Genetic variability among *Beauveria brongniartii* (Saccardo) Petch isolates from various geographical and host origins based on AFLP analysis

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Abstract: The entomopathogenic hyphomycete *Beauveria brongniartii* is a promising candidate for biocontrol of economically important agricultural and forest pests. Assessment of genetic relatedness of this species appears to be essential to gain insight into the monitoring of such biocontrol products. Distinction of *Beauveria* spp. strains with different virulence to target organisms revealed to be a serious constraint in the development of successful biocontrol using these important species. Thus, there is a need to find ways to monitor these strains when applied to natural agents. We have used amplified fragment length polymorphism (AFLPs) markers to estimate genetic variations among fourteen isolates (ten *B. brongniartii*, two *B. bassiana* (BALSAMO) VUILLEMIN and two *Nomuraea rileyi* (FARLOW) SAMSON) obtained from different geographical origins and hosts with differing virulence to scarabs. Seven different AFLP primer combinations yielded a total of 229 AFLP fragments comprised between 30 (*Eco*RI-ACA/*Tru*1l-C) to 57 (*Eco*RI-AAG/*Tru*1l-CTT) AFLP markers with an average of 54 amplified fragments per primer combination. Fragment size varied between 50-541 base pairs (bp) among the ten *B. brongniartii* isolates analysed in this study achieving a good resolution between the isolates. The cluster analysis based on genetic distance values clustered all isolates at above 0.40 similarity and demonstrated that some *B. brongniartii* isolates from distinct geographical origins and various hosts showed a greater genetic variability.

Key words: *Beauveria brongniartii*, *Melolontha melolontha*, *Holotrichia serrata*, AFLP, DNA fingerprinting, entomopathogenic fungi, Coleoptera, Scarabaeidae

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The fungus *Beauveria brongniartii* (SACCARDO) PETCH is one of the most virulent and widespread pathogens of soil-inhibiting scarabs including European cockchafer, *Melolontha melolontha* L. as well as *M. hippocastani* F. and *Holotrichia serrata* F. These scarab beetles are widespread throughout southern and central Europe and the Indian union, respectively, where the larvae cause considerable damage to a variety of important agricultural crops and forest trees (KELLER et al. 1986, Sharma et al. 1999). Recently, this entomopathogen has received special attention for field usage as a mycoinsecticide against *Melolontha* sp. Among isolates from Germany, some have been shown to be particularly pathogenic to *M. melolontha*, and these isolates were primary candidates for biocontrol agents (REINEKE & ZEBITZ 1996). However, it is important to determine first the natural variability within the fungal population so that individual strains can be characterized and monitored.

There is little information on the molecular biology of *B. brongniartii*, yet the development of DNA technology has provided a number of methods to detect the differences at DNA level. Genetic variability has been considered in many groups of entomopathogenic fungi and in some cases, genetic variability has been linked with pathogenicity (NEUVEGLISE et al. 1997). The internal-transcribed-spacer ribosomal region (ITS)-RFLP (restriction fragment length polymorphism) technique has led to separation of *B. brongniartii* populations from diverse geographical and biological origins in previous studies (NEUVEGLISE et al. 1994).

Although this technique was found to delineate intra-specific groups, it failed to distinguish between strains of *B. brongniartii* isolated from different *Melolontha* sp. A research based on RFLP and RAPD (random amplified polymorphic DNA) analysis showed clearer relationships between population structure of *B. bassiana* (Balsamo) Vuillemin and some defined host species (MAURER et al. 1997).

Compared to ITS-RFLP analysis, AFLP (amplified fragment length polymorphism) is a more suitable molecular marker technique for differentiation of *B. brongniartii* isolates as it encompasses the analysis of the total genome. In addition, it has been shown to be more reliable than RAPD markers. The AFLP technique has been successfully used to examine interspecific variation within different invertebrate mycopathogens (MURO et al. 2003). In this study, we have investigated the genetic variability in natural populations of *B. brongniartii* obtained from different insect hosts and geographical regions using AFLP technique.

Materials and Methods

Source of fungal pathogens

Ten *Beauveria brongniartii*, two *B. bassiana* (BALSAMO) VUILLEMIN and two *Nomuraea rileyi* (FARLOW) SAMSON representing different host and geographical origins, were analysed (Tab. 1). *B. brongniartii* and *B. bassiana* isolates were obtained from Germany (BBA, Darmstadt) and USDA-ARSEF collection (Ithaca, NY). Field isolates of *N. rileyi* were derived from natural epizootics on *Spodoptera litura* F. on a groundnut crop in India. To isolate DNA, the mycelium was obtained from a submerged culture of spores in Sabouraud medium with 1% peptone and shaking at 180 rpm at 25^0 C for 3-4 days. The fungal cells were collected by centrifugation (10,000 g, 20 min), lyophilized, weighed, and stored at - 20^0 C.

Tab. 1: Entomopathogenic fungal isolates, their host insects, geographical origin and virulence against *M. melolontha* and *H. serrata*

Isolate	Host	Origin	Virulence (% CM ± SE)*	
			M. melolontha	***H. serrata***
B. brongniartii				
Bbr 23	*Melolontha melolontha*	Stuttgart, Germany	82.14±5.36a	31.74±1.54c
Bbr 30	*M. hippocastani*	Freiburg, Germany	73.80±7.3ae	8.88±5.88cd
Bbr 41	*Holotrichia morosa*	China	22.02±4.8bd	46.66±3.85b
Bbr 50	*M. melolontha*	Stuttgart, Germany	95.2 ±4.76a	26.66±3.8c
Bbr 59	*M. hippocastani*	Darmstadt, Germany	60.7 ±7.44ac	24.12±7.7c
ARSEF 1072	*M. melolontha*	Thurgau, Switzerland	30.35±3.72bd	0.13±3.33d
ARSEF 1360	Unknown	Poland	14.13±0.90bd	3.10±5.24d
ARSEF 2660	Adult Coleoptera	India	17.85±0.00bd	52.70±3.9b
ARSEF 4384	*H. parallela*	Cangzhou, China	78.57±7.62ae	74.97±4.18a
ARSEF 5358	*Melolontha* sp.	Hessen, Germany	58.92±12.68ac	28.28±6.35c
B. bassiana				
NRRL 22865	Unknown	Iowa, USA	--	--
NRRL 22866	*Pachnaeus litus*	Florida, USA	--	--
N. rileyi				
Nr 18	*Spodoptera litura*	Guntur, India	--	--
Nr 32	*S. litura*	Guntur, India	--	--

* (CM ± SE with the same index are not significantly different (Tukey's HSD multiple comparison ($p<0.05$)); CM: corrected % mortality; SE: standard error of three assays.

Pathogenicity assays

All the isolates of *B. brongniartii* (2×10^7 conidia/ml) were tested for virulence against third instar larvae of *M. melolontha* and *H. serrata* in laboratory assays, as described previously (REINEKE & ZEBITZ 1996). The virulence of each strain was determined as percentage of larvae killed by the fungus within 30 days of inoculation.

DNA extraction

The fungal DNA was extracted using a modified CTAB (cetyltrimethyl-ammonium bromide) protocol with an additional spermine precipitation (REINEKE et al. 1998) with slight modification. The integrity and quantity of DNA was determined by electrophoresis as well as by measuring in a spectrophotometer. Only highly purified DNA-samples were used for further studies.

AFLP

AFLP fingerprinting was done using a modified protocol of REINEKE & KARLOVSKY (2000). No radioactivity was incorporated in the polymerase chain reaction but primers fluorescently labelled with Cy5 were used instead. All fungal isolates were evaluated using seven primer combinations; EAAG/MCTT, EAAG/MCA, EAAG/MCT, EAAG/MCGG, EACA/MC, EACA/MCA, EACC/MTCC. The amplification products were denatured at 80^0C, and loaded with 4µl into a 5% polyacrylamide gel (and analyzed on an ALFexpress II DNA Analysis System (Amersham Biosciences, Freiburg, Germany) in 5 x TBE electrophoresis buffer.

Data analysis

Virulence data were first subjected to angular transformation before analysis. Analysis of variance (ANOVA) using Systat statistical software (SPSS, 2004) was used to detect differences in mortality and mycosis. Tukey's HSD multiple comparison followed by significant difference were detected. Digitized photographs generated from AFLP gels were used for analysis using GelCompar 4.0 software (Applied Maths, Kortrijk, Belgium). A binary matrix was exported to the software AFLP-SURV v1.0 (VEKEMANS et al. 2002, available at http//:www.ulb.ac.be/sciences/lagev), and estimates of pairwise genetic distance were calculated between individuals according to LYNCH & MILLIGAN (1994). We bootstrapped over AFLP loci to generate 1,000 genetic distance datasets, based on all frequencies estimates by using Bayesian method with uniform prior distribution of allele frequencies (ZHIVOTOVSKY 1999). A genetic distance matrix was obtained and Neighbour-joining method was used to construct a tree using the software MEGA 3 (Molecular Evolutionary Genetics Analysis, available at http://www.megasoftware.net) considering *B. bassiana* and *N. rileyi* as an outgroups.

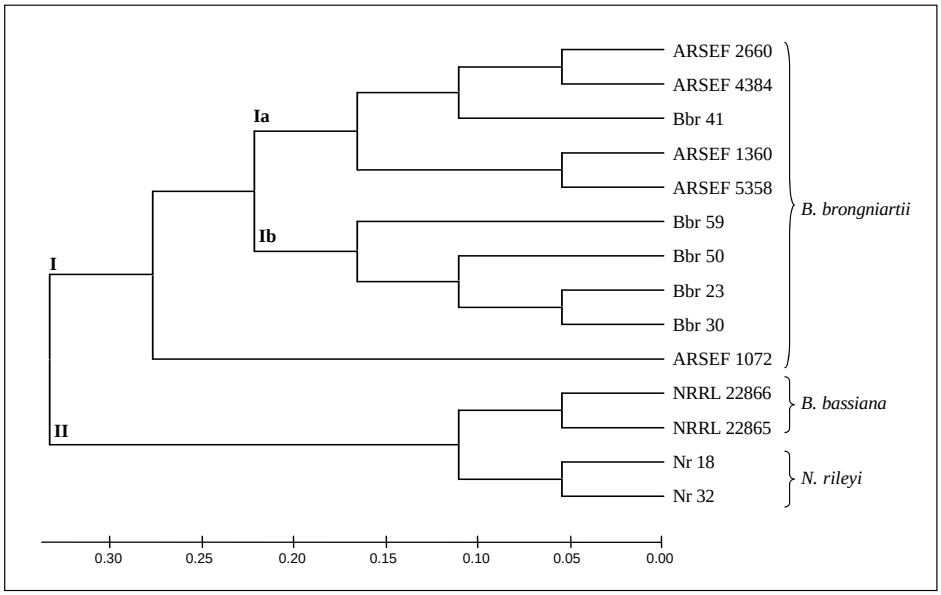

Fig. 1. Cluster analysis of entomopathogenic fungi isolates collected from different geographic and host origins, as revealed by neighbour-joining (NJ) from a matrix of pairwise genetic distance, based on AFLP data of 229 fragments amplified with 7 different primer combinations.

Results and discussion

Virulence assays

All isolates of *B. brongniartii* were found to be pathogenic with significant differences in their virulence to *M. melolontha* ($p<0.05$) and *H. serrata* ($p<0.05$) (Tab. 1). It was apparent that *B. brongniartii* isolates obtained from Germany were shown to be more virulent towards *M. melolontha* as compared to two isolates derived from Switzerland and Poland. KELLER (1998) reported that Swiss *B. brongniartii* isolates were more specific to *M. melolontha* larvae from the same region compared to larvae from Germany. Moreover, *B. brongniartii* isolates obtained from *Holotrichia* sp (Bbr 41 and ARSEF 4384) or from an undetermined coleopterous insect (ARSEF 2660) showed greater pathogenicity to *H. serrata* except for isolate ARSEF 4384. This indicated that *B. brongniartii* isolates obtained from different geographical and host origins showed higher host specificity to *M. melolontha* compared to *H. serrata*, indicating a narrow ecological host range. REINEKE & ZEBITZ (1996) classified 31 isolates of *B. brongniartii* according to differences in enzymatic patterns of the isolates. Similarly, there was a difference in pathogenicity of different *B. brongniartii* isolates tested against *M. melolontha* (DARWISH et al. 2000) and *H. serrata* (SHARMA et al. 1999). The last observation could partially be in agreement with our results concerning the selectivity between the isolates.

AFLP

An initial survey was conducted for a few number of *B. brongniartii* isolates with 21 AFLP primer combinations either with 1, 2 or 3 selective nucleotides at their 3'-end. Seven different AFLP primer combinations were selected, which yielded a total of 229 AFLP fragments among the *B. brongniartii, B. bassiana and N. rileyi* isolates analysed in this study, achieving a good resolution between the isolates. The number of AFLP markers amplified with the seven primers varied between 30 (*Eco*RI-ACA/*Tru*1l-C) to 57 (*Eco*RI-AAG/*Tru*1l-CTT), with an average of 54 amplified fragments per primer combination. The size of the fragments analyzed by fluorescence-based detection ranged from 50 to 541 base pairs. The number of loci as revealed by the presence of markers was 229, all of which were polymorphic (100%).

A cluster analysis on the basis of the obtained AFLP markers demonstrated that some *B. brongniartii* isolates from distinct geographical origins and various hosts showed a great amount of genetic variability (Fig. 1). Both the *B. bassiana* and *N. rileyi* isolates used as outgroup were clearly separated from the *B. brongniartii* cluster. In addition, with the exception of the Swiss isolate ARSEF 1072, the *B. brongniartii* isolates were separated into two main subclusters, Ia and Ib (Fig. 1). Except for ARSEF 5358, all isolates derived from *Holotrichia* sp. were placed in cluster Ia, while cluster Ib contained all isolates obtained from *Melolontha* sp. The isolate ARSEF 1072 originally collected in Switzerland showed a high degree of genetic distance to all other *B. brongniartii* isolates analysed in the present study and was therefore not included in any of the two *B. brongniartii* subclusters.

All isolates of *B. brongniartii* were found to be pathogenic to both insects tested with significant difference in their virulence to *M. melolontha* and *H. serrata*. Six isolates of *B. brongniartii* (Bbr 23, 30, 50, 59 and ARSEF 1072, and 5358) showed variation in virulence towards *M. melolontha* and no indication of clustering according to the pathogenicity of the test insects (Fig. 1) although isolates were obtained from *Melolontha* sp. The isolate ARSEF 1072, obtained from Switzerland, which was separated from all other *B. brongniartii* isolates in cluster analysis based on AFLP data, also showed less virulence towards both tested insects. It suggests that Swiss *B. brongniartii* isolates are likely to be more specific to *M. melolontha* of the same (Swiss) origin as compared to *Melolontha* larvae from other geographic regions and is in accordance with field observations reported by KELLER (1998). Within the cluster of isolates obtained from *Holotrichia* sp. two isolates were closely grouped together, of which isolate ARSEF 4384 also showed a greater pathogenicity towards *M. melolontha* larvae as compared to the other isolates within this cluster. This observation is partially supported by CRAVANZOLA et al. (1997), who reported that the most virulent strain isolated from Valle D'Aosta, causing 100% mortality compared to another strain obtained from *H. serrata* in India, was not virulent for larvae of *M. melolontha*. In addition, genetic variability has been detected in many groups of entomopathogenic fungi and in some cases, genetic variability has been linked with pathogenicity (BIDOCHKA et al. 1995, NEUVEGLISE et al. 1997). On the other hand, there are reports indicating clustering of fungi based on molecular data with similar virulence characters. Such clustering is more pronounced in plant

pathogenic fungi than in insect pathogenic fungi; several such instances have been reported (MALVICK et al. 1998). In the present investigation, isolates of *B. brongniartii* were grouped together according to their host origin. Thus, a relation between certain virulence characteristics and the respective genetic profiles could be envisaged.

In summary, the results obtained here have shown that differences in pathogenicity of *B. brongniartii* isolates from different geographical and host origins against larvae of *Melolontha* sp. and *Holotrichia* sp. were correlated with a great degree of genetic variability revealed by AFLP analysis and supports previous observations on genetically heterogeneous populations of this entomopathogen.

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