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Différentiation d'isolats du champignon entomopathogène *Beauveria bassiana* (Ascomycetes : Hypocreales) par PCR-RFLP

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Aller au sommaire du numéro

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Résumé de l'article

Le champignon entomopathogène *Beauveria bassiana* suscite de plus en plus d'intérêt en recherche et constitue une avenue intéressante en lutte biologique contre plusieurs insectes ravageurs en agriculture. Différentes approches (PCR, analyse des séquences d'ADN et PCR-RFLP) ont été utilisées lors de cette étude comme outils moléculaires d'identification de différents isolats de *B. bassiana*. Notre travail a consisté à identifier les régions 18S, ITS1, 5.8S, ITS2 et 28S de l'ADN ribosomal de *B. bassiana*. Les séquences d'ADN des régions amplifiées ont démontré que la région 18S de l'ADNr était la sous-unité la plus conservée, avec une homologie de 99,5 % entre les isolats étudiés, tandis que l'extrémité 3' du gène 28S a accumulé beaucoup de variabilité et peut donc être utilisée pour différencier les isolats de *B. bassiana*. La technique PCR-RFLP a été utilisée pour réaliser le suivi d'isolats de *B. bassiana* chez un ravageur ciblé, *Lygus lineolaris*, et pour les distinguer. Cette méthode comprenait deux étapes. Premièrement, la PCR était utilisée pour amplifier une région du gène 28S de *B. bassiana*. Deuxièmement, ce produit de PCR était digéré à l'aide des endonucléases de restriction et les fragments produits ont été comparés en utilisant l'électrophorèse sur gel. En raison de la grande spécificité et sensibilité de la PCR-RFLP, il a été possible de différencier les isolats de *B. bassiana* en utilisant comme échantillons des spores prélevées à la surface d'un insecte infecté.

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Differentiation of entomopathogenic fungus *Beauveria bassiana* (Ascomycetes: Hypocreales) isolates by PCR-RFLP

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The entomopathogenic fungus *Beauveria bassiana* is a promising biological control agent of several insect pests in agriculture. Molecular approaches (PCR, DNA sequence analysis and PCR-RFLP) were used in our research as tools for the identification of different *B. bassiana* isolates. Our work consisted in identifying the 18S, ITS1, 5.8S, ITS2 and 28S regions of *B. bassiana* ribosomal DNA. The DNA sequences of the amplified regions showed that the 18S rDNA is the most conserved unit, with a high homology (99.5%) between the isolates studied, while the 3′ end of the 28S rDNA has a great variability, which makes it possible to differentiate the isolates. The PCR-RFLP method was used to monitor isolates of *B. bassiana* and distinguish them in a target pest, *Lygus lineolaris*. This method involved two main steps. First, PCR was used to amplify a region of the 28S gene of *B. bassiana*. Second, this PCR product was digested using restriction endonucleases, and the fragments produced were compared using gel electrophoresis. Because of the high specificity and sensitivity of PCR-RFLP, it was possible to discriminate between *B. bassiana* isolates using spores scraped from the surface of an infected insect as samples.

Keywords: *Beauveria bassiana*, entomopathogenic fungus, nucleotide sequences, PCR-RFLP, ribosomal DNA, 28S gene.

[Différentiation d'isolats du champignon entomopathogène *Beauveria bassiana* (Ascomycetes : Hypocreales) par PCR-RFLP]

Le champignon entomopathogène *Beauveria bassiana* suscite de plus en plus d'intérêt en recherche et constitue une avenue intéressante en lutte biologique contre plusieurs insectes ravageurs en agriculture. Différentes approches (PCR, analyse des séquences d'ADN et PCR-RFLP) ont été utilisées lors de cette étude comme outils moléculaires d'identification de différents isolats de *B. bassiana*. Notre travail a consisté à identifier les régions 18S, ITS1, 5.8S, ITS2 et 28S de l'ADN ribosomal de *B. bassiana*. Les séquences d'ADN des régions amplifiées ont démontré que la région 18S de l'ADNr était la sous-unité la plus conservée, avec une homologie de 99,5 % entre les isolats étudiés, tandis que l'extrémité 3′ du gène 28S a accumulé beaucoup de variabilité et peut donc être utilisée pour différencier les isolats de *B. bassiana*. La technique PCR-RFLP a été utilisée pour réaliser le suivi d'isolats de *B. bassiana* chez un ravageur ciblé, *Lygus lineolaris*, et pour les distinguer. Cette méthode comprenait deux étapes. Premièrement, la PCR était utilisée pour amplifier une région du gène 28S de *B. bassiana*. Deuxièmement, ce produit de PCR était digéré à l'aide des endonucléases de restriction et les fragments produits ont été comparés en utilisant l'électrophorèse sur gel. En raison de la grande spécificité et sensibilité de la PCR-RFLP, il a été possible de différencier les isolats de *B. bassiana* en utilisant comme échantillons des spores prélevées à la surface d'un insecte infecté.

Mots clés : ADN ribosomal, *Beauveria bassiana*, champignon entomopathogène, gène 28S, PCR-RFLP, séquences nucléotidiques.

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INTRODUCTION

The entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin is a ubiquitous soil-borne pathogen isolated from a wide range of insect species, mostly pests of economic importance (Faria and Wraight 2001; Inglis *et al.* 2001; Jaronski and Goettel 1997). There is considerable interest in the development of this fungus for the control of insect pests because it is considered an environmentally benign alternative to chemical insecticides (Ludwig and Oetting 2002; Padjama and Kaur 2001; Wright and Chandler 1991). The potential of this fungus for biocontrol has been exploited by using local isolates collected from either the soil or dead insect hosts found in different geographical areas.

As it is the case with most mitosporic fungi, little is known about the basic genetics and/or genome organization of *B. bassiana*. One of the groups of genes that are most frequently targeted for phylogenetic studies is the one that codes for ribosomal RNA (Destéfano *et al.* 2004). However, there is concern about the release of *B. bassiana* isolates in the field, primarily because little is known about the fate of the inoculum and its impact on non-target organisms. This concern is exacerbated by the difficulty of tracking the released fungus in the field. In order to overcome this problem, a method needs to be devised that would discriminate between isolates and also allow the screening of large numbers of samples and detect the pathogen in infected hosts (Hajek *et al.* 1991). In turn, this should lead to a more efficacious use of entomopathogens in pest control programs. Highly specific markers would also be extremely useful for the patenting of commercially viable isolates.

Current methods (e.g. allozyme electrophoresis) only partly fulfil these requirements. They may fail because they can only distinguish between species (e.g. Hajek *et al.* 1991; Wilding *et al.* 1992) or require large quantities of purified fungal DNA (e.g. Hegedus and Khachatourians 1993), a time-consuming process that is unrealistic if large numbers of samples are to be processed. Polymerase chain reaction-random amplified polymorphic DNA (PCR-RAPD) has been used to characterize isolates of *B. bassiana* and has been shown to be highly discriminatory (Berretta *et al.* 1998; Castrillo and Brooks 1998; Urtz and Rice 1997). However, this method is very susceptible to contamination and depends on purified DNA extracted from axenic cultures.

Very similar problems to those encountered when identifying entomopathogenic fungi in infected insects are found when identifying plant pathogenic fungi from infected plants. Specific primers for PCR have been used effectively to detect and differentiate plant pathogenic fungi (e.g. Henson *et al.* 1993; Ouellet and Seifert 1993).

Genetic markers such as PCR-restriction fragment length polymorphism (PCR-RFLP) are much exploited in plants, in particular for the construction of genetic charts (Devey *et al.* 1994) or for the localization of QTLs (qualitative trait loci) related to resistance genes (Kicherer *et al.* 2000; Lübberstedt *et al.* 1999). In hyphomycetes, the PCR-RFLP technique makes it possible to differentiate, for example, isolates of *Metarhizium anisopliae* (Metsch.) Soroko (Destéfano *et al.* 2004), *Beauveria brongniartii* (Sacc.) Petch (Wada *et al.* 2003), *Paecilomyces farinosus* (Holmsk.) A.H.S. Br. & G. Sm. (Chew *et al.* 1997), and *Entomophaga maimaiga* Humber, Shimazu & R.S. Soper (Hajek *et al.* 1991).

Our work consisted in identifying the 18S, ITS1, 5.8S, ITS2 and 28S regions of *B. bassiana* ribosomal DNA. We describe a new method for the identification/characterization of *B. bassiana* isolates that are relevant to current research on the biocontrol potential of this fungus. The PCR-RFLP method is based on the amplification of a portion of the gene encoding the 28S rRNA from *B. bassiana* followed by restriction digestion of this PCR product.

MATERIALS AND METHODS

Fungal isolates and culture conditions

Six *B. bassiana* isolates came from the INRS-Institut Armand-Frappier (Laval, QC, Canada) collection and were derived from a variety of hosts and geographical origins (Table 1). Stock cultures of each isolate were kept frozen at -70°C in 70% glycerol. Isolates were growing on Sabouraud Dextose Agar (SDA) (10% neopeptone, 40% dextrose, 15% agar, pH 5.6) (Difco Laboratories, Germany) under controlled conditions in a growth chamber (MLR-350, Sanyo, Japan) at 25°C, 80% RH, in darkness.

Table 1. Host and geographical origin of the tested isolates of *Beauveria bassiana*

Isolate[a]	Host	Geographical origin
INRS-CFL	*Tomicus piniperda* (Coleoptera: Scolytidae)	Québec, Canada
INRS-IP	*Lygus* sp. (Heteroptera: Miridae)	Québec, Canada
ARSEF2991	*Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae)	Québec, Canada
DAOM195005	*Choristoneura fumiferana* (Lepidoptera: Tortricidae)	Québec, Canada
DAOM210087	*Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae)	Québec, Canada
DAOM216540	*Reticulitermes flavipes* (Isoptera: Rhinotermitidae)	Ontario, Canada

[a] INRS: Institut national de la recherche scientifique, Institut Armand-Frappier, Collection de champignons entomopathogènes, Laval, Québec, Canada; ARSEF: Agriculture Research Service of Entomopathogenic Fungi, USDA, Ithaca, New York, USA; DAOM: Eastern Cereal and Oilseed Research Centre, Ottawa, Ontario, Canada.

Table 2. Primers used in rDNA amplification of *Beauveria bassiana* isolates

Primer[a]	Sequence (5′->3′)	References
18S-ITS FW1	GTAGTCATATGCTTGTCTC	White *et al.* 1990
18S-ITS RV1	TCCTCCGCTTATTGATATGC	White *et al.* 1990
5′ end-28S FW1	CGGAGGAAAAGAAACCAACAGGAT	[a]
5′ end-28S RV1	CTCGAGTCATAGTTACTCCC	[a]
3′ end-28S FW1	CTGCCCAGTGCTCTGAATGTCAAAG	Neuvéglise and Brygoo 1994
3′ end-28S RV1	CCTCTCCGCAATGGTAATTCAGC	Neuvéglise and Brygoo 1994

[a] These primers were designed based on the nucleotide sequences obtained after sequencing the cloned PCR products. Two pairs of primers were used to amplify the complete 28S rDNA gene (this gene was divided into two regions: 5′ and 3′ end 28S).

DNA extraction from infected insects

The tarnished plant bug *Lygus lineolaris* (P. de B.) was infected with isolates of *B. bassiana* that have already been established as being highly virulent under laboratory conditions (Sabbahi *et al.* 2008). In our bioassays, the fungus-host contact was maximized by immersion of the host in a conidial suspension. The inoculation of insects was then performed by dipping 20 adults of *L. lineolaris* individually for 5 s in a 50 mL suspension at 1 x 10^7 conidia mL^{-1} (Butt *et al.* 1994). The insects were then kept individually on wet filter paper (Whatman International Ltd., Maidstone, England, UK) in a 9 cm diam Petri dish for incubation in a growth chamber at 25°C, 70% RH, and 16:8 h (L:D) photoperiod. The experiment was repeated three times. The replicated treatments were made from different stock cultures. Upon death, insects were kept in a growth chamber at 25°C, 90% RH, and darkness for 2 wk to promote fungal emergence and conidiation. Fungal isolates were re-selected from the dead individuals after sporulation on the surface of the cadavers using sterile sticks and spread on a *B. bassiana* selective medium (17.5 g oatmeal agar, 0.45 g dodine, 2.5 mg crystal violet, 0.2 g penicillin G, and 0.5 g streptomycin) (Chase *et al.* 1986). Each re-selected isolate was isolated by taking a single-conidium with sterile sticks and inoculated in Sabouraud Dextrose Broth (neopeptone 1%, dextrose 2%), and incubated at 25°C for 7 d at 160 rpm to produce spores. Details of the production of sterile spores and DNA extraction procedures are described by Pfeifer and Khachatourians (1993); 100 mg of *Aspergillus niger* Tiegh. cellulase and 50 mg of *Trichoderma harzianum* Rifai cellulase were used instead of 100 mg of *Trichoderma viride* Pers. cellulase and 100 mg of *Penicillium funiculosum* Thom cellulase. Extracted DNA was dissolved in 50 µL sterile distilled water and stored at -20°C until needed.

Amplification of SSU and LSU of rDNA

Amplification of specific portions of the rDNA small subunit (SSU) and large subunit (LSU) of six *B. bassiana* isolates was done by PCR in a DNA thermal cycler (Gene Amp® PCR System 9700, Applied Biosystems, Foster City, CA). The specific primers used to amplify the 18S-ITS region and 28S rRNA gene are shown in Table 2. Amplification was done in a 50 µL reaction mixture containing 100 ng of genomic DNA, 40 pmol of each primer, 1 x *Taq* polymerase buffer, 200 µmol L^{-1} deoxynucleotide triphosphate (dNTPs), and 2.5 U *Taq* polymerase. All PCR reagents were purchased from Sigma Aldrich (Oakville, ON). Amplification cycles were completed as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 1 min, extention at 72°C for 40 s, and final extension at 72°C for 10 min. Tubes were placed in the thermocycler once it had reached 60°C. Reaction products were analyzed by electrophoresis in 0.8% agarose gels in 0.5 x TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0). We used 1 kb plus DNA ladder (Gibco BRL, Burlington, ON, Canada) to reveal the PCR product size. Nucleic acids were visualised with ethidium bromide using Bio-Rad Gel Doc 1000 apparatus (Bio-Rad Laboratories Inc., Canada).

DNA sequencing and data analysis

PCR products were cloned in vector pCR®2.1 using the TA cloning kit (Invitrogen, Canada). Double-stranded DNA obtained by PCR amplification was used as template for sequence reactions. The reactions were performed by using either universal forward or reverse sequencing primers. In all cases, sequencing was performed in both directions for the entire sequence to avoid misreading. Sequence reactions were performed at the Genome Quebec Innovation Centre of McGill University (Montreal, Canada). The ends of the PCR products were identified by using internal primers based on the sequences obtained. DNA similarity searches were performed using Basic Local Alignment Search Tool (BLAST 1.4 10MP; Altschul *et al.* 1990), while DNA sequence alignment and pairing of the resulting nucleotide sequences were made using the Bio-Edit Program package v. 7.0.9 (Ibis Biosciences, Carlsbad, CA). These sequences were also compared with the published rDNA fragments of other hyphomycete species by conducting multiple sequence alignments using the BLAST program.

RFLP analysis of the 3′ end-28S rRNA region

Ten restriction endonucleases (NewEngland Biolabs Inc., ON, Canada) were screened to detect polymorphic restriction sites within the 3′ end of the 28S rDNA product amplified from six different isolates of *B. bassiana*. Digestion of the PCR products was carried out as follows: 5 µL of the PCR mixture containing about 100 ng of PCR product was digested by the addition of 5 units of restriction enzyme, 1 µL of appropriate buffer and 13.5 µL of distilled water. Reactions were incubated according to the manufacturer's instructions (NewEngland Biolabs

Table 3. Size (bp) of rDNA PCR products of *Beauveria bassiana* isolates

Isolate	18S-ITS	5′ end-28S	3′ end-28S
INRS-IP	2315	2178	2178
INRS-CFL	2315	2178	1265
ARSEF2991	2315	2178	1623
DAOM216540	2315	2178	1623
DAOM195005	2315	2178	822
DAOM210087	2703	2178	1754

Inc., ON, Canada). After digestion, the restriction fragments produced were separated by electrophoresis in 1.2% agarose gels as described above. We included 1 kb plus DNA ladder as DNA size markers.

RESULTS

We studied the rDNA of *B. bassiana* in order to identify variations in the 18S gene, ITS1, 5.8S gene, ITS2, and 28S gene. A method was used to detect the isolates from insect sampling. We used PCR, rDNA sequence data comparisons and RFLP analysis to confirm the identity of the isolates after bioassays. Our research made it possible to amplify and sequence, for the first time, the complete sequences of the rDNA of six isolates of *B. bassiana* (Table 3). These isolates were recorded in GenBank under the following accession numbers: EU334674, EU334675, EU334676, EU334677, EU334678 and EU334679.

We demonstrated that the rDNA contains preserved and variable areas. Precisely, the SSU of the rDNA was the most preserved unit, with a homology higher than 99.5% between the six studied isolates (Table 4). The analysis of the nucleotidic sequences of the 18S gene revealed some substitutions and deletions or insertions for the six isolates, and the presence of an intron of 387 bp only for isolate *DAOM210087* (Table 4). However, when compared with other GenBank nucleotide sequences of some hyphomycete species such as *B. brongniartii*, *M. anisopliae* and *Tolypocladium cylindrosporum* W. Gams, the internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene sequences make it possible to distinguish *B. bassiana* from these hyphomycete species.

Our results demonstrated for the first time that the 5′ end of the rRNA 28S gene was 100% homologous among the six isolates studied. However, the 3′ end of this gene accumulated much variability. In an attempt to explain the nature of the LSU rRNA gene size polymorphism of certain *B. bassiana* isolates, we analyzed the PCR products of the 3′ end of this gene region and we can report the presence of four group-I introns (Table 5). Their presence or absence in the isolates could explain the variation in the size of the amplified fragments of the rRNA 28S gene. This variation made it possible to establish the techniques of an approach based on the comparison of the profiles following digestion by the restriction enzymes, which will cross to specific sites in the variable areas of the 28S gene (Table 6). The use of the PCR-RFLP technique made it possible to discriminate the studied isolates according to their genetic profiles (Table 6). Results were reproducible when the DNA was extracted from conidia taken from axenic cultures.

The PCR-RFLP method, which includes PCR of primers at the 3′end of the 28S rRNA gene, was used with six *B. bassiana* isolates that produced different bands varying from 822 to 1754 bp (Table 3). Half of the endonucleases studied were able to digest the 3′-28S-PCR product and exhibited polymorphism between the six isolates (Table 6). Restriction patterns obtained using the endonucleases *Sal* I, *Acc* I and *Afl* II poorly discriminated between isolates while those produced using *Cla* I, *Sma* I and *EcoR* I were

Table 4. Genomic analysis of the rDNA SSU of *Beauveria bassiana* isolates (letters indicate substitutions, deletions or insertions)

SSU	Position	INRS-IP	INRS-CFL	ARSEF 2991	DAOM 216540	DAOM 195005	DAOM 210087
18S	409	G	G	G	G	G	A
	732	A	G	A	A	A	A
	970	G	A	A	A	A	A
	1028	A	G	G	G	G	G
Intron	1145…1532	—	—	—	—	—	387 bp
	1735	G	G	A	A	G	G
ITS 1	2181	T	T	T	T	T	C
	2204	T	T	T	C	T	T
	2275	T	T	T	T	T	C
5.8S	2379	C	C	T	T	C	T
	2453	C	T	C	C	C	C
ITS 2	2495	C	C	T	T	C	T
	2501	T	T	G	G	T	T

Table 5. Genomic analysis of the rDNA LSU of *Beauveria bassiana* (data represents the position, size and type of introns observed in *B. bassiana* isolates)

	Type and position of Group-I introns		
Isolate	Type 1 (388 bp)	Type 2 (505 bp)	Type 3 (358 bp)
INRS-IP	73-461 1183-1571		
INRS-CFL	73-461		
ARSEF2991	73-461		661-1019
DAOM216540	73-461		661-1019
DAOM195005			
DAOM210087	73-461	1050-1555	

Table 6. Size (bp) of DNA fragments obtained after endonuclease digestion of the 3′ end-28S of rDNA from *Beauveria bassiana* isolates

	Size of the fragments for each enzyme									
Isolate	*Acc* I	*Afl* II	*Afl* III	*Ava* I	*Bgl* I	*Bst* XI	*Cla* I	*EcoR* I	*Sal* I	*Sma* I
INRS-IP	705 486 284 107 72	916 474 216 48	1333 321	759 515 307 73	1260 377 17	1027 627	1366 257	1152 502	1262 392	834 820
INRS-CFL	781 484	745 473 48	944 321	757 435 73	873 392	640 625	1008 257	1150 115		832 433
ARSEF2991	668 484 231 215 25	745 473 48	1302 321	1115 435 73	1231 392	998 625	1010 627	1508 115	929 668 25	1190 433
DAOM216540	668 484 231 215 25	745 473 48	1302 321	1115 435 73	1231 392	998 625	1010 627	1508 115	929 668 25	1190 433
DAOM195005		745 77		435 387		640 182	565 257	707 115		433 389
DAOM210087	1022 467 265		1325 320 109	658 376 365 253 72	1290 464	1146 608	991 763	1638 116		939 450 365

moderately to highly discriminatory (Table 6). The restriction patterns of all isolates predominated. However, similar patterns for the *ARSEF2991* and *DAOM216540* isolates were noted.

DISCUSSION

Comparisons of the 18S rRNA sequences have been performed to assess the relationships between the major groups of living organisms (Coates *et al.* 2002a; Woese *et al.* 1990). For phylogeny of filamentous fungi, the 18S sequence is mostly used (Bruns *et al.* 1992). In the 18S gene, the variable domains mostly provide insufficient information for diagnostic purposes (de Hoog and Gerrits van den Ende 1998). The ITS regions are much more variable, but sequences can be aligned with confidence only between closely related taxa. These regions are generally used for species differentiation (Guarro *et al.* 1999). For example, strains of *Beauveria* were identified by PCR-RFLP of nuclear rRNA internal transcribed spacer regions (Coates *et al.* 2002b; Glare and Inwood 1998). In our study, the ITS region did not show any polymorphism within the *B. bassiana* isolates, but only differentiated *B. bassiana* from the other hyphomycete species. In contrast, 5.8S rDNA is too small and has the least variability and is therefore also inadequate for use in isolate differentiation.

Recently, several molecular approaches have been used in a number of reports to detect polymorphisms within the *Beauveria* species, e.g. RFLP analysis (Chew *et al.* 1997; Maurer *et al.* 1997; Pfeifer *et al.* 1993), rDNA sequence data comparisons (Destéfano *et al.* 2004; Rakotonirainy *et al.* 1991), and RAPD (Berretta *et al.* 1998; Castrillo and Brooks 1998; Urtz and Rice 1997). All these approaches undoubtedly helped to detect some polymorphism among isolates of the species and the genus but, in general, it was evident that the rRNA gene complex repeat region was rather conserved.

In this study, PCR-RFLP analysis of the 28S rRNA gene provided some levels of polymorphism indicating size differences in this region among the six *B. bassiana* isolates tested. The detection of group-I introns within the 28S region of *B. bassiana*, in combination with our observation of size enlargements of the relevant amplified region, led us to conduct an investigation into the nature of the recorded polymorphism in six *B. bassiana* isolates. Polymorphisms in the rDNA gene region have been attributed to small insertions/deletions, multiple duplications or, most often, to the presence of group-I introns. These introns are found in a diverse range of higher organisms, including fungi, protists and green algae, where they occur in the nuclear, mitochondrial and chloroplast genomes (Belshaw and Bensasson 2006; Bhattacharya *et al.* 2005; Haugen *et al.* 2005; Kupfer *et al.* 2004; Mattick 1994).

Considerably fewer cases are reported where group-I introns have been identified in the nuclear LSU rDNA genes, e.g. *B. brongniartii* (Neuvéglise *et al.* 1994, 1997). In many cases, the introns appear in a limited number of discrete DNA sequence positions, as reported for nuclear SSU rDNA genes (Gargas *et al.* 1995) or LSU rDNA genes (Neuvéglise *et al.* 1997). The distribution of group-I introns is, in general, extremely irregular, with introns being optional, i.e. present in some isolates and absent from others (Haugen *et al.* 2005). In an attempt to explain the nature of LSU rRNA gene size polymorphism of certain *B. bassiana* isolates, we analyzed their PCR products at the 3′ end of this gene region and discovered four group-I introns. The possible mobility of these introns as a source of molecular genetic variation should be discussed in future studies.

Although it is not the only tool for detecting absolute genetic relatedness, the PCR-RFLP technique can be used to get a rough estimate of the genetic relatedness of groups of *B. bassiana* isolates. Recent papers have used microsatellite and minisatellite markers to uncover both inter- and intraspecific variation within *Beauveria* (Enkerli *et al.* 2004). Specifically, Coates *et al.* (2002c) identified one polymorphic minisatellite locus, and Rehner and Buckley (2003) characterized eight highly polymorphic microsatellite loci for *B. bassiana*. In addition to suggesting relatedness, the markers will also be useful for conducting field tests to determine the impact of various isolates regarding the mortality and infection of insects. For example, Takatsuka (2007) developed molecular markers based on a sequence-characterized amplified region (SCAR) to monitor the presence of the *B. bassiana* F-263 strain, which is used to control the Japanese pine sawyer, *Monochamus alternatus* Hope.

We describe a robust method, PCR-RFLP, for the identification of *B. bassiana* isolates. However, in this study, two isolates were found to have similar profiles; this should not lead to an underestimation of the potential of this method because some isolates collected from the same or different locations could be clones and, therefore, might have been inadvertently replicated. For instance, the two Canadian isolates (*ARSEF2991* and *DAOM216540*), which had similar PCR-RFLP profiles, were isolated from *Leptinotarsa decemlineata* (Say) [Coleoptera: Chrysomelidae] and *Reticulitermes flavipes* (Koll.) [Isoptera: Rhinotermitidae] in Quebec and Ontario, respectively. This highlights the need for caution: exotic and native isolates should not automatically be assumed to possess different types of profile. Future studies should consider a larger sample number of *B. bassiana* isolates from different host and geographical origins to examine the genetic diversity between them.

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