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Evaluation of *Beauveria bassiana* isolates for virulence against *Spodoptera litura* (Fab.) (Lepidoptera: Noctuidae) and their characterization by RAPD-PCR

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Beauveria bassiana **is a known natural enemy of a number of insect pests of crop plants. Molecular markers provide a means for constructing the molecular phylogeny, diversity and link to virulent phenotypes in order to screen different isolates of any given entomopathogens. Twenty-seven isolates of *B. bassiana* isolated from different insect hosts and from different geographical origins were characterized by PCR-based RAPD markers. DNA markers provide more detailed genomic information and are highly rewarding in view of stability and reliability of the parameter. Bioassays were conducted by using second instar larvae of *Spodoptera litura* in order to categorize the isolates based on virulence. The different isolates were arbitrarily rated as more virulent, moderately virulent and less virulent based on the speed of kill. Genetic relatedness between the different isolates based on RAPD analysis and pathogenicity was studied. Clustering pattern to a certain extent reflected the genetic resemblance between the isolates based on geographical distribution as the isolates, which were not isolated from India, did not show any pairing with other isolates. On the other hand, no correlation was found between the pathogenicity of the isolates and the relatedness of the original insect host.**

Key words: *Beauveria bassiana*, isolates, *Spodoptera litura*, DNA fingerprinting, RAPD-PCR.

INTRODUCTION

The indiscriminate use of chemical pesticides is assuming a serious cause of concern to human health and environment safety. A viable alternative to chemical pesticides is integrated pest management. The entomopathogenic fungus *Beauveria bassiana* is a promising and extensively researched biocontrol agent that can suppress a variety of economically important insect pests (Coates et al., 2002; McGuire et al., 2005). *B. bassiana* has a wide host range, but differences in both host specificity and virulence among isolates has been reported (Ferron et al., 1991). However, it is increasingly being realized that this fungus is rather a generalist, with no strict host specificity (Rehner and Buckley, 2005). There are also several B. *bassiana*- based mycoinsecticide cur - rently registered or under commercial development for agricultural pests (Hajek et al., 2001).

To determine its efficacy, host specificity, survival and partial temporal distribution in the field, distinctive markers are needed for the individual strains (Leal et al., 1994). DNA markers provide more detailed genomic information than do isozymes and they are not influenced by environmental or culture conditions (Tigano et al., 1995). Molecular markers have been utilized to assess genetic variation among isolates of *B. bassiana* and other entomopathogenic fungi, thereby providing a means to identify strains of interest, determine the origin of isolates, or study the population structure (Castrillo et al., 2003). Arbitrary primed PCR is relatively easy to perform and the markers are rapid and reliable tools for resolving ambiguity based on morphological characterization. For population level analysis, arbitrary primed PCR is generally used (Welsh and McClelland, 1990). Kosir et al. (1991) reported altered RFLP patterns in a less virulent strain compared to the wild-type strain of *B. bassiana*.

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RAPD has been used to estimate the diversity of a population, for genotype characterization or constructing the molecular phylogeny of closely related taxons (Vakalounakis and Fragkiadakis, 1999). Bielikova et al. (2002) stated that the differentiation of fungal strains and species by RAPD is certainly an easy tool to detect polymorphisms in a large number of samples at relatively low cost. This technique utilizes short primers of arbitrary sequences that anneal to multiple target sequences, thus producing diagnostic patterns (Willaims et al. 1990). Besides these methods, Inglis et al. (1999) used telomeric fingerprinting and unambiguously differentiated several Brazilian strains of *M. flavoviride*, as well as strains from Africa and Australia. Genetic diversity, reproductive biology and speciation in *B. bassiana* were studied using methods like AFLP and SSCPs (Uma Devi et al., 2006). Genetic diversity of *Nomuraea rileyi* isolates was also evaluated using AFLP analysis (Sirima et al., 2005) and RAPD analysis (Tigano and Aljanabi, 2000). Castrillo et al. (2003) developed strain-specific molecular markers based on PCR of sequence- characterized ampli-fied regions (SCAR) in *B. bassiana.* Marilena Aquino de Muro et al. (2003) studied the use of AFLP for molecular analysis of *B. bassiana* isolates from Kenya and other countries. Molecular diversity of different isolates of *B. bassiana* has also been assessed using microsatellites (Estrada et al., 2007). The abundance and genetic diver-sity of *M. ansiopliae* var. *anisopliae* in southwestern Bri-tish Columbia and Southern Alberta has been examined (Inglis et al., 2008). *Spodoptera litura* is an important lepidopteron pest on a wide range of crops including tobacco, cotton and tomato throughout Europe, Asia, Africa and Australia (Anonymous, 2000). The pest has developed resistance to many of the conventional insect-cides (Kranthi et al., 2002).

In the present study RAPD analysis of *B. bassiana* isolates has been carried out with 10-mer operon primers belonging to the group OPAA and OPAB. The amplify-cation pattern in each of the primers that showed polymorphisms among the isolates and among the primers studied was recorded and subjected to statistical evaluation. Laboratory bioassays with twenty-four isolates of *B. bassiana* against the second instar larvae of *S. litura* were carried out to identify virulent isolates. These isolates were characterized and genetic relatedness based on RAPD-PCR analysis was carried out. The present communication deals with the results of our estimation of genetic relatedness based on RAPD-PCR analysis of twenty-seven isolates of *B. bassiana,* which were isolated from different hosts and geographical regions.

MATERIALS AND METHODS

Fungal cultures

The twenty-seven isolates of *B. bassiana* used in the present study included 3 isolates from USDA-ARS, 3 from EMBRAPA-Brazil and 21 local isolates collected from different cotton growing areas of Andhra Pradesh, India (Table 1). The insect pests that showed sporadic instances of natural epizootics were *Helicoverpa armigera* and *S. litura* on cotton, tomato and chilli fields. The fungus was isolated from insect cadavers obtained from six agroclimatic zones of Andhra Pradesh (numbered consecutively from 1 to 6) and had a distance of approximately 200 km away from each other (Table 1). Pure cultures were maintained on Sabouraud dextrose yeast agar (Dextrose – 4%; peptone – 1% and yeast extract – 1%) slants for further use.

Insect

The second instar larvae of *S. litura* were obtained by breeding the field-collected insects in the laboratory. Female moths lay up to 300 eggs in masses, which hatch within 3 - 5 days. The egg patches laid on castor leaves by the moths were carefully transferred onto fresh castor leaves for the eggs to hatch. The neonate larvae were later transferred to and maintained on fresh castor leaves until they reached the second instar stage. Bioassays were done with these larvae and a homogenous population was maintained.

Bioassays

Virulence of *B. bassiana* isolates were tested at second instar stages of the pest. Fifteen larvae were taken for each treatment in a plastic container of 8 cm in diameter and 10 cm in height, lined with moistened filter paper and small pieces of castor leaves. Two (2) ml of conidial suspension at a concentration of 1 x 10^8 spores/ml was sprayed using a hand atomizer. The number of spores available per larvae may be less than the theoretical expectation, since some of the spores may adhere to the walls of the container and some may be lost during spraying. The spores that are consumed through the feeding on leaves are not likely to play a role in the infection process as the digestive enzymes of the host are likely to degrade them. Three replicates were maintained for treatment with each isolate. Control insects received a spray of 0.02% Tween- 80 solution in sterile distilled water. The treated larvae were kept in the plastic containers and fed with castor leaves during incubation at 25 $\pm$ 2°C. During the incubation period the relative humidity was maintained at about 95%. The dead larvae before 24 h were removed from the experiment. The larval mortality was recorded at 6-hourly intervals. The mummified larvae, if any, were kept for reisolation of the fungus in Petri plates lined with moistened filter paper.

DNA extraction

For DNA extraction, the isolates were grown in Sabouraud dextrose yeast peptone broth. Conidia from 7-day old pure slants of *B. bassiana* were scrapped and suspended in 0.02% Tween-80 solution, and the concentration was adjusted to 10^8 spores/ml. Conical flasks containing 75 ml of Sabouraud dextrose yeast peptone broth were inoculated with 2 ml of 10^8 spores/ml of conidial suspension and incubated in an orbital shaker (200 rpm) at 25^oC for a period of 3 - 4 days. The mycelium was harvested after 72 h and washed 3 - 4 times with sterile distilled water and ground to a fine powder in liquid nitrogen. DNA was extracted according to the method of Lee and Taylor (1990) with slight modifications. Mycelia (500 mg) were ground in 5 ml of lysis buffer (50 mM Tris HCl, 50 mM EDTA, 3% SDS and 1% β - mercaptoethanol). The mixture was incubated at 65°C for 30 min. The mixture was centrifuged and an equal volume of phenol:chloroform at a ratio of 1:1 was added to the supernatant, mixed gently for 1 min and then centrifuged. This was done twice. To the aqueous phase, an equal volume of chloroform:isoamyl alcohol at a ratio of 24:1 was added, mixed gently and then centrifuged. To the recovered aqueous phase, $1/10^{th}$ volume of sodium acetate (3 M) and an equal volume of

Table 1. Source of *Beauveria bassiana* isolates.

Isolate	Code NO. / Accession NO.ARSEF/ Local	Host insect	Geographical location
4-6	USDA-ARS (1314)	*Helicoverpa virescens*	France
4-7	USDA-ARS (2427)	*Nilaparvata lugens*	Indonesia
4-8	ITCC (4668)	Lepidoptera	India
4-11	EMBRAPA-BRAZIL, CG-482	Lepidoptera	Brazil
4-12	EMBRAPA-BRAZIL, CG-151	*Spodoptera frugiperda*	Brazil
4-13	Zone 4, Guntur district	*Helicoverpa armigera*	India
4-14	Zone 1, Guntur district	*Helicoverpa armigera*	India
4-15	Zone 1, Guntur district	*Helicoverpa armigera*	India
4-16	Zone 2, Guntur district	*Spodoptera litura*	India
4-17	Zone 2, Guntur district	*Helicoverpa armigera*	India
4-18	Zone 1, Guntur district	*Helicoverpa armigera*	India
4-19	Zone 2, Guntur district	*Helicoverpa armigera*	India
4-20	Zone 3, Guntur district	*Spodoptera litura*	India
4-22	Zone 2, Guntur district	*Helicoverpa armigera*	India
4-23	Zone 5, Guntur district	*Helicoverpa armigera*	India
4-24	Zone 4, Guntur district	*Spodoptera litura*	India
4-25	Zone 2, Guntur district	*Spodoptera litura*	India
4-26	Zone 1, Guntur district	*Spodoptera litura*	India
4-27	Zone 1, Guntur district	*Spodoptera litura*	India
4-28	Zone 1, Guntur district	*Helicoverpa armigera*	India
4-29	Warangal district	*Spodoptera litura*	India
4-30	Warangal district	*Helicoverpa armigera*	India
4-31	Warangal district	*Spodoptera litura*	India
4-32	Warangal district	*Spodoptera litura*	India
4-35	EMBRAPA – BRAZIL, CG-458	*Anthronomus grandis*	India
4-36	Warangal district	*Spodoptera litura*	India
4-37	Warangal district	*Spodoptera litura*	India
4-39	Unknown	Unknown	India
4-40	Unknown	Unknown	India

chilled isopropanol was added. After incubation at -20° C for 30min, the DNA strings that appeared were recovered by centrifugation and washed with 70% ethanol. The DNA pellet was dried in vacuum for 20 min and the pellet was suspended in 50 – 100 µl of TE buffer (20 mM Tris HCl, 1 mM EDTA) or in sterile double distilled water, and stored at -20° C until further use.

DNA amplification

A total of 40 primers of the OPAA and OPAB group (Operon Technologies) were screened for amplification and polymorphism using a Perkin-Elmer Cetus Gene Amplification PCR system, Model 9600. The 25 µl reaction mixture consisted of 2.5 µl of PCR buffer (PCR buffer and 1.5 mM $MgCl_2$), 1.25 µl of 100 mM dNTP mixture, 0.5 µ l of primer (10 mM),, 0.125 µl of *Taq* DNA polymerase, 1 µl of DNA (25 ng) and 19.625 µl of double distilled water. The selected temperature profile included initial denaturation for 3 min at 94^{O}C, denaturation for 40 s at 94^{O}C, annealing for 40 s at 37^{O} C, extension for 1 min at 72^{O}C, and a final extension for 7 min at 72^{O}C for 30 cycles. The amplified PCR products were electrophoresed on a 1.5% agarose gel in TAE buffer. 100-bp ladder maker was included to serve as marker. The gels were stained with ethidium bromide and were photographed under UV illumination using a Pentax camera with ORWO film.

Statistical analysis

Bioassays

The data was subjected to Probit Analysis (Finney, 1964) for calculating the regression lines, LT_{50} values and fiducial limits. Heterogeneity among the populations was performed by the chi square test.

Cluster analysis

A binary matrix for presence or absence (1/0) of all the polymorphs characterized by specific molecular weights in the isolates with each primer was compiled. Based on the matrix, simple matching coefficients (similarity values) between all possible paired combinations of the isolates were calculated, as suggested by Sokal and Michener (1958). The similarity values were subjected to the sequential agglomerative hierarchical nested (SAHN) clustering using the unweighed pair group method (arithmetic average; UPGMA), employing NTSYS PC Version 2.0 to generate dendrograms. Bootstrap values for the branches of the phenogram were generated using the Win boot program.

Table 2. Evaluation of *Beauveria bassiana* isolates for virulence against second instar of *Spodoptera litura*

Isolate	LT_{50} (in hours)	Chi^2	Slope	Fiducial limits	Arbitrary rating[a]
4-6	146.67±5.5761	0.2878	12.7012	136.11 to 156.96	Less virulent
4-7	144.42±4.6544	0.2316	14.0940	135.58 to 153.81	Less virulent
4-8	154.89±6.3801	0.6050	13.3129	142.88 to 167.91	Less virulent
4-11	140.06±3.2303	2.4457	19.3463	133.84 to 146.52	Moderately virulent
4-12	138.73±4.7454	0.4128	12.4894	129.71 to 148.32	Moderately virulent
4-13	144.41±4.0317	0.9537	16.4778	136.70 to 152.51	Less virulent
4-14	136.26±4.2005	0.4756	14.3885	128.23 to 144.71	Moderately virulent
4-15	140.36±4.0530	0.4528	14.9457	132.61 to 148.49	Moderately virulent
4-16	138.09±3.7446	0.7352	16.5948	130.91 to 145.07	Moderately virulent
4-17	136.92±3.5648	0.7151	17.3753	130.07 to 144.07	Moderately virulent
4-18	140.77±5.8894	0.5817	10.0908	129.68 to 152.79	Moderately virulent
4-19	133.52±3.9388	0.5046	16.5699	126.00 to 141.44	Moderately virulent
4-20	132.15±0.0339	0.2634	12.5906	122.20 to 142.88	Moderately virulent
4-22	140.30±4.2107	0.3433	14.3332	132.28 to 148.79	Moderately virulent
4-23	136.49±4.1116	0.6532	14.6349	128.64 to 144.71	Moderately virulent
4-24	149.71±4.8942	0.8149	13.4761	140.43to 159.62	Less virulent
4-25	148.86±4.6830	0.5701	13.4323	139.97 to 158.33	Less virulent
4-26	149.175±4.0845	0.8934	15.6714	141.38 to 157.39	Less virulent
4-27	152.42±5.4316	0.3003	13.7434	142.14 to 163.44	Less virulent
4-28	149.14±4.0830	0.6028	15.6681	141.35 to 157.35	Less virulent
4-29	122.92±4.2197	1.0259	14.5531	-	Highly virulent
4-30	115.36±401211	0.8184	15.7508	107.55 to 123.72	Highly virulent
4-31	123.74±9.8312	2.4115	7.4991	105.90 to 144.59	Highly virulent
4-32	149.22±4.2634	0.3727	15.0631	141.09 to 157.81	Less virulent

[a]: Highly virulent – LT_{50} between 115-130 h, moderately virulent - LT_{50} between 131-140 h and less virulent - $LT_{50} > 141$ h.

Table 3. List of RAPD primers which showed amplification

Primer details	Sequence
OPAA – 04	AGGACTGCTC
OPAA - 06	GTGGGTGCCA
OPAA – 14	AACGGGCCAA
OPAA – 16	GGAACCCACA
OPAB – 11	GTGCGCAATG
OPAB – 12	CCTGTACCGA
OPAB – 18	CTGGCGTGTC

RESULTS

Bioassays

The pathogenicity test, which was carried out on second instar larvae of *S. litura*, showed differences in mortality rates among the twenty- four isolates, studied. The least LT_{50} values of 115, 122 and 123 h was recorded in isolates 4 - 29, 4 - 30 and 4 - 31, and were thus categorized as highly virulent isolates (Table 2). The highest LT_{50} values of 152 and 154 h were recorded in isolates 4 - 8 and 4 - 27. The isolates showing a range of LT_{50} from 130 to 140 h were categorized as moderately virulent, and eleven isolates were included in this category. The isolates which showed an LT_{50} value between 141 - 154 h were categorized as less virulent, and ten isolates showed this pattern. Regression equation, chi-square, slope and fiducial limits were calculated by probit analysis. Chi square test showed no heterogeneity among the populations tested. LT_{50} values showed a good fit and were well within the range of fiducial limits. The isolates that were more effective in the second instar were 4 - 29, 4 - 30 and 4 - 31.

Cluster analysis

Seven of the forty primers (Table 3) showed amplification and polymorphism. The experiment was repeated using the primers showing amplification for confirming the re-

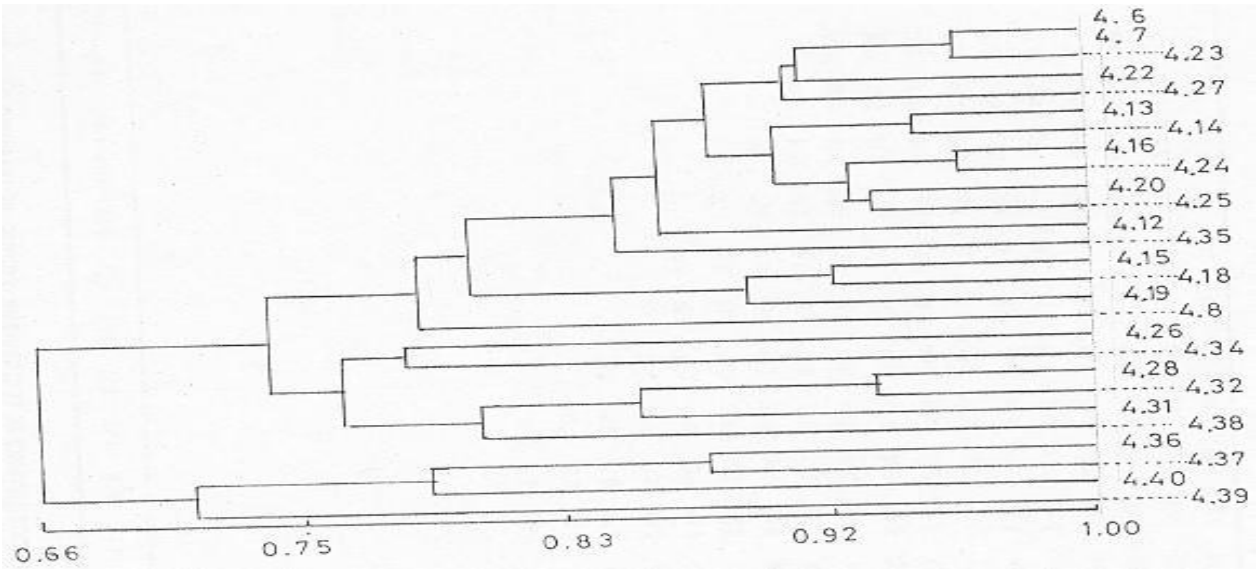

Figure 1. Phenogram of 27 isolates of *B. bassiana* using seven primers by UPGMA based on simple matching coefficients.

results. Amplification patterns of the seven primers were pooled and a total of 73 bands with molecular weights ranging from 320 to 2300 bp were analyzed. Binary data was obtained and used for carrying out cluster analysis and for arriving at the phenograms and simple matching coefficients.

The similarity coefficient values were calculated using SAHN clustering and the phenogram (Figure 1). The simple matching coefficient values (Table 4) were generated and the data in Table 4 represents similarity values between the isolates. The similarity coefficients among RAPD profiles of *B. bassiana* were higher than 52%. The maximum similarity value of (1.0) or 100% was observed between isolates 4 - 6 and 4 - 7. A similarity value of 0.96 was observed between isolates 4 - 23 and 4 - 6, 4 - 23 and 4 - 7, 4 - 24 and 4 - 6. The lowest similarity value of 0.52 was recorded between isolates 4 - 40 and 4 - 19. Similarity values of 0.53 were observed between 4 - 40 and 4 - 15, 4 - 40 and 4 - 18, 4 - 40 and 4 - 27. The rest of the isolates showed values ranging from 0.56 to 0.96. The isolates that showed less than 60% similarity were 4 - 40 and 4 - 8, 4 - 40 and 4 - 12, 4 - 39 and 4 - 15, 4 - 37 and 4 - 15, 4 - 40 and 4 - 16, 4 - 37 and 4 - 18, 4 - 40 and 4 - 20, 4 - 40 and 4 - 22, 4 - 40 and 4 - 24, 4 – 40 and 4 - 25. The values representing the simple matching coefficients are presented in Table 4. The SAHN/UPGMA tree grouped the isolates into three clusters with most basal bifurcation at 0.66 similarities (Figure 1). One cluster had the isolates branching from each other between 0.90 and 1.0 similarity. Five of the isolates (4 - 6, 4 - 7, 4 - 12, 4 - 35 and 4 - 8), on the other hand, did not pair with any of the clusters. Bootstrapping of the values was used to determine the robustness of the data (Figure 2). The numbers at the forks show the percentage of times that the group consisting of the species, which is to the right of that fork, occurred.

The isolates under study were from different geographical regions (Table 1). Cluster analysis by SAHN/UPGMA tree showed three clusters bifurcating at 0.66 similarities. The first cluster had nine isolates and all were from India or the same geographical region but from different zones. The second and third clusters had isolates from different geographical regions (Table 5). Interestingly, it was noticed that the five isolates, which did not pair with any of the three clusters, were exotic isolates from the USDA or from EMBRAPA, except isolate 4 - 8 which was from the ITCC collection from India. Thus, geographical distribution and clustering pattern did not reveal relatedness, as is evident from the clustering pattern.

Regarding the original insect host of the isolates, eleven of the isolates were isolated from *S. litura*, and nine from *H. armigera*. The isolates, which did not show any pairing, were from different host, e.g. isolate 4 - 6 was from *H. virescens*. Isolate 4 - 7 was from *Nilaparvata lugens*, isolates 4 - 8 and 4 - 11 from an unknown lepidopteran host, 4 - 35 was from *Anthronomus grandis* and isolate 4 - 12 was from *Spodoptera frugiperda*. All three clusters showed isolates from different hosts, either from *S. litura* or *H. armigera* (Table 5).

On the other hand, isolates obtained from different hosts were grouped in the same clusters, indicating no relatedness between the original host and DNA markers of the isolates. The pathogenicity against second instar larvae of *S. litura* did not reveal any relatedness with the clustering pattern, as each cluster had all three categories of isolates categorized depending on their studies.

Table 4. Simple matching coefficients of *B. bassiana* isolates as revealed by SAHN/ UPGMA tree analysis.

	4-6	**4-7**	**4-8**	**4-12**	**4-13**	**4-14**	**4-15**	**4-16**	**4-18**	**4-19**	**4-20**	**4-22**	**4-23**	**4-24**	**4-25**	**4-26**	4-27	4-28	4-31	4-32	4-34	4-35	4-36	4-37	4-38	4-39	4-40
4-6	1.00																										
4-7	1.00	1.00																									
4-8	0.84	0.84	1.00																								
4-12	0.89	0.89	0.78	1.00																							
4-13	0.90	0.90	0.82	0.88	1.00																						
4-14	0.88	0.88	0.79	0.88	0.95	1.00																					
4-15	0.75	0.75	0.70	0.75	0.85	0.88	1.00																				
4-16	0.92	0.92	0.81	0.89	0.93	0.93	0.84	1.00																			
4-18	0.78	0.78	0.78	0.75	0.88	0.88	0.92	0.86	1.00																		
4-19	0.82	0.82	0.77	0.82	0.89	0.86	0.88	0.85	0.90	1.00																	
4-20	0.86	0.86	0.78	0.86	0.88	0.88	0.81	0.95	0.84	0.82	1.00																
4-22	0.90	0.90	0.74	0.82	0.84	0.86	0.77	0.88	0.77	0.75	0.85	1.00															
4-23	0.96	0.96	0.79	0.85	0.89	0.89	0.77	0.93	0.79	0.78	0.88	0.92	1.00														
4-24	0.93	0.93	0.82	0.90	0.92	0.89	0.79	0.96	0.82	0.86	0.90	0.84	0.92	1.00													
4-25	0.88	0.88	0.79	0.85	0.89	0.89	0.79	0.93	0.82	0.81	0.93	0.89	0.92	0.92	1.00												
4-26	0.78	0.78	0.67	0.73	0.74	0.74	0.67	0.75	0.70	0.66	0.75	0.79	0.79	0.71	0.77	1.00											
4-27	0.89	0.89	0.73	0.78	0.82	0.82	0.70	0.86	0.73	0.71	0.81	0.90	0.93	0.85	0.88	0.78	1.00										
4-28	0.84	0.84	0.73	0.75	0.79	0.79	0.70	0.84	0.73	0.74	0.81	0.82	0.85	0.79	0.82	0.78	0.78	1.00									
4-31	0.73	0.73	0.67	0.70	0.71	0.74	0.67	0.73	0.67	0.66	0.73	0.74	0.74	0.68	0.74	0.75	0.67	0.84	1.00								
4-32	0.82	0.82	0.71	0.79	0.78	0.81	0.71	0.82	0.71	0.73	0.79	0.84	0.84	0.78	0.81	0.79	0.77	0.93	0.88	1.00							
4-34	0.70	0.70	0.62	0.67	0.68	0.68	0.64	0.70	0.67	0.66	0.67	0.68	0.71	0.66	0.68	0.78	0.64	0.78	0.78	0.79	1.00						
4-35	0.92	0.92	0.75	0.84	0.85	0.82	0.73	0.84	0.75	0.82	0.81	0.85	0.88	0.85	0.82	0.81	0.81	0.78	0.75	0.77	0.73	1.00					
4-36	0.73	0.73	0.64	0.70	0.71	0.74	0.62	0.73	0.62	0.63	0.70	0.71	0.74	0.68	0.71	0.70	0.67	0.81	0.81	0.85	0.70	0.70	1.00				
4-37	0.66	0.66	0.60	0.63	0.64	0.67	0.58	0.66	0.58	0.56	0.63	0.64	0.67	0.62	0.64	0.66	0.60	0.71	0.79	0.75	0.68	0.66	0.88	1.00			
4-38	0.78	0.78	0.67	0.70	0.74	0.74	0.62	0.78	0.64	0.66	0.73	0.74	0.79	0.74	0.74	0.70	0.73	0.86	0.73	0.82	0.70	0.70	0.84	0.74	1.00		
4-39	0.73	0.73	0.62	0.67	0.71	0.68	0.56	0.70	0.59	0.63	0.70	0.66	0.71	0.68	0.66	0.67	0.64	0.81	0.73	0.74	0.67	0.70	0.75	0.74	0.75	1.00	

DISCUSSION

In view of the potential insecticidal properties of *B. bassiana*, initial selection of a truly aggressive isolate is of utmost importance for the success of a biocontrol program. Variation between isolates from single or multiple geographical locations and from different hosts necessitates the need for specific parameters for their identification. A wide range of variation was recorded in the degree of virulence among the 24 isolates tested. Chemical constituents of the larval cuticle vary with age, leading to progressive hardening of the cuticle and increased humoral defense mechanisms to the microbial infection (Boman, 1981). Vimala Devi (1994) recorded lower susceptibility of older larvae of *S. litura.* Devi et al. (2001) reported no correlation between aggressiveness of the isolates and the relatedness of the original insect host. However, a correlation was found between the RAPD grouping and the phenotypic classification of the isolates. Rivera et al. (1997) and Valderrama et al. (2000) found no correlation between the clusters obtained by RAPD analysis of the insect host and the pathogenicity of *Hypothenemus hampei* (Coffee berry borer). Luz et al. (1998) indicated that the virulent isolates against *T. infestans* were homogenous and could not be distinguished by RAPD analysis. The high similarity could be related to the original host of the strains analyzed. Marilena Aquino de Muro et al. (2003) revealed no significant correlation between the isolates and host and geographical origin, but the AFLP technique used did reveal clonal populations of *B. bassiana* within Kenya. The phylogenetic characterization of *B. bassiana* at the molecular level is of immense importance for understanding genomic organization, genetic variation and gene stability (Kosir et al. 1991). Characterization of the isolates based on morphological or biochemical parameters alone would be

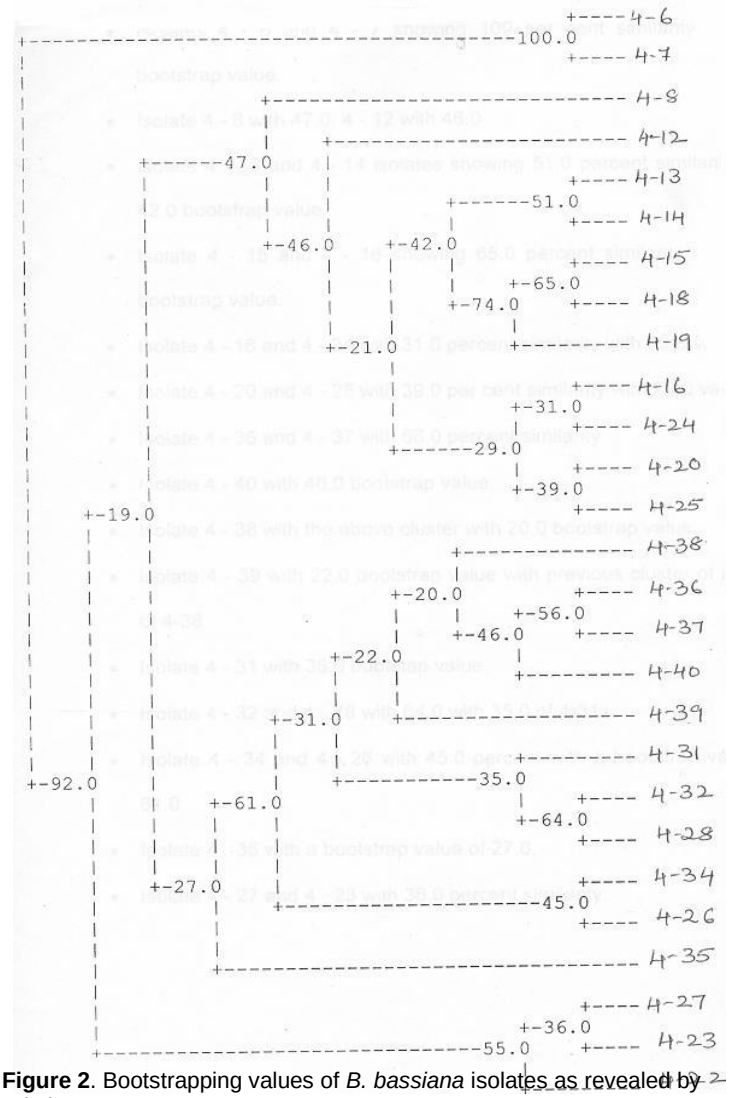

Figure 2. Bootstrapping values of *B. bassiana* isolates as revealed by Win boot program.

inadequate for understanding the genetic basis for character differentiation. Tigano et al. (1995) showed that overall genetic variability revealed by DNA markers permit finer taxonomic resolution than do morphological characters of conidia, which are currently used in taxonomic studies. Fegan et al. (1993) stated that PCR-based technology provides an additional means to resolve taxonomic problems in entomopathogenic fungi, and also provide markers for genetic analysis and population studies in the field. Bidochka et al. (1994) used PCR-based technology for assessing the genomic variability between 24 isolates of deuteromycetous fungi, *Metarhizium* spp. and *B. bassiana*, and recorded distinct markers that led to resolving the taxonomic classification of the species. Castrillo et al. (1998) used isozymes and RAPD and detected variation among 24 *B. bassiana* isolates. Further, they reported better resolution of the differences between the strains with respect to RAPD markers. *B. bassiana* isolates in several instances collected from the same insect species and from the same region were genetically dissimilar (Berretta et al. 1998 and Urtz and Rice, 1997) or similar genetic types were described from widely separated geographic locations (Bidochka et al. 1994). Maurer et al. (1997) has shown by RFLP and RAPD analysis, clear relationship between the population structure of *B. bassiana* and some defined host species.

Among *B. bassiana* isolates of the present study, an overall lack of relationship between the clustering pattern and pathogenicity and the original insect host from which the isolates were isolated was recorded. However, incomplete relationship was evident between clustering pattern and geographical distribution of the isolates. The isolates of *B. bassiana* under study were isolated from different hosts. Eleven of the isolates were isolated from

Table 5. Clustering pattern and the relationships between isolates of *B. bassiana*

Cluster	Virulence (Arbitrary rating)	Host Insect	Geographical Location
Cluster I			
4-23	Moderately virulent	*Helicoverpa armigera*	India
4-22	Moderately virulent	*Helicoverpa armigera*	India
4-27	Less virulent	*Spodoptera litura*	India
4-13	Less virulent	*Helicoverpa armigera*	India
4-14	Moderately virulent	*Helicoverpa armigera*	India
4-16	Moderately virulent	*Spodoptera litura*	India
4-24	Less virulent	*Spodoptera litura*	India
4-20	Moderately virulent	*Spodoptera litura*	India
4-25	Less virulent	*Spodoptera litura*	India
Cluster II			
4-15	Moderately virulent	*Helicoverpa armigera*	India
4-18	Moderately virulent	*Helicoverpa armigera*	India
4-19	Moderately virulent	*Helicoverpa armigera*	India
4-26	Less virulent	*Spodoptera litura*	India
4-34	-	-	India
4-28	Less virulent	*Helicoverpa armigera*	India
4-32	Less virulent	*Spodoptera litura*	India
4-31	Highly virulent	*Spodoptera litura*	India
4-38	-	-	
Cluster III			
4-36	-	*Spodoptera litura*	India
4-37	-	*Spodoptera litura*	India
4-40	-	Unknown	India
4-39	-	Unknown	India
No Pairing			
4-6	Less virulent	*Helicoverpa virescens*	France
4-7	Less virulent	*Nilaparvata lugens*	Indonesia
4-8	Less virulent	Lepidoptera	India
4-12	Moderately virulent	*Spodoptera frugiperda*	Brazil
4-35	-	*Anthronomus grandis*	India

S. litura and nine from *H. armigera*. RAPD markers recorded in *B. bassiana* isolates appear to be reliable tools, as there is no ambiguity in the results obtained. Other methods like RFLP, AFLP (Marilena Aquino de Muro et al. 2003), minisatellite locus markers, telomere fingerprinting and SCAR markers (Castrillo et al., 2003) can be used to provide more accurate results for differentiating the isolates and characterizing their phylogenetic relationships.

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