

**THE MOLECULAR DIVERSITY OF DIFFERENT ISOLATES OF
Beauveria bassiana (BALS.) VUILL. AS ASSESSED USING INTER-
MICROSATELLITES (ISSR_s)**

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Abstract: Inter-microsatellite PCR (ISSR-PCR) markers were used to identify and to examine the genetic diversity of eleven *Beauveria bassiana* isolates with different geographic origins. The variability and the phylogenetic relationships between the eleven strains were analyzed using 172 ISSR-PCR markers. A high level of polymorphism (near 80%) was found using these molecular markers. Seven different isolates showed exclusive bands, and ISSR primer 873 was able to distinguish between all the strains. The dendrogram obtained with these markers is robust and in agreement with the geographical origins of the strains. All the isolates from the Caribbean region were grouped together in a cluster, while the other isolates grouped in the other cluster. The similarity exhibited between the two clusters was less than 50%. This value of homology shows the high genetic variability detected between the isolates from the Caribbean region and the other isolates. ISSR-PCR markers provide a quick, reliable and highly informative system for DNA fingerprinting, and allowed the identification of the different *B. bassiana* isolates studied.

Key words: *Beauveria bassiana*, Strain identification, Inter-microsatellite, Phylogenetic relationships

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Abbreviations used: AFLP – amplified fragment length polymorphism; EST – expressed sequence tag; ISSR – inter simple sequence repeat; ITS – internal transcribed spacer; PCR – polymerase chain reaction; RAPD – random amplified polymorphic DNA; RFLP – restriction fragment length polymorphism; Rp – resolving power; SSCP – single-strand conformation polymorphism; SSR – simple sequence repeat

INTRODUCTION

The haploid imperfect filamentous fungus *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales) has both endophytic and entomopathogenic characteristics. It is a ubiquitous, insect-pathogenic, hemibiotrophic, mitosporic fungus with a very diverse and large host range, and is a beneficial fungus infecting a wide range of insects of the order Lepidoptera, Coleoptera and Hymenoptera, most of which are agricultural pests [1, 2]. The potential of this fungus in biocontrol has been exploited by making use of local isolates collected from either the soil or dead insect hosts found in different geographic areas.

This fungus is also used in the sugar cane culture in Cuba to control the larval populations of *Diatraea saccharalis* (Fabricius) (Lepidoptera: Pyralidae) [3]. It is currently recognized that *B. bassiana* is a species complex with several cryptic species, and that it is phylogenetically affiliated to the Ascomycotina (the Clavicipitaceae family of Hypocreales of the Pyrenomycetes) [4, 5].

The characterization of *B. bassiana* was based on the description and measurement of structures of agronomic interest [6], on its physiological [7] and pathogenic characteristics, [8] and on the results of isozymatic studies [9].

The characterization of *B. bassiana* was carried out using different kinds of DNA markers [10-12], RFLPs and RAPDs [13, 14], ITSs [15], microsatellites (SSRs) and minisatellites [15-17]. The molecular variation found between *Beauveria* isolates is related to the insect host range, and was assessed using RFLP, ITSs [18, 19] and isozymes [20, 21]. PCR-RAPD genotyping has also been used with *B. bassiana* [22, 23] and *B. brongniartii* isolates [24, 25]. Molecular markers were employed to assess genetic differentiation in asexual fungi and to identify phylogenetic species, defined as the smallest diagnosable taxonomic unit with a clear pattern of paternal ancestry [5, 26-31]. AFLPs, SSCPs and the DNA sequences of three genes (large and small subunits of rRNA and β -tubulin) were also evaluated for *B. bassiana* [32].

The ISSRs (inter-simple sequence repeats) are PCR products obtained using primers based on dinucleotide, trinucleotide, tetranucleotide and pentanucleotide repeats [33]. ISSRs are based on the amplification of regions between inversely oriented, closely spaced microsatellites. They have been used for cultivar identification in maize [34, 35], trifoliate orange [36], wheat [37], potatoes [38], *Diploaxis* [39], bean [40], rye [41] and barley [42], and also employed in the detection of genetic variability in the charcoal root rot pathogen *Macrophomina phaseolina* [43]. However, ISSRs have not yet been used to study isolates of *B. bassiana*.

The objectives of this study were to develop a set of ISSR markers, to determine their variability and to apply them for genome analyses, distinguishing between genotypes of *B. bassiana* isolates with different geographic and entomopathogenic origins, and to establish phylogenetic relationships.

MATERIALS AND METHODS

Fungal strains

Eleven isolates of *Beauveria bassiana* with different geographic origins and with different pathogenic characteristics were used (Tab. 1). Seven isolates came from Cuba. The isolates were grown in static liquid medium (Adamek) [44] and incubated at 25°C for four days. The fresh mycelia were lyophilized for 24 hours.

Tab. 1. The eleven *Beauveria bassiana* isolates used in this study.

Isolate	Strain	Entomopathogenic origin	Geographic origin
1	Quivicam	<i>D. sacchrallis</i>	Cuba
2	252	<i>A. floridus</i>	USA
3	MG1	<i>D. sacchrallis</i>	Cuba
4	18	<i>D. sacchrallis</i>	Cuba
5	India	Insecta	India
6	Borer	<i>D. sacchrallis</i>	Cuba
7	PCC	<i>D. sacchrallis</i>	Cuba
8	93	Insecta	Guadalupe
9	60	Insecta	Bulgaria
10	CC	<i>D. sacchrallis</i>	Cuba
11	156	<i>D. sacchrallis</i>	Cuba

Genomic DNA extraction

The lyophilized mycelia were used for the genomic DNA extraction. The extraction was carried out using a small-scale DNA isolation method (Dneasy Plant Mini Kit, from Qiagen).

Primers used in the PCRs

One hundred primers based on dinucleotide, tetranucleotide or pentanucleotide repeats were used. These oligonucleotides were obtained from UBC primer set 100/9 (University of British Columbia). A total of fourteen oligonucleotides from UBC primer set 100/9 (University of British Columbia) were selected according to the number and consistency of the amplified fragments (Tab. 2).

Tab. 2. ISSR primer set #9 UBC (University British Columbia) and the respective oligonucleotide sequence. Note: B = C, G, T; D = A, G, T; H = A, C, T; R = A, G; V = A, C, G; Y = C, T.

Primer	Sequence	Primer	Sequence	Primer	Sequence	Primer	Sequence
808	(AG) ₈ C	821	(GT) ₈ T	849	(GT) ₈ YA	885	BHB(GA) ₇
809	(AG) ₈ G	828	(TG) ₈ A	850	(GT) ₈ YC	889	DBD(AC) ₇
810	(GA) ₈ T	842	(GA) ₈ YG	873	(GACA) ₄	891	HVH(GT) ₇
818	(CA) ₈ G	846	(CA) ₈ RT				

Polymerase chain reactions (PCRs), resolving power and band profile reproducibility

For the amplification reactions, we adapted the protocol of Zietkiewicz et al. [33]. We used total genomic DNA extracted from lyophilized mycelia, and each amplification reaction (final volume of 20 μ l) consisted of 1 μ l of total genomic DNA (10 ng/ μ l), 1 μ l of the corresponding primer (5 μ M), 10 μ l of Taq-PCR master mix (Qiagen), and 8 μ l of ultra-pure distilled water (Qiagen). DNA amplifications were performed in a PTC-100 thermocycler (MJ Research Inc) with an initial step of 5 min at 94°C, followed by 45 cycles of 30 s at 94°C, 45 s at 52°C and 2 min at 72°C, with a final 6 min extension at 72°C. The amplification reactions were stored at 4°C until their resolution by electrophoresis. Samples of 20 μ l PCR products were analyzed on 1.5% agarose gels running at 90 V for 2 hours. The gels were stained using ethidium bromide. According to Prevost and Wilkinson [38], the resolving power (Rp) of a primer is:

$$R_p = \sum I_b,$$

where I_b (band informativeness) takes the value of: $1 - (2 \times [0.5 - p])$ and p is the proportion of the 11 isolates containing the band.

Two replicate DNA extractions from the same *B. bassiana* isolate were used to assess the consistency of the band profiles. Two different extractions of strain 18 from Cuba were analyzed in all the amplification experiments (Figs 1 and 2, lines 2 and 12). Moreover, each PCR with a given ISSR primer was repeated three times to assess the consistency of the band profiles, and only the repetitive PCR products were scored.

Analysis of the amplification profiles

ISSR markers behave as dominant markers. They were scored for the presence (1) or absence (0) of homologous bands (bands with the same size) for all the isolates. The dendrograms were constructed by UPGMA cluster analysis using three different coefficients – the simple matching coefficient (SM), the DICE coefficient and the Jaccard coefficient (J) [45] – and using the NTSYS-pc version 1.6 package [46]. $SM = m/n$, where m = shared present fragments (11) + shared absent fragments (00), and n = the total of obtained fragments. $DICE = 2a/(2a+b+c)$, where a = shared present fragments, b = fragments present in the Operative Taxonomic Unit (OTU) X and absent in the OTU Y, and c = fragments present in the OTU Y and absent in the OTU X. $J = a/(n-d)$, where a = shared present fragments, d = shared absent fragments, and n = the total of obtained fragments. To evaluate support for the branches, a bootstrap analysis [47] was performed with a branch and bound algorithm using the majority rule option. The analyses were repeated 10,000 times with random addition of isolates using the WinnBoot software [48].

RESULTS

ISSR amplification

The 14 ISSR primers used here for the PCR amplifications of DNA from 11 isolates of *B. bassiana* were selected from the primers from the set 100/9 UBC, which gave rise to reproducible amplification products (Fig. 1). The sequences of these 14 primers seem to indicate that the microsatellites that are more frequent in *B. bassiana* contain the repeated dinucleotides (AG)_n, (AC)_n and (GT)_n (Tab. 2).

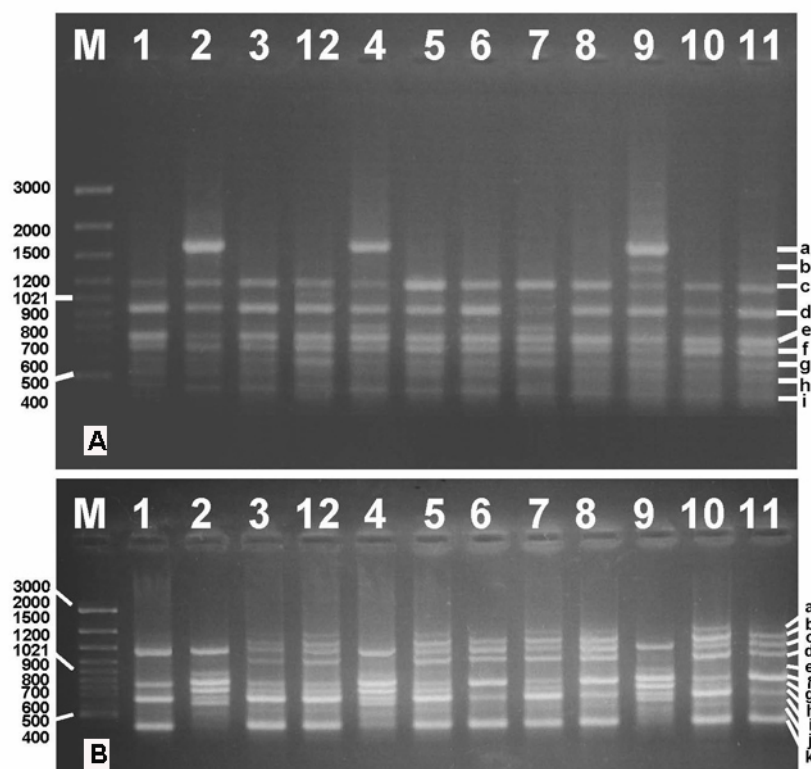


Fig. 1. The ISSR amplification products obtained from the eleven isolates studied. A – 846 ISSR primer. B – 889 ISSR primer. 1) MG1, 2) strain 252 (USA), 3) strain 18, 4) India, 5) Bórer, 6) Quivicam, 7) PCC, 8) strain 93 (Guadalupe), 9) strain 60 (Bulgaria), 10) CC, 11) strain 156, 12) repetition of the strain 18 (another extraction). M = The molecular weight marker was Gene Ruler 100 bp Plus-Fermentas.

The total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (P%), number of different genotypes (NG), Resolving Power (Rp) and number of exclusive bands (NEB) obtained with each primer are shown in Tab. 3. The total number of amplified products was 172

(an average of 12.28 bands per primer), ranging from 300 to 3000 bp, with 135 (78.49%) polymorphic DNA fragments. The maximum number of amplified products was 18 (primers 809 and 810) and the minimum was 6 (primer 818) (Tab. 3). Seven different isolates (five isolates from Cuba and the isolates from Bulgaria and the USA), showed a total of 11 exclusive bands that could be transformed in STSs.

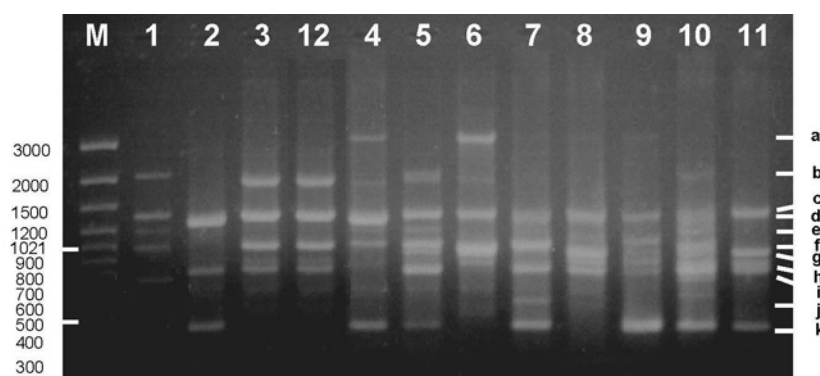


Fig. 2. ISSR amplification products obtained from the eleven isolates studied with the 873 ISSR primer. 1) MG1, 2) strain 252 (USA), 3) strain 18, 4) India, 5) Bórer, 6) Quivicam, 7) PCC, 8) strain 93 (Guadalupe), 9) strain 60 (Bulgaria), 10) CC, 11) strain 156, 12) repetition of the strain 18 (another extraction). M – see Fig. 1 legend

Tab. 3. The total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (P%), number of different genotypes or isolates identified (NG), Resolving Power (Rp) and number of exclusive bands (NEB) obtained with each ISSR primer.

Primer	TNB	NPB	P%	NG	Rp	NEB
808	15	12	80	8	6.3	1
809	18	15	83.4	8	7.9	0
810	18	16	88.9	7	6.6	3
818	6	4	66.7	4	2.0	0
821	11	9	81.8	7	4.4	1
828	11	8	72.7	6	4.2	1
842	14	8	67.2	10	3.4	0
846	10	4	40.0	6	1.4	1
849	14	13	92.8	10	5.6	2
873	11	11	100	11	6.2	1
885	10	10	100	6	6.6	0
888	16	16	100	7	8.9	1
889	11	7	63.6	4	4.7	0
891	7	2	28.6	3	1.1	0
Total	172	135	78.5	97	69.5	11

The resolving power (Rp) of the 14 ISSR primers ranged from 1.1 for primer 891 to 8.9 for primer 888. Four ISSR primers (888, 809, 885 and 810) had the highest Rp values (8.9, 7.9, 6.6 and 6.6, respectively), and 873 was the only primer able to distinguish between the eleven isolates analyzed (Fig. 2). The average Resolving Power (Rp) was 5.0.

Dendrogram obtained

In order to study the genetic relationships between the eleven isolates and to obtain a robust dendrogram, we used the ISSR markers obtained with all the primers. The dendrogram obtained using only primer 873 showed low bootstrap values, probably due to the low number of ISSR products used to construct the dendrogram [11].

The dendrograms obtained using the SM, DICE and J coefficients showed the same structure and the same clusters. Therefore, we have only presented the results obtained with the SM coefficient. The dendrogram obtained using SM (matrix available on request) has two main clusters (A and B), one with eight and one with three isolates (Fig. 3).

All the isolates from the Caribbean region belong to cluster A. This cluster is divided into two subclusters (A₁ and A₂). Subcluster A₁ has a unique Cuban isolate, MG1, and subcluster A₂ contains the rest of the Cuban isolates – 18, Bórer, Quivucam, PCC, CC and 156 – and the isolate from Guadalupe. All the isolates of non-Caribbean origin (India, USA and Bulgaria) are in Cluster B.

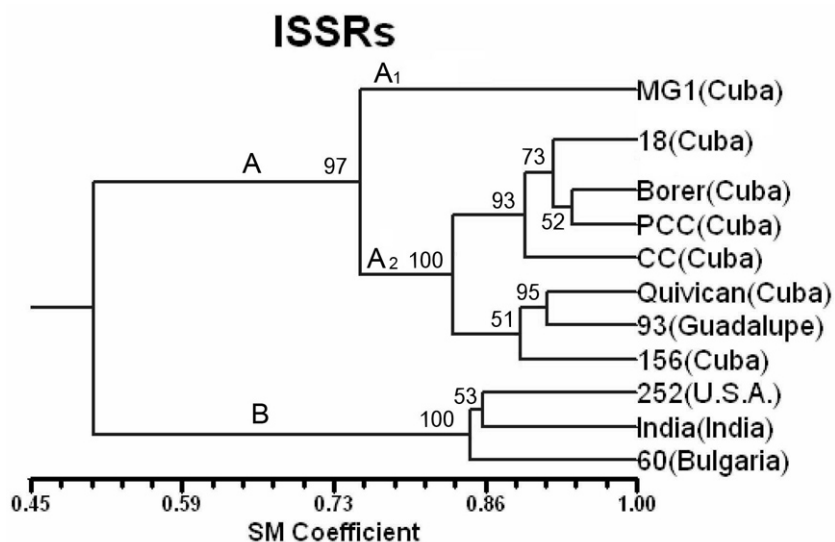


Fig. 3. UPGMA dendrograms showing the genetic relationships between the eleven isolates based on the ISSR-PCR markers (SM coefficient). Node numbers at the forks refer to bootstrap values. These numbers show the percentage of times the group consisting of the isolates which are to the right of that fork occurred.

DISCUSSION

Different molecular markers were employed to study the genetic variability and to identify different isolates of *Beauveria bassiana*. As far as we know, this is first time that ISSR markers have been used on isolates of *B. bassiana*. ISSR is easy to employ and highly reproducible, and uses very robust markers compared with other techniques in which previous knowledge of the genome is not necessary, such as RAPD [49].

In our case, nearly of 80% of the bands generated using ISSR were polymorphic. This shows the high level of genetic variation that exists between the different isolates. These fungi display parasexual reproduction, which normally confers high genetic variability. In the same way, the ISSR markers proved to be an efficient marker system because of their capacity to reveal several informative bands in a single amplification (a mean of 9.6 bands per primer). Furthermore, it was possible to identify all the isolates with a single primer (873) (Fig. 2).

The mean Resolving Power (Rp) obtained (5.0) was lower than that obtained in previous studies on other species (9.8 for barley cultivars [42] and 8.8 for potato cultivars [38]). One reason for this lower value could be the lower resolution power of agarosa gels against the resolution power in PAGE electrophoresis. However, this value (5.0) is higher than that obtained with barley cultivars [42] using RAPD and PAGE (3.85).

The three dendrograms generated using the SM, DICE and J coefficients showed the same structure and the same clusters (Fig. 3). The dendrograms generated have two distinct clusters. Cluster A contained the isolates from the Caribbean region and B had the isolates from other geographical locations. The robustness of the tree was estimated by bootstrap resampling [47], and the bootstrap values for the Cluster A and B were very high, 97% and 100%, respectively. This data concurs with that from other studies in which it was possible to generate dendrograms with different clusters depending on the sample geographical origin [50]. However, in other studies, it was not possible to obtain clusters related to geographical origin using different molecular markers like minisatellites [15], RAPD with fluorescent labels [14] or rDNA intron [51].

The different isolates of cluster A exhibited nearly 75% similarity. Subcluster A₁ contained just one isolate, MG1 from Cuba. This isolate is clearly different from the rest of the Caribbean isolates. This is valuable data because this isolate shows higher virulence than any of the other isolates used in Cuba. Also, it has been used in the sugar cane culture in Cuba to control the larval populations of *Diatraea saccharalis*. A diagnostic band for this isolate was obtained, and in future it will be possible to obtain a specific molecular marker for this important isolate. The other Caribbean isolates exhibited a similarity greater than 82%, and are grouped in subcluster A₂. It also had a high bootstrap value (100%).

The isolates from regions other than the Caribbean are in cluster B, and displayed more than 80% similarity. Between the two clusters, the similarity exhibited is less than 50%. This is a low value of homology, and showed the

high genetic variability present between the isolates from the Caribbean region and the rest of the isolates. These values of similarity are in concordance with other previous studies [9, 11, 14]. The worldwide distribution of the *B. bassiana* isolates analyzed using AFLPs were found to be very dissimilar [32]. In another previous study of 50 *B. bassiana* isolates of worldwide distribution, a very close similarity (~80%) in AFLP fingerprints was reported [52]. However, the level of similarity between isolates varied with the technique used [53, 54]. The lack of correlation between different DNA marker/fingerprinting techniques in the analysis of buffalo grass was attributed to the highly heterogeneous nature of the species and its diverse genetic background [55]. Another explanation is that the different markers analyzed detected polymorphisms in different regions of the genome.

ISSR fingerprints provide a useful tool for establishing a rapid and rational approach for differentiating between isolates of entomopathogenic fungi. These markers can be used to evaluate the efficacy of the use of different isolates and the persistence of the fungus in the sugar cane agronomic ecosystem. Using these markers, we obtained a genetic profile that identifies the most important isolates used actually in the biocontrol of the sugar cane borer in Cuba.

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