Discrimination of Japanese isolates of *Beauveria brongniartii* (Deuteromycotina: Hyphomycetes) by RFLP of the rDNA-ITS regions

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(Received 17 March 2003; Accepted 19 July 2003)

Abstract

Isolates of the entomopathogenic fungus *Beauveria brongniartii* differ in the shape of the conidia and the amount of red pigment production, which are associated with the host insects they were obtained from. We investigated intraspecific polymorphisms among 38 isolates of *B. brongniartii* by using restriction fragment length polymorphisms (RFLP) of the internal transcribed spacer regions (ITS1 and ITS2), 5.8S rDNA, and small portions of the 18S and 28S rDNAs. Based on the RFLP patterns, the isolates were divided into two distinct groups. One group was composed mainly of isolates from longicorn beetles (Coleoptera: Cerambycidae), whereas the other group mainly contained isolates from scarab beetles (Coleoptera: Scarabaeidae). The DNA sequences of the amplified regions showed high homology between the groups, with only 7 substitutions in 852 nucleotides, and just 1 of the 7 substitutions accounted for the difference in the RFLP patterns between the two groups. Furthermore, RFLP successfully distinguished all of the *B. brongniartii* isolates from the 5 tested isolates of *B. bassiana* and the 1 of *B. amorpha*.

Key words: *Beauveria brongniartii*; RFLP; rDNA-ITS region; longicorn beetle; scarab beetle

INTRODUCTION

The fungus *Beauveria brongniartii* is mainly used to control a pest, *Melolontha melolontha* (Coleoptera: Scarabaeidae), inhabiting the soil in Europe, (Gillespie, 1988). In Japan, some strains of *B. brongniartii* isolated from the yellowspotted longicorn beetle, *Psacotha hilaris* (Coleoptera: Cerambycidae), have been used to control a mulberry and fig tree pest, *P. hilaris*, and the whitespotted longicorn beetle, a citrus tree pest, *Anoplophora malasiaca*. Use of the *B. brongniartii* strains in mulberry fields is allowed because of its low pathogenicity to the silkworm *Bombyx mori* (Kawakami, 1978). Some characteristics of *B. brongniartii* isolates differ depending on whether they were obtained from longicorn beetles or scarab beetles. For example, the shape of conidia of isolates from longicorn beetles are ovoid, whereas those from scarab beetles are long-ovoid (Kawakami, 1978; Samson et al., 1988). Isolates from scarab beetles generally produce red pigment in culture media, but those from longicorn beetles

rarely produce pigment (Kawakami, 1978).

The internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA (rDNA) repeat units evolve rapidly and may vary among species within a genus or among populations (White et al., 1990). Polymerase chain reaction (PCR) amplification has been used to detect polymorphisms in the ITS regions of fungi (Curran et al., 1994; Feibelman et al., 1994; Henrion et al., 1994; Matsumoto et al., 2000; Coats et al., 2002). Neuvéglise et al. (1994) showed restriction fragment length polymorphisms (RFLP) in the rDNA-ITS regions of isolates of *B. brongniartii* from different geographical and biological origins. DNA sequence data also confirmed the intraspecific genetic variability among the *B. brongniartii* strains.

In this study, we characterized 38 isolates of *B. brongniartii* from various insect hosts, including longicorn beetles and scarab beetles, together with 5 isolates of *B. bassiana* and 1 isolate of *B. amorpha*, on the basis of PCR-RFLP analysis.

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MATERIALS AND METHODS

Fungal isolates and culture conditions. The *Beauveria* isolates used in this study were from the collection maintained at the National Institute of

Agrobiological Sciences and Forestry and Forest Products Research Institute, both in Tsukuba, Ibaraki, Japan. Most of the isolates were collected in Japan, covering the mainland from Hokkaido to Kyushu districts. One isolate was from China

Table 1. List of *Beauveria* spp. isolates studied

Species	Isolate ^a	Source of isolate	Family of host ^b	Geographical origin	Year of isolation
<i>B. brongniartii</i>	614	<i>Psacotheta hilaris</i>	C	Gifu	1976
	677	<i>Psacotheta hilaris</i>	C	Gifu	1977
	678	<i>Psacotheta hilaris</i>	C	Gifu	1977
	769	<i>Psacotheta hilaris</i>	C	Yamanashi	1978
	782	<i>Psacotheta hilaris</i>	C	Gunma	1978
	793L	<i>Psacotheta hilaris</i>	C	Kanagawa	1978
	853	<i>Psacotheta hilaris</i>	C	Nagano	1980
	854	<i>Psacotheta hilaris</i>	C	Nagano	1980
	858	<i>Psacotheta hilaris</i>	C	Nagano	1980
	864	<i>Psacotheta hilaris</i>	C	Nagano	1980
	867	<i>Psacotheta hilaris</i>	C	Ibaraki	1980
	876	<i>Psacotheta hilaris</i>	C	Gunma	1980
	900	<i>Psacotheta hilaris</i>	C	Tottori	1981
	914	<i>Psacotheta hilaris</i>	C	Yamanashi	1982
	1002	<i>Psacotheta hilaris</i>	C	Kumamoto	1986
	B921	<i>Psacotheta hilaris</i>	C	Ibaraki	1992
	672	Cerambycidae	C	Kanagawa	1977
	676	<i>Apriona japonica</i>	C	Gunma	1977
	860	<i>Apriona japonica</i>	C	Ibaraki	1980
	774	Soil	—	Kanagawa	1978
	995	<i>Pterolophia annulata</i>	C	Kumamoto	1986
	1024	<i>Anoplophora malasiaca</i>	C	Nagasaki	1987
	B931	<i>Mesosa longipennis</i>	C	Ibaraki	1993
	*F237	<i>Moechotypa diphyis</i>	C	Nagasaki	1978
	*F402	<i>Mesosa longipennis</i>	C	Unknown, Japan	Unknown
	*F666	<i>Uraecha bimaculata</i>	C	Miyagi	1988
	*F1118	<i>Holotrichia parallela</i>	S	Cangzhou, China	1994
	*F77	<i>Mimela costata</i>	S	Nagano	1976
	*F614	Soil	—	Ibaraki	Unknown
	*F621	<i>Anomala cuprea</i>	S	Iwate	1987
	*F638-1	<i>Anomala cuprea</i>	S	Unknown, Japan	1988
	*F638-2	<i>Anomala cuprea</i>	S	Unknown, Japan	1988
	*F876-1	<i>Mimela testaceipes</i>	S	Hokkaido	1990
	*F876-2	<i>Mimela testaceipes</i>	S	Hokkaido	1990
	*F1003-1	<i>Mimela testaceipes</i>	S	Yamagata	Unknown
	*F1003-2	<i>Mimela testaceipes</i>	S	Yamagata	Unknown
	*F1120	<i>Popillia japonica</i>	S	Hokkaido	1994
	*F389	Unknown	—	Nagasaki	1978
<i>B. bassiana</i>	32	<i>Bombyx mori</i>	B	Kanagawa	1960
	*F263	<i>Monochamus alenatus</i>	C	Unknown, Japan	Unknown
	*F760	<i>Bombyx mori</i>	B	Unknown, Japan	Unknown
	1018	<i>Psacotheta hilaris</i>	C	Gunma	1987
	1022	<i>Episomus mundus</i>	Cu	Kagoshima	1988
<i>B. amorphia</i>	*F953	Scarabaeidae	S	Tokyo	1991

^a Isolates prefixed “*F” indicate those maintained at the Forestry and Forest Products Research Institute (Tsukuba, Ibaraki, Japan). Underlined isolates were used in nucleotide sequence studies.

^b C, Cerambycidae; S, Scarabaeidae; B, Bombycidae; Cu, Curculionidae.

(Table 1). The conidia of each isolate were cultured in SMY medium (4% maltose, 1% peptone, and 1% yeast extract) on a shaker (120 rpm) at 25°C for 3 to 5 days.

DNA extraction. The cultured mycelia were harvested, washed with distilled water by filtration, and freeze-dried. Approximately 1 g of dried sample was ground in liquid nitrogen and then resuspended in 16 ml of buffer (100 mM citric acid, 100 mM phosphoric acid [pH 6.0], and 100 mM EDTA). Then 2 ml of 10% SDS was added, the mixture was placed on ice for 15 min, 4.5 ml 5 M NaCl was added, and the mixture was stored overnight at 4°C. After centrifugation at $10,000\times g$ for 40 min, the supernatant was transferred to a fresh tube, and a 0.6-volume of isopropanol was added to precipitate the DNA. The sample was centrifuged at $10,000\times g$ for 10 min, and the pellet was washed with 70% ethanol, dried, and resuspended in 5 ml TE-sarcosinate buffer (20 mM Tris HCl [pH 8.0], 0.25 mM EDTA, and 0.4% sodium N-lauroyl sarcosinate). Then 5 g CsCl and 285 μ l ethidium bromide (10 mg/ml) were added, and the sample was centrifuged at $300,000\times g$ for 18 h. The DNA band was recovered and, after several washes with n-butanol, 3 volumes of TE buffer were added. The DNA was precipitated with 2 volumes of ethanol and was recovered by centrifugation at $5,500\times g$ for 10 min. The pellet was washed, dried, and resuspended in TE buffer.

PCR amplification. The region of the ribosomal repeat, including a small portion of 18S rDNA, ITS 1, 5.8S rDNA, ITS2, and a small portion of the 28S rDNA, was amplified by PCR. The primers used, PN3: 5'-CCGTTGGTGAACCAGCGGAGGGATC-3' and PN16: 5'-TCCCTTTCAACAATTCACG-3', were described by Neuvéglise et al. (1994). PCR reactions were carried out in a 100- μ l volume containing 200 μ M of each dNTP, 100 pmol of each primer, 2.5 U *Taq* DNA polymerase (Takara Bio-medicals, Japan), and 0.1 μ g of DNA template. Cycling parameters consisted of an initial denaturation at 95°C for 3 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min, by using a thermal cycler (PC800, Astec, Japan).

RFLP analysis. The amplified DNA was digested with the 4-base recognition restriction enzymes *AluI*, *HaeIII*, *MspI*, *HhaI* (Toyobo Biochemicals, Japan), *MboI*, and *AccII* (Takara) according

to the manufacturer's instructions. Digested fragments were separated on 3% agarose (NuSieve 3:1 agarose, FMC BioProducts, Maryland, USA) by gel electrophoresis in 0.5 $\times$ TBE buffer at 50 V, stained with ethidium bromide, and photographed under UV light. A 100-bp DNA ladder (Takara) was used as size markers.

DNA sequencing. The sequences of the ITS regions of 4 isolates—F402 and F638-1 (*B. brongniartii*), F760 (*B. bassiana*), and F953 (*B. amorpha*)—were analyzed. To increase the cloning efficiency of the blunt-ended DNA, fragments polished by using a PCR Polishing Kit (Stratagene, California, USA) were cloned into pCR-ScriptTM SK(+) (Stratagene). The cloned plasmid DNA was extracted by using the alkali-SDS lysis method. Sequencing reactions were carried out by dideoxy chain termination using an AutoReadTM Sequencing Kit (Amersham Pharmacia, Buckinghamshire, UK) with primers PN16, PN12 (5'-CAATGTGCGTTCAAAGATTCGAT-3'), and PN10 (5'-TCCGCTTATTGATATGCTTAAG-3'), as described by Neuvéglise et al. (1994). Sequence information was obtained by using an ALF DNA Sequencer II (Amersham Pharmacia). Sequences of the 4 isolates were manually aligned for comparison.

RESULTS

Separation of species and isolates

Amplification of the ITS regions including the 5.8S rDNA from all isolates of *B. brongniartii*, *B. bassiana*, and *B. amorpha* resulted in approximately 950-bp products. Polymorphisms among the amplified DNAs were detected by digestion with *AluI*, *HaeIII*, *MspI*, *HhaI*, and *AccII* (Table 2); no variations were observed in the restriction pattern with *MboI*. The restriction patterns with *AluI*, *HaeIII*, and *HhaI* discriminated all isolates of *B. bassiana* from all isolates of *B. brongniartii* and *B. amorpha*. Similarly, the *B. amorpha* isolate was discriminated from all isolates of the other *Beauveria* species by the restriction patterns obtained with *MspI*. The restriction patterns from *AccII* discriminated all isolates of *Beauveria* species from each other (Fig. 1) and grouped most isolates of *B. brongniartii* into isolates from longicorn beetles and those from scarab beetles (Table 3). According to the RFLP patterns, F1118 (isolated from scarab beetle in China) was categorized with isolates from

Fig. 1. Restriction patterns of rDNA-ITS regions of *B. brongniartii*, *B. bassiana*, and *B. amorpha* digested with *AccII*. Lanes 1–4, *B. brongniartii* isolated from Scarabaeidae (1: F77, 2: F876-1, 3: F638-1, 4: F1120); 5–8, *B. brongniartii* isolated from Cerambycidae (5: 867, 6: 1024, 7: F237, 8: F666); 9, *B. amorpha* (F953) and 10–12, *B. bassiana* (10: F263, 11: 32, 12: F760). Lane M are 100 bp-ladder DNA size markers.

longicorn beetle, and 774 and F614, which were obtained from soil, belonged to distinct RFLP pattern groups of *B. brongniartii*.

Sequence of rDNA-ITS region

We then sequenced the rDNA-ITS regions (about 880 nucleotides) of the 4 isolates of *Beauveria* spp. that are distinguishable from each other by RFLP analysis. (Fig. 2). Each nucleotide sequence dataset for F402, F638-1, F760, and F953 was deposited in the DNA Databank of Japan under the accession numbers AB100036, AB100037, AB100038, and AB100039, respectively. All isolates revealed similar sequences, and the differences were concentrated within ITS regions. The difference between *B. brongniartii* F402 (isolated from *M. longipennis*) and F638-1 (isolated from *A. cuprea*) was 7 of the 852 nu-

Table 2. Sizes (bp) of DNA fragments obtained after endonuclease digestion of the ITS regions of rDNA from *Beauveria* spp. isolates

Restriction pattern no.	Sizes of fragments with each enzyme					
	<i>MboI</i>	<i>AluI</i>	<i>HhaI</i>	<i>MspI</i>	<i>HaeIII</i>	<i>AccII</i>
1	660	630	545	465	280	520
	190	145	320	265	200	185
	60	100	75	95	155	145
		65		60	120	105
				25	90	
				15	55	
					20	
					10	
2		470	405	290	290	480
		160	320	265	200	330
		145	140	175	155	105
		100	75	95	120	45
		65		60	90	
				25	55	
				15	20	
3					280	480
					190	185
					165	145
					120	105
					90	45
					55	
					20	
4					10	
						520
						330
						105

Table 3. Restriction pattern groups of *Beauveria* spp. isolates

Restriction pattern groups	Restriction patterns with each enzyme ^a						Isolates of the same restriction pattern group
	<i>Mbo</i> I	<i>Alu</i> I	<i>Hha</i> I	<i>Msp</i> I	<i>Hae</i> III	<i>Acc</i> II	
Group A	1	1	1	1	1	1	<i>B. brongniartii</i> from Cerambycidae: 614, 677, 678, 769, 782, 793L, 853, 854, 858, 864, 867, 876, 900, 914, 1002, B921, 672, 676, 860, 995, 1024, B931, F237, F402, and F666 <i>B. brongniartii</i> from Scarabaeidae: F1118 <i>B. brongniartii</i> from soil: 774
Group B	1	1	1	1	2	2	<i>B. brongniartii</i> from Scarabaeidae: F77, F621, F638-1, F638-2, F876-1, F876-2, F1003-1, F1003-2, F1120, and F389 <i>B. brongniartii</i> from soil: F614
Group C	1	2	2	1	3	3	<i>B. bassiana</i> : 32, F263, F760, 1018, and 1022
Group D	1	1	1	2	1	4	<i>B. amorphae</i> : F953

^aNo. of the restriction pattern type refers to that in Table 2.

cleotides sequenced (0.82%). A single base substitution (the 223rd base from the beginning of the sequences in Fig. 2), which changes a recognition site of *Acc*II, was responsible for the discrimination between the longicorn beetle isolates and the scarab beetle isolates.

DISCUSSION

We used RFLP analysis to reveal variations among rDNA amplified fragments of *B. brongniartii*. The patterns of the 38 isolates investigated allocated them into 2 groups correlated with their host insects: one group comprised isolates from the Cerambycidae, and the other, those from the Scarabaeidae. Nucleotide differences between the 2 groups accounted for only 0.82% of the region sequenced, and a single base change was responsible for the RFLP grouping. The identities of the resulting RFLP bands are unknown, so their suitability for determining the relationship among isolates is generally only assumed (Maurer et al., 1997). Thus far, many fungi have been analyzed by using RFLP of the ITS region of the rDNA, because this method is simple and useful for examining intraspecific polymorphisms and for distinguishing morphologically similar species (Matsumoto et al., 2000). For example, Neuvéglise et al. (1994) investigated 28 *B. brongniartii* strains isolated from coleopterans and lepidopterans collected mainly from Europe and Madagascar, and divided the

fungi into 6 RFLP-associated groups. All isolates from the white grub *Hoplochelus marginalis* (Coleoptera) were in Group 1, which also contained 1 isolate from another source. Differences in the target DNA sequence between group 1 and the other groups ranged from 2.6% to 9.1%—all exceeding the value we obtained. In the study, geographical origins of fungal isolates were not correlated with the RFLP groups. We similarly observed no polymorphisms associated with geographical origins of the Japanese isolates. The isolate list of Neuvéglise et al. (1994) contained 2 Japanese strains isolated from *Mimela costata* (Coleoptera: Scarabaeidae) and *Pissodes nitidus* (Coleoptera: Curculionidae). These strains were assigned to RFLP group 2, which consisted of strains from scarab beetles and weevils (Coleoptera: Curculionidae) mainly from Europe. When we compared the sequences of the rDNA-amplified fragments of the isolate Bt33 belonging to group 2 with that of our isolate F638-1 from scarab beetle, 17 of the 805 nucleotides differed (2.1%); this value is higher than the difference between our 2 RFLP groups. Furthermore, the assumed band pattern of group 2 isolates digested with *Acc*II was different from that of our scarab beetle group. In our study, F1118 from scarab beetles in China showed atypical RFLP patterns, but it is unclear whether this exception was due to the geographical origin of the isolate. Further investigation with more isolates from Japan and neighboring countries is needed to

Fig. 2. Sequence alignments of ITS regions, 5.8S rDNA, and the 5' end of the 28S rDNA of *Beauveria* spp. isolates: F402, *B. brongniartii* isolated from *M. longipennis*; F638-1, *B. brongniartii* isolated from *A. cuprea*; Bt33, *B. brongniartii* isolated from *Melolontha melolontha* (Coleoptera: Scarabaeidae) in France (Neuvéglise et al., 1994); F760, *B. bassiana* isolated from *B. mori*; F953, *B. amorpha* isolated from a scarab beetle. Only the nucleotides differing from those of sequences of F402 are shown. Identities, deletions, and undetermined nucleotides are noted by dots, stars and n, respectively. Nucleotides of ITS1 and ITS2 are shown in italics.

analyze the genetic divergence of *B. brongniartii* with regard to geographical origin.

In general, fungal isolates are more virulent to the original host than to other hosts (Tanada and Kaya, 1993). In a bioassay, 1.8×10^4 conidia/ml of the 782 isolate from *P. hilaris* (longicorn beetle) killed all *P. hilaris* tested, whereas 1.4×10^8 conidia/ml of F1120 from *Popillia japonica* (scarab beetle) did not kill *P. hilaris* (data not shown). If the RFLP patterns reflecting the host insect taxa can serve as an index of the virulence of the isolates, this information will be useful in selecting

isolates for effective pest control.

In addition to the differentiation of isolates within *B. brongniartii*, our RFLP analysis clearly differentiated *B. bassiana*, *B. amorpha*, and *B. brongniartii*, which are morphologically similar species. Several attempts have been made to differentiate strains of *B. bassiana* (St. Leger et al., 1992; Hegedus and Khachatourians, 1996). Coats et al. (2002) identified polymorphism in the rDNA and ITS regions of *B. bassiana* isolates by using PCR-RFLP and DNA sequencing, but found no noteworthy correlation between the RFLP and host

range. Likewise, we could not detect RFLP in similar DNA regions of *B. bassiana* investigated by Coats et al. (2002) despite the different original hosts. Using RFLP and random amplified polymorphic DNA (RAPD) techniques on total genomic DNA, Maurer et al. (1997) showed clear relationships between the genotype of *B. bassiana* and various defined host species. Therefore, analysis of total genomic DNA can be useful when examining the polymorphism of *B. brongniartii* isolates, for example in relation to geographical origins. Although *B. brongniartii* is important as a pest control agent, knowledge of its geographical distribution, pathogenicity, genetic diversity, and other attributes is more limited than for those of *B. bassiana*, especially for isolates from Asian countries. For the development of microbial control by *B. brongniartii*, it is indispensable to monitor the released strain and distinguish it from the other isolates in target insects, nontarget insects, and the environment. Therefore, additional genetic experiments are necessary to identify individual isolates of *B. brongniartii*.

ACKNOWLEDGEMENTS

We are grateful to Dr. M. R. Goldsmith for her critical reading of this manuscript. We also thank Dr. H. Tamura for his advice.

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