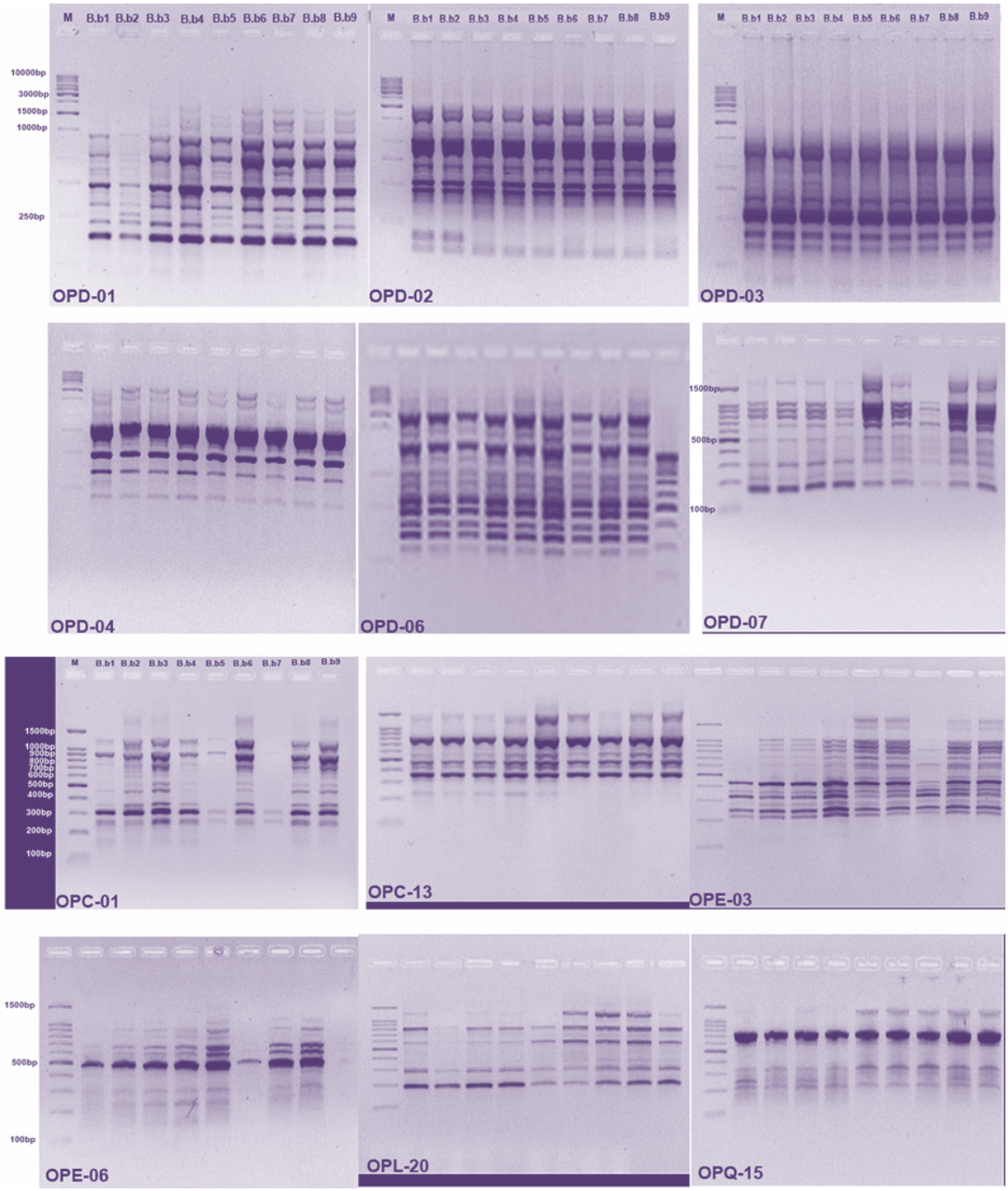

Fig. 1: Agarose electrophoresis showing the random amplified polymorphic DNA (RAPD) among isolates of *Beauveria sp*. Banding patterns amplified with 12 of the arbitrary primers used in this study: (plateA, Primer OPD01; OPD-02 and OPD-03); (Plate B, Primer OPD- 04;OPD-06 and OPD-07); (Plate C, Primer OPC-01; OPC-013 and OPE-03) and (Plat D,Primer OPE -06, OLP-20 and OPQ-15) MM – molecular marker: 100bpDNA ladder Marker, lanes 2 to 10 represent the isolates of *Beauveria sp.*

Table 2 : Similarity Matrix based on RAPD markers of nine *Beauveria* species.

Case	Matrix File Input								
	B1	**B.2**	**B3**	**B.4**	**B.5**	**B6**	**B.7**	**B.8**	**B.9**
B.1	**1.000**								
B.2	0.737	**1.000**							
B.3	0.615	0.650	**1.000**						
B.4	0.557	0.539	0.693	**1.000**					
B.5	0.526	0.548	0.699	0.781	**1.000**				
B.6	0.568	0.560	0.648	0.728	0.685	**1.000**			
B.7	0.568	0.547	0.606	0.590	0.576	0.615	**1.000**		
B.8	0.545	0.569	0.614	0.633	0.629	0.764	0.757	**1.000**	
B..9	0.561	0.531	0.574	0.582	0.579	0.746	0.752	0.876	**1.000**

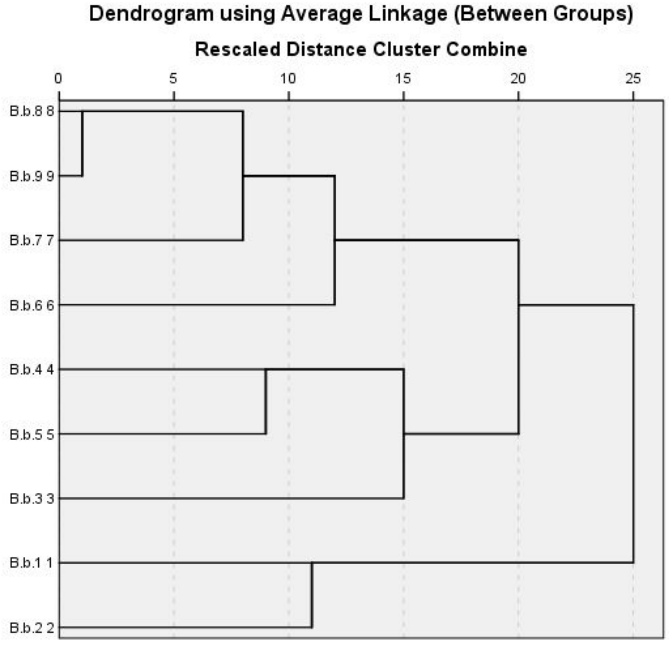

Fig. 2: UPGMA dendrogram indicating the genetic relationships among Beauvaria species based on RAPD markers.

Molecular markers by using ISSR analysis: Five oligonucleotide primers were used to establish ISSR-PCR fingerprints of the nine *Beauvaria* genotypes isolates to detect molecular markers for differentiation. These primers were HB09, HB11, HB12, HB13 and HB14. Both the number and size of the amplified products varied considerably with the different primers. The results of ISSR-PCR of the studied Beauvaria genotypes are given in table (3). From this table it is clear that 23 polymorphic bands were generated with a percentage of polymorphism 17%. Thirteen unique bands were identified of them. Figure (3) and table (3) exhibited the ISSR profile produced by five primers**.** The obtained results revealed that the primer HB-09 and HB-13 have amplified the maximum number of bands, while the primer HB-14 has amplified the least number of bands. Such results indicate that primer HB-09 and HB-13 repeats are more frequent in the Beauvaria genome than the HB-12 and HB-14 repeats[20] .The highest percentage of polymorphism (94.12%) was detected with the primer HB-09, while the least one was recorded in the primer HB-12 and HB-14. Thirteen unique bands were identified among the total bands, and could be considered for marker assisted selection. Among these, 11 unique bands were characteristic for the most Beauvaria genotypes (B2 and B1) and were detected by primer HB-09. In addition, six unique bands were characteristic for the genotypes (B2), five of them were detected in B1 by the primer HB-09 while the third one was scored in B1 and B2 by the primer HB-13. Moreover, one unique band was characteristic for the Beauvaria genotype (B1) by primer HB-13 at molecular size 1010 bp and the other at 996 bp with B2 isolate. Furthermore, there were 10 shared bands could be used as markers and were found only in the three primers HB-09, Hb-11 and HB-13. Applications of the ISSR technique in gene tagging and marker assisted selections are becoming more popular. The results showed that the ISSR primers are

informative markers, which can be examined to correlate banding patterns[21]. The results indicated that ISSR markers have been successfully utilized for assessing the genetic diversity and revealed a remarkable molecular discrimination between the nine isolates under study. Similar results were reported and found that ISSR markers detected a very high level of polymorphism between and among *Beauveria* isolates. These informative primers could be easily developed for population genetic analysis, epidemiological, and ecological studies of *B. bassiana*. Additionally, ISSR analysis can be utilized to generate unique PCR products or amplicons in different Beauveria species or strains of interest, and then may be converted into species – or strain- specific sequence-characterized amplified region (SCAR) markers[22].

Table 3 : List of primers, their sequence, numbers and size of the amplified fragments (bands) generated by ISSR primers in *Beauvaria spp.*

Primer code	Sequence (5′ to 3′)	Monomorphic Bands*	Polymorphic bands**		Total bands	Percent Polymorphism%	Size range (bp)
			Shared bands	Unique bands			
HB09	**$(GT)_6GC$**	1	5	11	17	**94.12%**	192-1072
HB11	**$(GT)_6CC$**	7	2	0	9	**22.225**	205-951
HB12	**$(CAC)_3GC$**	10	0	0	10	**0.00%**	209-2783
HB13	**$(GAG)_3GC$**	9	3	2	14	**35.71%**	245-1346
HB14	**$(GTG)_3GC$**	7	0	0	7	**0.00%**	267-1450
	Total	34	10	13	57	**152%**	

Monomorphic Bands → Same Bands (similar bands) .

Polymorphic Bands → Different Bands (present in few but absent in others /not present in all).

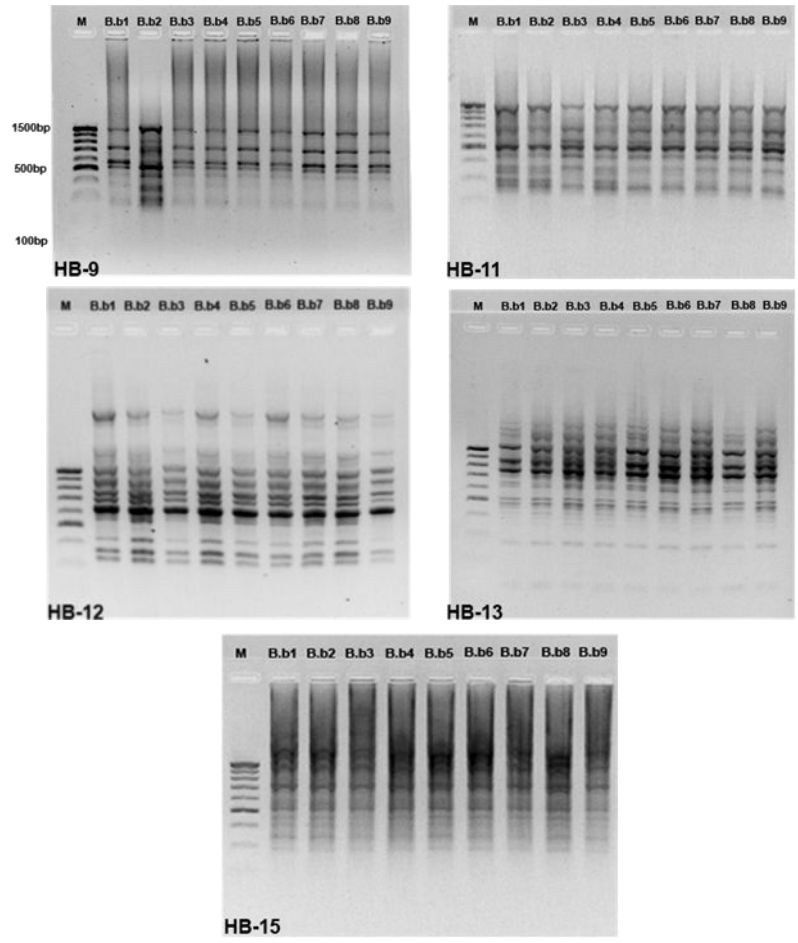

Fig.3: ISSR profile for nine strains of *Beauvaria* sp. Lane(1) 100bp DNA ladder marker and lane (2-10) *Beauvaria* isolates.

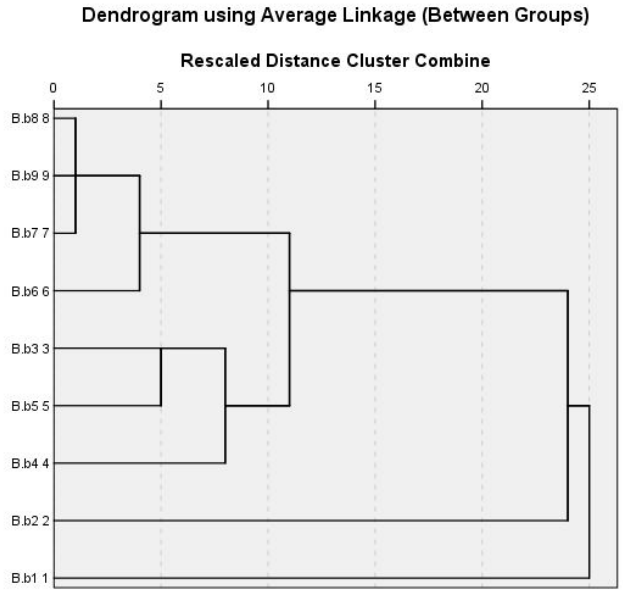

Fig 4: Dendrogram derived from the analysis of nine isolates of *Beauvaria* using ISSR markers and based on distances obtained from the dissimilarity matrix and neighbour-joining clustering method.

RAPD and ISSR analysis:

The similarity coefficients of the nine *Beauvaria* isolates based on RAPD and ISSR markers ranged from 0.450 to 0.030 among the nine genotypes. Similarity index between B8 and B9 showed the highest value (0.907), while the lowest value was (0.596) between B9. and B2. Table (5). Cluster analysis performed from combining data of both markers generated a dendrogram separated the genotypes into three clusters. The first cluster included B8, B9, B7and B6, where B8 Was highly related to B9. and closely related to B7. The second cluster comprised B4,B5 and B3. then come B1 and B2 in the third cluster. Fig (5).

The present work used a combination of RAPD and ISSR markers to determine the further genetic affinities between *Beauvaria* species at the DNA level, the results indicated that, close correspondence between the similarity matrices of both RAPD and ISSR individually or combined, hence both the marker systems can be effectively used in determination of genetic relationship among *Beauvaria* species. Similar studies have been widely applied in a variety of plant genera, such as *Cicer* [12] ,and *Vicia*[23] .The results indicated that RAPD, ISSR and combined RAPD and ISSR analysis showed in fig (5) provided the possibility of identifying the investigated *Beauvaria* genotypes. The three dendrogram showed minor differences in the cluster pattern of the different *Beauvaria* isolates as revealed by RAPD, ISSR and combined RAPD and ISSR.

Both RAPD and ISSR markers contribute a significant number of polymorphic markers, which could be useful in identifying lentil genotypes, contributing to saturate genetic maps and in marker-assisted selection were reported[24].

Table 5: Similarity coefficients of the Beauvaria species based on RAPD and ISSR markers.

Case	Matrix File Input								
	B1	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**	**B9**
B1	**1.000**								
B2	0.728	**1.000**							
B3	0.642	0.674	**1.000**						
B4	0.610	0.592	0.735	**1.000**					
B5	0.569	0.590	0.749	0.809	**1.000**				
B6	0.590	0.588	0.703	0.743	0.728	**1.000**			
B7	0.591	0.581	0.674	0.638	0.635	0.692	**1.000**		
B8	0.573	0.596	0.678	0.670	0.676	0.807	0.817	**1.000**	
B9	0.585	0.567	0.646	0.630	0.636	0.794	0.815	0.907	**1.000**

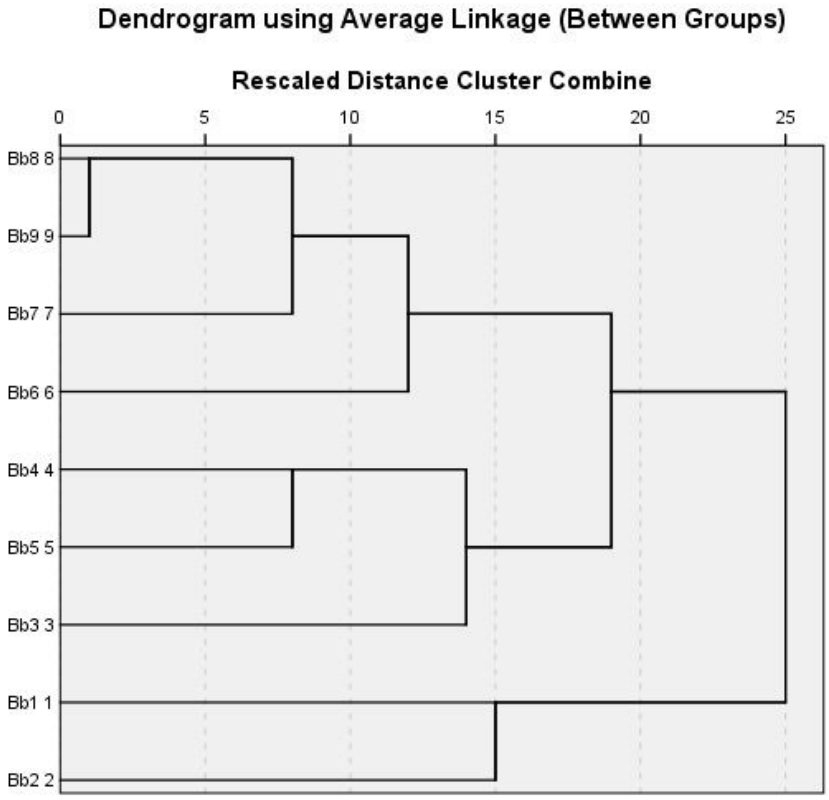

Fig.5: The differences in the cluster pattern of the different *Beauvaria* isolates as revealed by Mix RAPD and ISSR.

Conclusion:

Genetic similarity measured through analysis of RAPD data of nine *Beauveria sp* revealed varying degree of genetic relatedness among them belonging to different species.

The present study was preliminary attempt to develop RAPD and ISSR primers to distinguish the nine *Beauveria* isolates genotypes shown that more difficult screening of primers has to be done before RAPD and ISSR markers can be developed. This study showed a significant morphological variation and a large genetic diversity within and among isolates. Lately, this technique has been used to study the genetic relations between the different fungi isolates and to determine the relationship between them.

These results highlight the distinctive nature of the RADD marker compared to ISSR marker as a powerful procedure to survey the genetic diversity of *Beauvaria* species. These results are in agreement with the result observed before[25] .In the present study, ISSR markers were found less reliable for detecting genetic relatedness among the *Beauvaria sp*. than RAPD markers. The use of more ISSR primers may improve the reliability of this approach for characterizing isolates at the molecular level.

In conclusion, RAPD and ISSR markers differed in their ability to differentiate individuals and for detecting polymorphisms. They can complement each other, although this was not the case in the present study. However, these markers did provide sufficient variation to identify *Beauvaria* isolates.

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