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ASSESSING THE MOLECULAR GENETIC VARIATION OF SOME LOCAL ISOLATES OF THE FUNGUS *BEAUVERIA BASSIANA* (BALS.) USING RAPD PCR

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ABSTRACT

The results of the genetic analysis using 15 primers for RAPD technique showed a difference in the number of bands. The number of polymorphic bands was 127 for all primers, and many of them showed unique bands for some isolates, which are a fingerprint to distinguish the isolates. The isolate 100Bb was the highest number of bands these primers have given 34 bands with different molecular weights. To find the percentage of genetic affinity between fungal isolates, the data were introduced in SPSS program. The results showed a difference in similarity percentage between isolates. The highest was among the isolates 17Bb and 47Bb (0.437) they were the closest genetically and the lowest between 100Bb and 17Bb were 0.039, the results of the dendrogram analysis showed isolation of the isolates into two main groups, the first group included the isolates 17Bb and 47Bb, and in the second group the isolate 100Bb.

KEYWORDS: *Beauveria bassiana*, RAPD PCR, Molecular Variation.

INTRODUCTION

The genus Beauveria is known for its extensive genetic variability, facilitating the selection of isolates with high virulence. Additionally, this genus adapts to the climatic conditions of areas where biological control is applied. While Beauveria can be morphologically distinguished as a genus, species identification proves challenging due to its ease of composition and the absence of distinct morphological differences. Recent studies have utilized both morphological and molecular methods to diagnose diversity within the Beauveria genus.[1]

In a study utilizing primers OPX7, OPX13, OPE11, OPE14, and OPE15 to distinguish genetic variations among 42 isolates of *B. bassiana* using RAPD and Amplified Fragment Length Polymorphism (AFLP) techniques, isolates were categorized into two genetic groups based on RAPD and three genetic groups based on AFLP (Ferri et al., 2012).

Another study investigated the genetic variability among 50 isolates of *B. bassiana* using 6 primers for RAPD analysis. The results of The RAPD analysis divided the isolates into seven genotypes. It was noted that the level of genetic differences among isolates was low, which was expected as most isolates belonged to the same insect type and location (Fernandes et al., 2005).

Twenty isolates of *B. bassiana* were collected from different countries and classified based on their virulence into three categories: highly virulent, moderately virulent, and weakly virulent. RAPD analysis was conducted on these isolates using 20 primers, resulting in their classification into three groups based on the pattern of distribution of DNA bands. The observed genetic variation among the isolates was at a rate of 30%. Interestingly, one highly virulent isolate shared a similar DNA band distribution pattern with weakly virulent isolates, while another highly virulent isolate grouped with moderately virulent isolates. This indicated that there was no direct correlation between a specific level of virulence and a particular pattern of DNA band distribution (Devi et al., 2001).

In this study examined the genetic diversity of *B. bassiana* isolates from various ecosystems in Tamil Nadu using 15 isolates. Random Amplified Polymorphic DNA (RAPD) markers were employed, and among the 15 used, six markers displayed 100% polymorphism. Analysis through UPGMA clustering revealed similarity coefficients ranging from 42.00% to 86.00% among the

isolates, with the highest similarity (85%) observed between isolates B23 and B24. The results indicated significant genetic variation among all isolates, suggesting RAPD as an effective method for assessing genetic diversity in *B. bassiana* isolates (Prabhukarthikeyan et al., 2017).

To diagnose and differentiate local fungal isolates of the same *B. bassiana* species, they were genetically studied using Polymerase Chain Reaction (PCR) technique (Ferri et al., 2012). This study aimed to assess the molecular genetic variability of *B. bassiana* isolates utilizing the RAPD-PCR technique.

MATERIALS AND METHODS

Source of fungal isolates

In this study, three locally selected isolates were used from isolates preserved at the Agricultural Research Department / Ministry of Science and Technology. These isolates were previously isolated from orchard soils and farms in Iraq using the Bait Trap Technique employing *Galleria mellonella* (L.) caterpillars as bait. Post purification using the single spore technique, the isolates Bb17, 47Bb, and 47Bb were obtained from palm and citrus farms in Nahrain, Nasiriya, and Madain, respectively. The fungal isolates were activated by taking 0.5 cm sections from the stored fungal cultures and placing them onto freshly prepared Potato Dextrose Agar (PDA) plates, which were then incubated in a temperature controlled incubator at 25±2°C for one week.

Extraction of DNA

The fungal yarn was taken with cream from the surface of the PDA plate a week after the mushrooms were grown on them and incubated in the incubator, and transferred to the Abendorov tubes. The method was used (Safavi, 2010) with modifications.

The fungal mycelium was scraped from the surface of the PDA plate after one week of fungal growth and incubation. It was transferred to Eppendorf tubes and the method by (Safavi, 2010) was used with modifications.

1-Addition of 500 µl of CTAB to the Eppendorf tube containing the fungal mycelium, followed by vortexing with a vortex mixer. Then, the tube was placed in a water bath at 65°C for 90 minutes.

2-Add 300 µl chloroform Absolut to the tube and centrifugation at 5,000 rpm for five minutes.

3-Collecting 350 µl of the supernatant from the top of the Eppendorf tube and transferring it to a new tube. Then, adding 50 µl of Sodium Acetate and 450 µl of isopropanol with gentle hand mixing.

4-Placing the tube in a refrigerator for 30 minutes, followed by centrifugation at 12000 rpm for 10 minutes using a centrifuge, then pouring off the isopropanol.

5-Adding 600 µl of 70% alcohol, then centrifuging at 12000 rpm for 5 minutes. This step was repeated twice. Afterward, the alcohol was poured off, and the sample was air-dried at room temperature for 30 minutes. Then, adding 50 µl of D.D. water and gently mixing by hand.

Indicators of random DNA chain reaction

The reaction was carried out using a PCR PreMix kit reaction mixture. Fifteen random primers were used, as detailed in Table (1), manufactured by Bioneer according to Operon company specifications. The primers were diluted with sterile distilled water to obtain the recommended concentration following the manufacturer's instructions. Subsequently, original solutions for each primer were prepared at a concentration of 100 nanograms per 1 microliter.

Table 1: Random Prefixes and their Sequences.

Sr. No.	Primer Name	primer sequence 5⟶3
1	OP A-01	CAGGCCCTTC
2	OP A-05	AGGGGTCTTG
3	OP A-11	CAATCGCCGT
4	OP A-15	TTCCGAACCC
5	OP A-20	GTTGCGATCC
6	OP C-04	CCGCATCTAC
7	OP D-02	GGACCCAACC
8	OP E-08	TCACCACGGT
9	OP E-12	TTATCGCCCC
10	OP F-12	ACGGTACCAG
11	OP H-16	TCTCAGCTGG
12	OP H-09	TGTAGCTGGG
13	OP O-16	TCGGCGGTTC
14	OP S-12	CTGGGTGAGT
15	OP Z-11	CTCAGTCGCA

Subsequently, the samples were placed into a Thermocycler and the program was executed following the method by (Kaur & Padmaja, 2008). The initial denaturation temperature for the DNA strand was set at 95°C for four minutes. Denaturation of the template occurred at 94°C for one minute, followed by primer annealing at 55°C for one minute. Primer extension was performed at 72°C for one minute. The final extension

was carried out at 72°C for a duration of six minutes. Both template denaturation and primer extension were repeated for 35 cycles.

PCR electrophoresis of the thermal polymerase reaction mixture

performed gel electrophoresis for DNA samples using a Agarose gel. Four µl of the 100 bp DNA ladder standard marker, which ranges from 100 to 2000 base pairs in molecular weight.

Analysis of the results

1-The software Photo Capt version 10.01 (Cerasela et al., 2011) was used to calculate the molecular weight of DNA bundles resulting from polymerase chain reaction (PCR) by comparing them with the size of the standard 100bp DNA ladder marker that runs alongside the isolated DNA.

2-Using the statistical program SPSS version 23 to calculate the percentage of similarity and draw the dendogram (Genetic Relationship Tree) Complete linkage method, which is one of the hierarchical agglomerative method, was followed to facilitate presentation of data by grouping isolates in clusters and according to similarity of response pattern and based on Sokal and Sneath2 equation.

The statistical software SPSS version 23 was utilized to compute the percentage of similarity and construct the dendrogram (Genetic Relationship Tree). The complete linkage method, one of the agglomerative hierarchical clustering methods, was employed to cluster the isolates based on their response patterns using the Sokal and Sneath equation.

3-The percentage (Polymorphsim, Aptitude, Discriminant Power) was calculated for each initiator (AL-Judy and Majeed, 2013) as in the following equations.

Percentage calculations for polymorphism, efficiency, and discriminatory power for each primer were derived from the equations proposed by (AL-Judy and Majeed, 2013) as follows.

a. Percentage of polymorphism = (number of differing bands in the primer / total number of primer bands) x 100.

B. Percentage of primer efficiency = (total number of primer bands / total number of bands for all primers) x 100.

C. The percentage of the starter discriminant ability = (number of varied packages of initiator / number of varied packages of all prefixes x 100).

Percentage of primer discriminant ability = (number of differing bands for the primer / total number of differing bands for all primers) x 100.

RESULTS AND DISCUSSION

Polymorphism, primers competence and discriminant capacity

In this study, 15 primers were employed, most of which exhibited high polymorphism while some showed low polymorphism. One of these primers, namely OPH-9, did not yield any bands, as indicated in Table (2). These primers collectively produced 136 bands, of which 127 were polymorphic, demonstrating the polymorphism percentages. The primers varied in the number of visible bands, with the lowest band count of 4 observed in the OPH-16 primer, and the highest count of 18 bands appeared in the OPZ-11 primer. Additionally, the primers differed in the number of polymorphic bands, with the minimum count of 2 polymorphic bands in the OPA-20 primer and the maximum count of 17 polymorphic bands in the OPZ-11 primer.

RAPD marker properties were computed by determining the percentage of polymorphism, efficiency, and discriminatory power for each primer. Primers OPD-02, OPE-12, OPA-11, OPA-01, OPA-15, OPS-12, and OPE-08 exhibited the highest polymorphism percentage, reaching 100%. The lowest polymorphism percentage was recorded at 40% for primer OPA-20. The highest efficiency of 13.2% was achieved by primer OPZ-11, and similarly, the highest discriminatory power of 13.3% was observed for primer OPZ-11 as well.

Table 2: Percentage of Polymorphism, Efficiency, and Discriminatory Power for Primer.

Sr. No.	Primer Code	Total Number of Bands	Primer Discriminatory Power	% Polymorphism of the primer	% Primer Efficiency	%Primer Discriminatory Ability
1	OP A-05	13	10	76.9	9.5	7.8
2	OP D-02	10	10	100	7.3	7.8
3	OP E-12	14	14	100	10.2	11
4	OP O-16	14	14	100	10.2	11
5	OP Z-11	18	17	94.4	13.2	13.3
6	OP A-11	12	12	100	8.8	9.4
7	OP C-04	9	8	88.8	6.6	6.2
8	OP A-20	5	2	40	3.6	1.5
9	OP A-01	10	10	100	7.3	7.8
10	OP A-15	9	9	100	6.6	7
11	OP F-12	8	8	100	5.8	6.2
12	OP H-16	4	3	75	2.9	2.3

13	OP S-12	5	5	100	3.6	3.9
14	OP E-08	5	5	100	3.6	3.9
15	Total	136	127			

Discuss the outcome of RAPD interactions

Most of the primers used in the study exhibited distinct bands for all studied isolates, serving as genetic fingerprints to distinguish each isolate from the others (Table 3). Isolate 100 stood out with the highest number of distinct bands when using the primers OPA-01, OPA-05, OPA-11, OPA-15, OPC-04, OPE-12, OPF-12, OPH-16, OPO-16, OPS-12, and OPZ-11, providing 34 distinct bands with varying molecular weights (Table 4-18). In contrast, isolate 47 showed the lowest number of distinct bands, presenting 16 bands with differing molecular weights when using the primers OPA-01, OPA-05, OPA-11, OPA-15, OPC-04, OPD-02, OPE-12, OPH-16, and OPZ-11.

Although the polymorphism percentages for the primers OPD-02, OPE-12, OPO-16, OPA-11, OPA-01, OPA-15, OPF-12, OPS-12, and OPE-08 were the highest at 100%, they did not display the highest percentages for efficiency and discriminatory power. This indicates a correlation between each of these factors with the number of polymorphic bands. Consequently, an increase in the number of polymorphic bands using a primer implies an increased ability to differentiate isolates from each other using that primer. These distinct bands serve as valuable tools in environmental studies to identify highly pathogenic isolates, monitor them over the long term, and determine the stability of their efficiency after introduction to the environment by forming specific primers. Strain-specific sequence Characterized Amplified Region (SCAR) for these isolates (Ferri et al., 2012; Castrillo et al., 2003; Carneiro et al., 2008).

Table 3: the distinctive bands for each isolation for all the primers used.

	Isolate100	**Isolate 47**	**Isolate17**	
OPA-01	190، 122	1470، 589	629	visible bands
	1955، 948، 761، 449، 231			absent bands
OPA-05	1000، 739، 588 ,167	1745	487	visible bands
	929، 700، 563,380			absent bands
OPA-15	623، 458، 177	916	885	visible bands
	580، 418، 328، 155			absent bands
OP C-04	100	1042، 651	985، 600	visible bands
	509، 449، 346			absent bands
OP D-02	408، 190	453، 327	550، 491	visible bands
	661، 378، 287، 185			absent bands
OP E-12	379، 282، 106	1600، 1150، 700، 634	900، 584، 465، 224،115	visible bands
	310		235	absent bands
OP H-16	349	1000، 448		visible bands
				absent bands
OP O-16	900، 687، 600، 344،307			visible bands
	1271،869،649، 577، 481،314		426	absent bands
OP Z-11	612، 468، 400، 314، 238	1223	1200،714،623، 378، 335، 185	visible bands
	1429، 963، 531، 428		171	absent bands
OP F-12			266	visible bands
			189	absent bands

Estimating the genetic dimension between the studied isolates

Genetic similarity, in this context, represents the degree of genetic resemblance between two groups, individuals, isolates, or even among species belonging to the same genus. In this study, the computer program SPSS was utilized to determine the genetic similarity based on RAPD results, relying on the percentages that reveal similarities in bands produced by all used primers. The results exhibited variations in similarity ratios among the isolates, as shown in the table below. The highest similarity ratio reached 0.437 between isolates 17Bb and 47Bb, indicating they are genetically closer to each other. Conversely, the lowest ratio was 0.039 between isolates 100Bb and 17Bb, signifying that they are genetically more distant from each other.

These findings align with the results of numerous researchers who also demonstrated differences in genetic similarity ratios among isolates of the fungus *B. bassiana* (Golshan et al., 2014; Bhadauria et al., 2013; Santoro et al., 2008). The increased appearance of shared bands among isolates reduces the genetic distance between them. These shared bands indicate the similarity in genetic material in that specific genomic region among the studied isolates. This similarity may manifest in phenotypic traits or in genetic adaptations to

environmental requirements. It could also denote similarities in non-coding DNA regions (Yassin, 2011).

Cluster Analysis

Introducing the studied isolates into the cluster analysis method generates a dendrogram, resembling a tree and known as a genetic similarity tree, as depicted in Figure (1). It displays principal clusters that may further divide into smaller clusters, based on the genetic proximity among these groups. Thus, the presence of a subset of isolates within a particular cluster indicates the genetic extent of similarity among those isolates within that group (Elmeer et al., 2011; El Kichaoui et al., 2013; Khierallah et al., 2011). The dendrogram illustrates the separation of isolates into two main clusters: the first cluster comprises isolates 17Bb and 47Bb, while the second cluster includes isolate 100Bb.

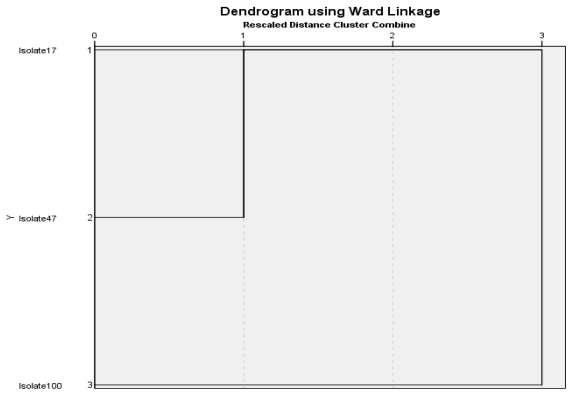

Figure 1: Genetic Similarity Dendrogram.

According to the RAPD analysis, genetic diversity was found to be associated with the geographic origin of the isolates, which aligns with previous studies (Poeaim et al., 2014; Kaur and Padmaja, 2008). The impact of isolates from various regions on both the adult and larval stages of the cucurbit fruit fly was investigated in a preceding study. Specifically, the current isolates Bb100, Bb47, and Bb17 were examined. Among these isolates, Bb100 demonstrated the highest efficacy against adult female insects in comparison to the other isolates. Furthermore, these isolates caused a noteworthy decrease in adult emergence, particularly at a concentration of 10^9 spores/mL (Faleh et al., 2017).

Previous studies have suggested that there might be an increase in genetic variation among isolates with increasing geographic distance between isolated regions (Rehner et al., 2006; Fernandes et al., 2009). On the other hand, the isolates in the current study were isolated from soil and exhibited significant genetic variation among them. This was also evident in the findings of (Urtz and Rice, 1997) who observed greater genetic diversity among fungal isolates obtained from soil compared to those isolated from insect cadavers. Similarly, (Fungaro et al. 1996) noted a similar trend in isolates of the fungus Metarhizium anisopliae.

The possibility of a parasexual cycle occurring in the fungus *B. bassiana* involves genetic material recombination by the fusion of cells from different fungal hyphae. This process involves the dissolution of the dividing wall between them, leading to a plasmic fusion followed by nuclear fusion, resulting in a single diploid nucleus. This is accompanied by a nuclear division process termed parameiosis within the fungal hyphae, without causing morphological changes. Consequently, the diploid phase becomes a transient and unstable phase (Paccola-Meirelles and Azevedo, 1991, 1994; Viaud et al., 1996). Therefore, this phenomenon has not been observed in laboratory settings for the fungus *B. bassiana* (Paccola-Meirelles and Azevedo, 1991; Bello and Paccola-Meirelles, 1998).

It is also possible for genetic recombination to occur through crossing over during mitotic division, termed mitotic crossing over. Additionally, intra-hyphae transformation might occur (Glass et al., 2000). In this process, when two cells from different non-identical strains of fungal hyphae, termed heterokaryon, fuse, one of the strains releases genetic material fragments, which get incorporated into the genome of the other strain. This process is referred to as interstrain gene transfer and has been observed in some fungi such as Trichoderma (Stasz and Harma, 1990; Harman et al., 2002). The high genetic variation among *B. bassiana* isolates isolated from nature indicates the potential occurrence of these recombination processes in the field during concurrent insect infection by different isolates, as demonstrated by (Leal-Bertioli et al., 2000) in the insect-pathogenic fungus M. anisopliae.

CONCLUSION

the study indicated that genetic diversity was linked to the geographical origin of isolates. However, no relationship was observed between genetic diversity and virulence of the isolate, aligning with some previous studies.

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