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Research Article

Analysis of genetic diversity among different isolates of *Beauveria bassiana* by RAPD-PCR

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ABSTRACT: The genetic diversity of *Beauveria bassiana* isolated from the different ecosystem of Tamil Nadu was analyzed. Totally fifteen isolates were used in this study. The genetic variability and relationship among 15 isolates were analyzed using 15 Random Amplified Polymorphic DNA (RAPD) markers. Among 15 primers used, six primers *viz*., OPA02, OPF01, OPX07, OPA03, OPZ19 and OPG19 showed 100 percent polymorphism. The relationships among the isolates were examined and represented as dendrogram by using UPGMA clusters. Results revealed that similarity coefficients of *B. bassiana* isolates based on RAPD markers ranged from 42.00 to 86.00 percent. The maximum of 85 percent similarity was observed between the isolates B23 and B24. All isolates have significantly different from one another. Overall we concluded that RAPD was found to be better in assessing genetic diversity among *B. bassiana* isolates.

KEY WORDS: *Beauveria bassiana,* clustering, Genetic diversity, RAPD, UPGMA

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INTRODUCTION

Beauveria bassiana is called as white muscardine fungus, because of its white powdery appearance. It infects a variety of insects from different orders and has the potential for insect pest control (Meyling *et al.,* 2009). *B. bassiana* based mycoinsecticides are used against agricultural, veterinary and medical insect pests. In recent years, *B. bassiana* considered as a dual purpose bioagent against both insect pests and plant pathogens. Isolates of *B. bassiana* inhibited mycelial growth of an array of soilborne and foliar plant pathogens *in vitro* (Ownley *et al*., 2008). Hence, it is essential to assess genetic variation among *B. bassiana* isolates and to determine their distribution, abundance and potential for genetic exchange between and within populations. The biodiversity of the entomopathogenic fungal genetic resources has been studied using morphological, biochemical or molecular markers (Castrillo *et al*., 1999).

To determine its effectuality, host specificity, survival and partial temporal distribution within the field, proper markers are required for the individual strains (Leal *et al.,* 1994). DNA markers can give more précised genetic information compared to isozymes and they are not changed by environmental conditions (Tigano *et al.,* 1995). A number of markers, like AFLPs, RFLPs, RAPDs, DNA barcoding, and microsatellites, are currently available to detect polymorphisms in genomic DNA. Among them, RAPD-PCR technique has been used successfully to measure genetic diversity among fungal entomopathogens, particularly with isolates belonging to the genus *Beauveria.* RAPD markers are an easy tool to study the genetic differentiation in large samples of fungal species at low cost (Bielikova *et al*., 2002). Also, the technique uses short arbitrary sequences that anneal to multiple target sequences, thus producing diagnostic patterns (Willaims *et al.,* 1990). In this study, we aimed to investigate the genetic diversity of *Beauveria* isolates using RAPD-PCR.

MATERIAL AND METHODS

Isolation of *Beauveria*

The insect-bait method was used for the isolation of entomopathogenic fungi existing in the soil. *Helicoverpa armigera* was used as insect bait. Isolation was made according to the procedure of Shimazu (1993). Dead insect samples were surfaced sterilized and placed in SDY medium (Sabouraud's dextrose with 1 percent yeast extract

medium) (Barley flour 50 g; Dextrose 10 g; Neo peptone 4 g; Yeast extract 2 g; Agar Agar 18 g; Distilled water 1000 ml). Then the plates incubated at room temperature (28 ± 2 ^{0}C) for 7 days, the colonies obtained were transferred to SDY slant for preservation. All the isolates were confirmed based on morphological colony characters.

Preparation of condial suspension and bioassay against *Helicoverpa armigera*

The fungal pathogenic isolates were cultured on SDY medium and the Petri dishes were incubated for one week at 25 ± 1 ^{0}C. A quantity of 25-30 ml of sterilized distilled water (containing 0.05 per cent Tween 20) was used to harvest the fungal spores. Neubaur haemocytometer was used to estimate the spore count. The spore concentration of the suspension was adjusted to 10^8 spores/ml with sterile distilled water and they were used for bioassay against tomato fruit borer (*H. armigera*). Third instar larvae of *H. armigera* were bioassayed for their susceptibility to *B. bassiana*. Ten larvae were taken in a Petri dish which was lined by a filter paper at the bottom for absorbing excess moisture. Ten ml of ($1x10^8$ conidia/ml) conidial suspension was sprayed on the larvae using a hand atomizer. Three replicates were maintained each contained ten third larvae of *H. armigera*. Ten ml of sterilized distilled water with 0.05 per cent Tween 20 served as control. Petri dishes were kept in laminar air flow chamber for five minutes to remove the excess moisture and then carefully transferred to individual clean sterile plastic Petri dish containing fresh leaves of tomato. Then kept inside the BOD incubator at 25 ± 1 ^{0}C. Regular larval mortality was noticed at 24 h interval until 10 days of treatment.

Molecular characterization

DNA was isolated according to the procedure of Zolan & Pukkila (1996). To extract the DNA, 1 g of frozen mycelium was ground to fine powder in liquid nitrogen and incubated in 5 ml of 2 percent CTAB extraction buffer at 65°C for 1 h. The suspension was added with equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) mixture. It was vortexes to mix two phases, followed by a centrifuge at 12,000 rpm for 5 min. The supernatant was transferred to clean tube and mixed with an equal volume of ice cold isopropanol. It was incubated at 25°C for DNA precipitation. The precipitate was collected by centrifugation and the pellet was washed with 0.1 M ammonium acetate in 70 percent ethanol. Again incubation was given for 15 min. The pellet was resuspended in TE buffer and the DNA concentration was estimated spectrophotometrically. *B. bassiana* specific primer combination of ITS 1: 5' – TCCGTAGGTGAACCTGCGG – 3' and PN16: 5' – TCCCTTTCAACAATTTCACG – 3' was used to identify the isolates at species level. The PCR reaction mixture of 20 µl contained approximately 50 ng of total DNA, 5 mM each dNTPs, 20 pmol of each forward and reverse primer and 0.5 U of Taq DNA polymerase (Bangalore Genei, India). PCR conditions were 2 min preheating step at 95 ^{0}C followed by 35 cycles consisting of denaturing at 95 ^{0}C for 1 min, 50 ^{0}C annealing for 30 sec, extension at 72 ^{0}C for 2 min and with a final extension at 70 ^{0}C for 7 min (White *et al*., 1990).

RAPD -PCR

Out of 28 RAPD primers, 15 primers were selected to detect polymorphic RAPD bands among the fifteen isolates of *B. bassiana*. The PCR was carried out in a Mastercycler gradient. PCR for RAPD analysis was carried out in 0.2 ml PCR tubes containing 50-80 ng genomic DNA, 10 µM of primer, 0.25 mM dNTP, 1.5 mM $MgCl_2$, 2 U of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India) and 1X PCR reaction buffer. For RAPD, DNA amplification was performed using the following parameters: 40 cycles of 94^0 C for 1 min, 36^0 C for 1 min and 72^0 C for 2 min. The PCR reactions were repeated at least three times to confirm the reproducibility of each PCR band.

Electrophoresis

The RAPD-PCR products were separated in 1.5 percent (w/v) agarose gel in 1x TAE buffer (0.4 M Tris, 0.2 M acetic acid, 10 mM EDTA; pH 8.4) containing 0.5 µg/ml ethidium bromide. The PCR product along with gel loading buffer (6x containing 0.25% bromophenol blue, 0.25% xylene, cyndol FF, and 3% glycerol) was loaded and electrophoresis was carried out at 50 V. Then, the gel was viewed in an UV illuminator.

Data Analysis

The banding patterns were scored for RAPD primers in each *B. bassiana* isolate starting from the small size fragment to large sized one. Polymorphic DNA bands were documented as presence (1) and absence (0). The genetic similarity coefficients between each isolate were calculated by the Numerical Taxonomy Multivariate Analysis System (NTSYS-PC), version 2.10 (Exeter Software, Setauket, New York) software package (Rohlf, 1990). A dendrogram was constructed based on Jaccard's similarity coefficient using the marker data from the *B. bassiana* with unweighted pair group method (UPGMA) (Jaccard, 1912).

The data for pathogenicity study was analysed using the IRRISTAT v.92-1 programme developed by the Biometric Unit, International Rice Research Institute (IRRI, Philippines). Data were subjected to analysis of variance (ANOVA). Data in percentages were arcsine transformed before analysis. The treatment means were compared by Duncan's multiple range test (DMRT) (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

The entomopathogenic fungal isolates were isolated from soil samples and insect cadavers collected from various places of Tamil Nadu (Prabhukarthikeyan *et al.,* 2014). Among the isolates, B2 was found to be more effective against *H. armigera* which was recorded 73.33 percent larval mortality (Table 1). Similarly, thirteen isolates of *B. bassiana* was isolated from silk worm, *Spodoptera litura* and soil (Bala Naik *et al.,* 2015). *Beauveria* strains were recovered from soil samples collected from *Argania spinosa* forests in Morocco using *Galleria mellonella* baiting method (Imoulan *et al.,* 2016). The molecular characterization of entomopathogenic fungi *B. bassiana* is gaining immense importance for understanding the genomic organization, gene stability and genetic variation (Kosir *et al.,* 1991). The morphological or biochemical characterization alone would be inadequate for understanding the genetic profile and differentiation of isolates. The overall genetic variability revealed by DNA markers permit finer taxonomic resolution than by morphological characterization (Tigano *et al.,* 1995). The genetic variability of *Metarhizium* spp. and *B. bassiana* were assessed using PCR based technology. (Bidochka *et al.,* 1994).

Table 1. Isolation source, morphological characters and entomopathogenicity

Isolates	Place	Source	Colony observation			*Larval mortality (%)*
			Growth pattern	Colour	Surafce	
B1	Coimbatore	Soil	Disperse	White	Smooth and raised	23.33 (28.88)^{i}
B2	Arachalore	Insect	Disperse	Yellowish white	Smooth	73.33 (58.94)^{a}
B3	Madurai	Soil	Disperse	White	Flat	50.00 (44.99)^{c}
B6	Agasthiar Hills	Soil	Disperse	White	Smooth	40.00 (39.23)^{de}
B9	Cumbum	Soil	Disperse	White	Smooth	43.33 (41.16)^{d}
B13	Periyar reserve	Insect	Dense	White	Smooth and raised	23.33 (28.88)^{i}
B20	Cherrapunji	Insect	Disperse and dense	White	Flat	53.33 (46.91)^{c}
B21	Ooty	Insect	Disperse	Yellowish white	Smooth and raised	63.33 (52.75)^{b}
B22	Kovilpatti	Soil	Disperse	White	Smooth	26.67 (31.09)^{hi}
B23	Coimbatore	Insect	Disperse	White	Flat	33.33 (35.26)^{fg}
B24	Periyakulam	Soil	Disperse	White	Smooth and raised	36.67 (37.27)^{ef}
B25	Kohima	Soil	Disperse	Yellowish white	Smooth	60.00 (50.78)^{b}
B26	Udumalai	Insect	Disperse and dense	White	Smooth	30.00 (33.21)^{gh}
B27	Pollachi	Soil	Disperse and dense	White	Smooth	50.00 (44.99)^{c}
B28	Theni	Soil	Disperse and dense	Yellowish white	Smooth	43.33 (41.16)^{d}
Control						0.00 (0.00)^{j}

*Values are mean of three replications.
*Tested at a concentration of 1×10^{8} spores/ml.
Figures in parentheses represent arcsine transformation. Means in a column followed by same superscript letters are not significantly different according to DMRT at $P \leq 0.05$

Table 2. Polymorphism detected by RAPD markers

S. No	Primers	Total allele	MB	PB	% MM	% PM	Total amplicons
1.	C3 - 5'CGGCTTGGGT 3'	4	1	3	25.00	75.00	33
2.	OPA02 - 5' TGCCGAGCTG 3'	11	0	11	0.00	100.00	122
3.	OPF01 - 5' ACGGATCCTG 3'	6	0	6	0.00	100.00	48
4.	OPC20- 5' ACTTCGCCAC 3'	5	1	4	20.00	80.00	51
5.	OPX07- 5' GAGCGAGGCT 3'	8	0	8	0.00	100.00	72
6.	OPA03 - 5'AGTCAGCCAG 3'	10	0	10	0.00	100.00	102
7.	OPA09 - 5'GGGTAACGCC 3'	8	2	6	25.00	75.00	68
8.	OPA13 - 5'CAGCACCCAC 3'	9	1	8	11.11	88.89	87
9.	OPQ04 - 5'AGTGCGCTGA 3'	6	1	5	16.67	83.33	30
10.	OPZ13 - 5' GACTAAGCCC 3'	4	2	2	50.00	50.00	25
11.	OPZ19 - 5'GTGCGAGCAA 3'	11	0	11	0.00	100.00	105
12.	OPE04 - 5'GTGACATGCC3'	8	1	7	12.50	87.50	40
13.	OPF06 - 5'GGGAATTCGG3'	5	2	3	40.00	60.00	19
14.	OPG19 - 5'GTCAGGGCAA3'	5	0	5	0.00	100.00	32
15.	OPH-19 - 5'CTGACCAGCC3'	7	2	5	28.57	71.43	43

Table 3. Similarity matrix for *Beauveria* isolates generated using RAPD primers

Isolates	B1	B2	B3	B6	B9	B13	B20	B21	B22	B23	B24	B25	B26	B27	B28
B1	1.000														
B2	0.810	1.000													
B3	0.585	0.666	1.000												
B6	0.623	0.704	0.788	1.000											
B9	0.370	0.5	0.536	0.567	1.000										
B13	0.379	0.512	0.530	0.543	0.850	1.000									
B20	0.573	0.671	0.683	0.740	0.621	0.637	1.000								
B21	0.512	0.607	0.683	0.763	0.641	0.658	0.842	1.000							
B22	0.626	0.661	0.653	0.760	0.458	0.452	0.679	0.679	1.000						
B23	0.367	0.481	0.537	0.569	0.684	0.704	0.625	0.666	0.531	1.000					
B24	0.312	0.407	0.481	0.493	0.690	0.685	0.587	0.649	0.456	0.857	1.000				
B25	0.314	0.364	0.486	0.44	0.465	0.5	0.443	0.52	0.418	0.485	0.553	1.000			
B26	0.323	0.430	0.379	0.410	0.493	0.486	0.432	0.414	0.371	0.413	0.410	0.363	1.000		
B27	0.298	0.397	0.402	0.45	0.597	0.547	0.469	0.469	0.395	0.493	0.472	0.352	0.781	1.000	
B28	0.372	0.406	0.432	0.426	0.371	0.382	0.373	0.410	0.402	0.347	0.323	0.480	0.54	0.517	1.000

In the present study, all the *Beauveria* isolates amplified an ampicon size of 930 bp which confirmed that the isolates belong to *B. bassiana* (Fig 1). RAPD analysis of *B. bassiana* with different random primers amplified the DNA fragments with different molecular weights. The size of the amplified products varied from 100 to 2500 bp. Of the 15 primers used, six primers *viz.*, OPA-02, OPF01, OPX07, OPA03, OPZ19 and OPG19 were found to show 100 percent polymorphism (Table 2; Fig 2). RAPD markers are suitable for genetic variability analyses because they are randomly generated from many loci throughout the genome. Different level of genetic diversity of *Beauveria* sp. was reported in previous studies, involving different molecular techniques (Poeaim *et al.,* 2014; Garrido-Jurado *et al.,* 2015). Jie and Liangen (2010) reported the genetic relationship of *B. bassiana* isolates using RAPD-PCR. A total of 88 DNA bands were showed polymorphic out of 138 DNA bands amplified with 4 random decamer primers. Eight RAPD primers amplified 72 scorable bands. Out of that, only 5 (7%) were monomorphic bands among all the isolates of *B. bassiana*. The number of bands generated by each primer differed from 4 (OPA09) to 13 (OPA03 and OPQ01), with an average of 5.3 bands per primer. The size of the bands varied from 300 (OPZ19) to 3800 (OPA 13) base pairs (Carneiro *et al*., 2008).

In our study, the relationships among the isolates were examined and represented as dendrogram by using UPGMA. The *Beauveria* isolates were divided into two main groups, I and II which were further subdivided into different clusters. Among the isolates, 12 isolates were grouped under group I and three isolates were grouped under group II. The isolates B1, B2, B3, B6, B20, B21, B22, B9, B13, B23, B24 and B25 were placed in one group with approximately 44 percent similarity coefficient between them and B26, B27 and B28 were grouped in another group which showed 53.5 percent similarity. In group I, there was two clusters namely A and B. Cluster A was divided into sub cluster C and D with approximately 52 percent similarity. In the case of cluster C, two sub clusters namely E and F were noticed with 62 percent similarity. Sub cluster E had two isolates B1 and B2 sharing 81 percent similarity. Whereas, sub cluster F had B3, B6, B20, B21 and B22 having 69 percent similarity. Sub cluster D had four isolates, B9, B13, B23 and B24 sharing 69 percent similarity, among them B23 and B24 showed maximum similarity up to 85.7 percent (Table 3). Cluster B was separated with one isolate *i.e.,* B25. Group II had three isolates B26, B27 and B28 sharing 53.5 percent similarity among them. Overall, all isolates were significantly different from one another (Fig 3).

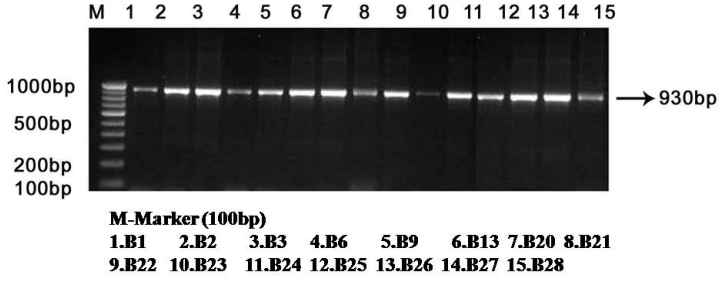

Fig. 1. PCR amplification *Beauveria bassiana* isolates using species specific primers.

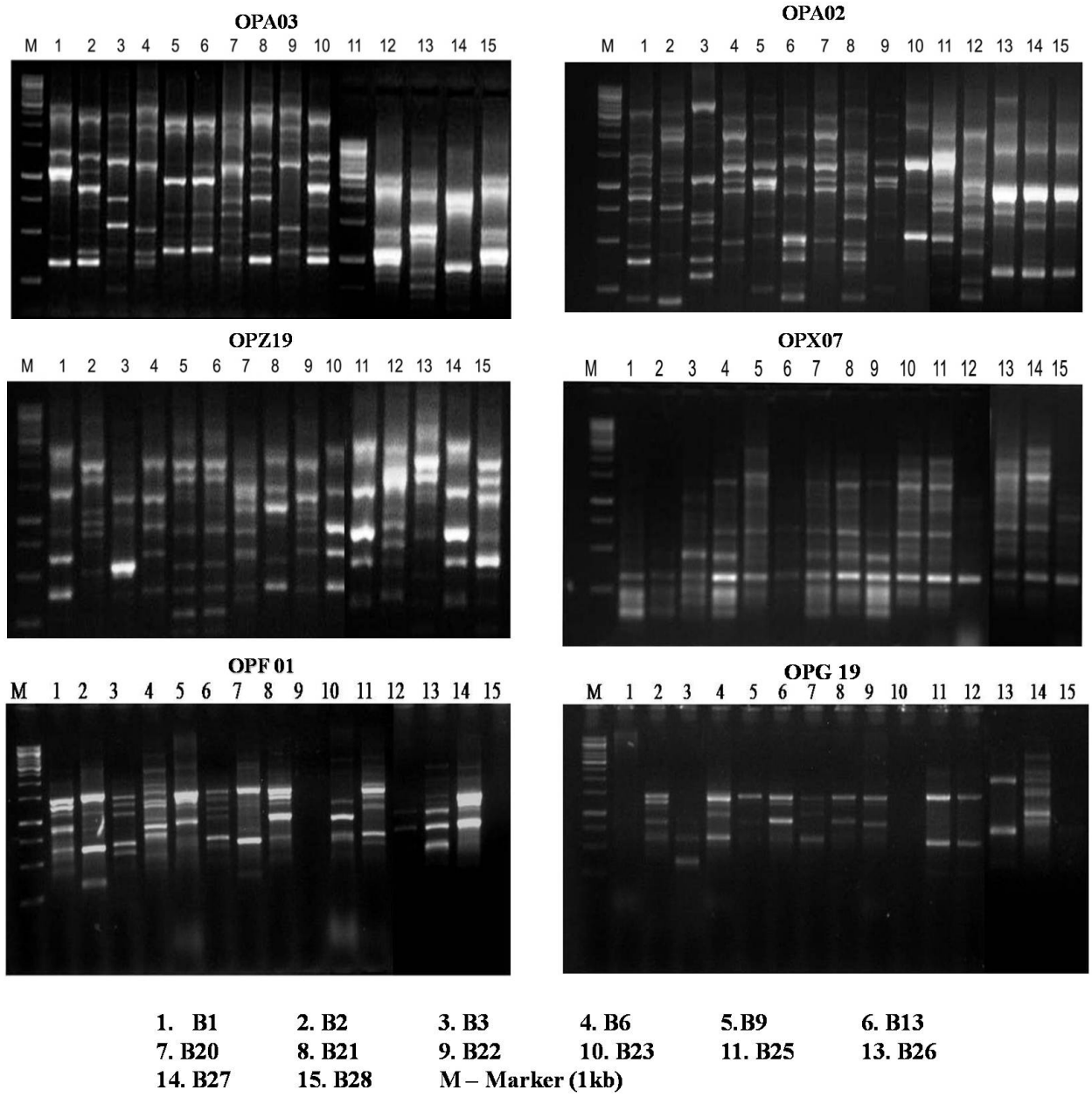

Fig. 2. Examples of RAPD polymorphism in isolates of *Beauveria bassiana* generated with random primers.

The results of present investigations are in accordance with works of Kaur and Padmaja (2008) who evaluated the genetic diversity of 27 *B. bassiana* isolates using RAPD analysis and reported that cluster analysis separated the 27 *B. bassiana* isolates into three clusters bifurcating at 0.66 similarities. Based on RAPD-PCR, 67 polymorphic RAPD fragments were capable of differentiating twenty four *B. bassiana* and grouped them based on host insect and the pathogenicity against maize fall armyworm larvae. Three RAPD markers were closely associated with the pathogenicity against coffee berry borer explaining 57 to 89 per cent phenotypic variation (Cruz *et al*., 2006). Besides, our results supported by Gaitan et al. (2002) who assessed the variability of 95 isolates of *B. bassiana* from different geographical regions and hosts using RAPDs and ITS- RFLP molecular markers in order to characterize its genetic variability. Furthermore, these results were in accordance with results of Imoulan et al. (2016) who characterized *B. bassiana* isolates using RAPD markers and reported that all isolates were significantly different from one another. The current study revealed that the genetic variability among the isolates was determined using PCR-RAPD technique and a high genetic diversity was detected. This information will improve the basic understanding of the genetic variability among *B. bassiana* isolates.

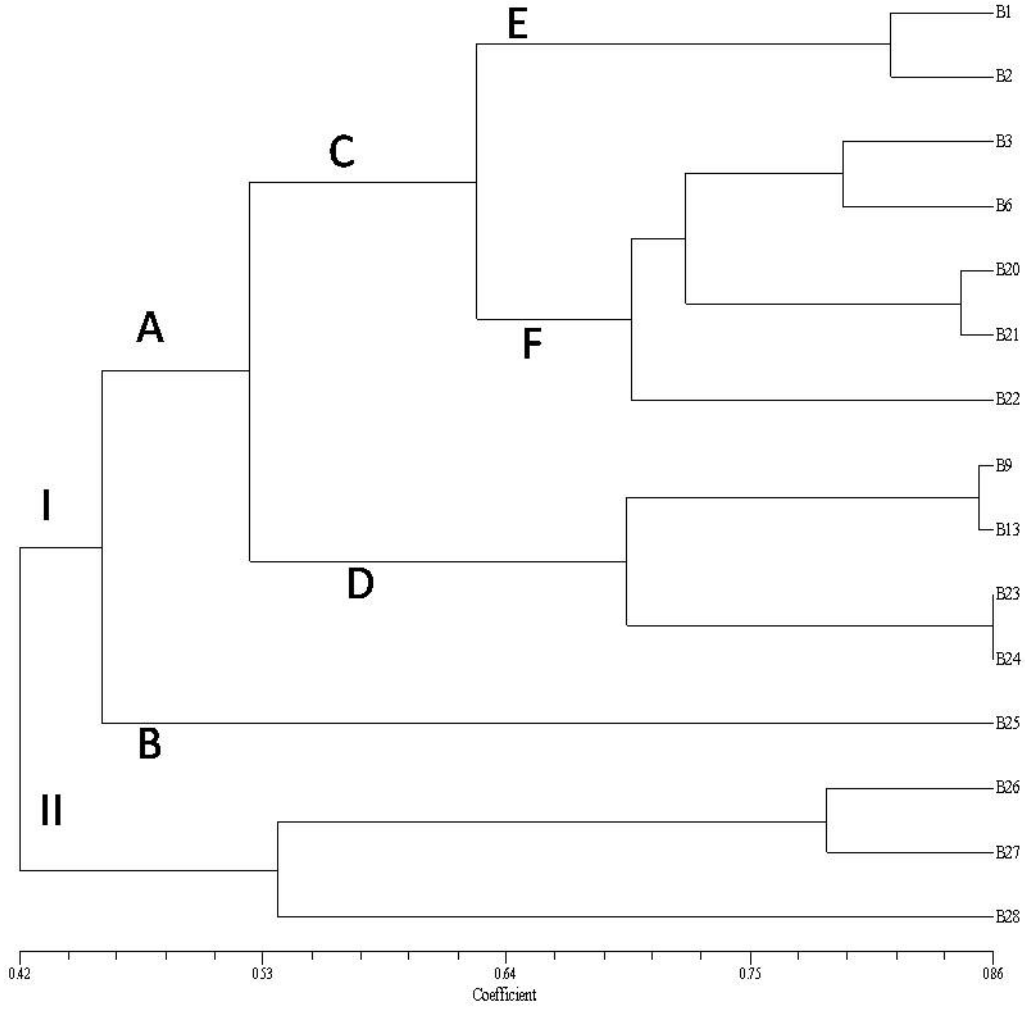

Fig. 3. UPGMA cluster analysis of *Beauveria bassiana* based on RAPD primers.

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