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Abstract
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Beauveria bassiana haplotype determination based on nuclear rDNA internal transcribed spacer PCR–RFLP

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DNA sequence alignment of the nuclear 5.8S ribosomal RNA (rRNA) gene and internal transcribed spacers (ITS) from *Beauveria bassiana* demonstrated that 6.62% sequence variation existed between nine isolates. A higher level of mutation was observed within the ITS regions, where 8.39% divergence occurred. Polymerase chain reaction restriction fragment length polymorphism, PCR–RFLP, and DNA sequence alignment of the ITS1 and ITS2 regions identified seven polymorphic restriction endonuclease sites, *Alu* I, *Hha* I, *Hinf* I, *Sin* I, *Tru* 9AI and two *Tha* I restriction sites. The allelic frequency of each genetic marker was determined from 96 isolates. PCR–RFLP defined 24 *B. bassiana* genotypes within the sample set, from which eight phylogenetic clusters were predicted to exist. AMOVA and Fst (θ) indicated that no significant correlation existed between *B. bassiana* haplotype and insect host range as defined by insect order from which each isolate was derived.

INTRODUCTION

The entomopathogenic fungus *Beauveria bassiana* (*Ascomycota*, *Hypocreales*) has shown use within insect biocontrol regimes for suppression of the crop pests *Ostrinia nubilalis* (Bing & Lewis 1991, 1992), *Diabrotica unidecimpunctata* (Krueger & Roberts 1997) and *Diabrotica virgifera virgifera* (Mulock & Chandler 2000). Success within biological control practices has spearheaded an interest in the documentation of molecular differences, and the genetic identification ('fingerprinting') of particular strains. Variable morphological characteristics, such as conidia size and shape, have been defined between *B. bassiana* (Brady 1979a), and *Beauveria brongniartii* (Brady 1979b). The presence of phenotypic diversity with respect to host specificity implies that genetic differences are present within the species (St Leger *et al.* 1992). Mutation detection techniques have proven useful in the differentiation of species of *Beauveria* (Neuveglise *et al.* 1994, Glare & Inwood 1998), yet, insufficient data exists regarding molecular differences among *B. bassiana* isolates.

Nuclear ribosomal RNA gene sequences are well described, with degrees of conservation observed throughout evolution (White *et al.* 1990) that have allowed for phylogenetic comparison among species (Guadet *et al.* 1989, Rakotonirainy *et al.* 1994). Defined regions within the initial transcripts have demonstrated differential rates of change at the nucleotide level, and intraspecies comparison of fungi has shown a preponderance of sequence polymorphism within two internal transcribed spacer (ITS) regions (Neuveglise *et al.* 1994, Buscot *et al.* 1996, Chew, Strongman & MacKay 1997). A moderate level of mutation was reported in the first internal transcribed spacers (ITS1) of *B. bassiana*, where two DNA sequences showed 91% similarity (Shih *et al.* 1995). The second *B. bassiana* internal transcribed spacer (ITS2) sequence was determined to be invariant, and the 5.8S rRNA gene showed 98% similarity (Shih *et al.* 1995). Additional nucleotide data have been provided from *B. bassiana* rRNA coding and ITS regions (Rakotonirainy *et al.* 1991, 1994, Neuveglise *et al.* 1994), but no intraspecies comparisons were made.

Mutation detection within DNA sequences can be facilitated by several means. Restriction endonuclease cleavage of PCR-amplified DNA fragments, PCR–RFLP, has proven useful in the intraspecies differentiation of *B. bassiana* (Glare & Inwood 1998) and *B. brongniartii* (Neuveglise *et al.* 1994). Based on a DNA fragment that contained the ITS1, 5.8S rRNA gene, and ITS2, Glare & Inwood (1998) reported five RFLPs that separated *Beauveria* species into two groups. The first contained *B. bassiana* and *B. brong-*

Table 1. Origin, subpopulation designation, and haplotype of 96 *Beauveria bassiana* isolates as determined by PCR–RFLP of the rRNA ITS regions 1 and 2. Four subpopulations, *Lepidoptera* (Lep.), *Coleoptera* (Col.), *Hymenoptera/Diptera* (HD), *Hemiptera/Homoptera* and others (HHO), based on insect order(s) from which the isolate was originally recovered (Humber 1992), are separated by horizontal spaces. Restriction fragments are labeled 1 or 0, as described in Table 2. Hi = *Hinf* I, Tr = *Tru* 9AI, Th = *Tha* I, Al = *Alu* I, Hh = *Hha* I, and Si = *Sin* I.

				ITS1			ITS2				
Isolate	Host	Geographic origin	Subpop.	Hi	Tr	Th	Al	Hh	Si	Th	Haplotype
Bb 151	*Cydia pomonella* (Lepidoptera; Torticidae)	France	Lep.	1	0	1	1	0	0	1	G
Bb 501	*Ostrinia nubilalis* (Lepidoptera; Crambidae)	PR China	Lep.	1	0	1	1	1	1	1	A
Bb 502	*Ostrinia nubilalis* (Lepidoptera; Crambidae)	PR China	Lep.	1	0	1	1	1	1	1	A
Bb 533	*Ostrinia nubilalis* (Lepidoptera; Crambidae)	PR China	Lep.	1	0	1	1	1	1	1	A
Bb 652	*O. nubilalis* (Lepidoptera; Pyralidae)	PR China, Beijing	Lep.	1	0	1	1	1	1	1	A
Bb 959	*Spordoptera frugiperda* (Lepidoptera; Noctuidae)	Brazil, Londrina, Parana	Lep.	1	0	1	1	1	0	1	C
Bb 1001	*Dioryctria sylvestrella* (Lepidoptera; Pyralidae)	Japan, Hachiojo, Tokyo	Lep.	1	0	1	1	1	1	1	A
Bb 1022	Maize: *O. nubilalis* infective (Lepidoptera; Crambidae)	USA, Champaign, IL	Lep.	1	0	1	1	1	1	1	A
Bb 1038	*Ostrinia nubilalis* (Lepidoptera; Crambidae)	USA, Tulley, NY	Lep.	1	0	0	0	1	1	1	N
Bb 1113	*Galleria mellonella* (Lepidoptera; Pyralidae)	Italy, Bologna	Lep.	1	0	0	1	0	0	1	L
Bb 1121	*Ostrinia nubilalis* (Lepidoptera; Crambidae)	Italy, Padus, Venetia	Lep.	1	0	0	1	0	0	1	L
Bb 1149	*Heliothis aramigera* (Lepidoptera; Noctuidae)	Spain, Cordoba	Lep.	1	0	1	1	1	1	1	A
Bb 1314	*Heliothis virescens* (Lepidoptera; Noctuidae)	France, La Miniere	Lep.	1	0	1	1	1	1	1	A
Bb 1564	*Hyphantria cunea* (Lepidoptera; Actiidiae)	Italy, Cade (Reggio)	Lep.	1	0	1	1	1	1	1	L
Bb 2297	*Brassolis* larva (Lepidoptera; Brassolidae)	Columbia, Cumaral, Meta	Lep.	1	0	1	1	1	0	1	C
Bb 2570	*Lymantria dispar* (Lepidoptera; Lymantriidae)	USA, Dover, DE	Lep.	1	0	1	1	1	1	1	A
Bb 2619	*Brassolis saphorae* (Lepidoptera; Brassolidae)	Brazil, Moju, Para	Lep.	1	0	0	1	0	0	1	L
Bb 2629	*Diatraea saccharalis* (Lepidoptera; Pyralidae)	Brazil, Usina Salgado, Upojuca	Lep.	1	0	0	1	0	0	1	L
Bb 2737	*Bombyx mori* (Lepidoptera; Bombycidae)	Phillipines, La Trinidad, Benguet	Lep.	1	0	1	1	1	1	1	A
Bb 2976	*Ostrinia nubilalis* (Lepidoptera; Crambidae)	USA, Centre Co., PA	Lep.	1	0	0	0	0	0	1	P
Bb 3113	Soil: *O. nubilalis* infective (Lepidoptera; Crambidae)	USA, Ames, IA	Lep.	1	0	1	1	1	1	1	A
Bb 3543	*Galleria mellonella* (Lepidoptera; Pyrallidae)	USA, Warren, VT	Lep.	1	0	1	0	0	0	0	I
EL03	*Ostrinia nubilalis* (Lepidoptera; Cramblidae)	USA, Ames, IA	Lep.	1	0	1	1	1	1	1	A
EL12	*Ostrinia nubilalis* (Lepidoptera; Cramblidae)	USA, Ames, IA	Lep.	0	1	0	0	0	0	0	X
EL13	*Ostrinia nubilalis* (Lepidoptera; Cramblidae)	USA, Ames, IA	Lep.	1	0	1	1	1	1	1	A
EL14	*Ostrinia nubilalis* (Lepidoptera; Cramblidae)	USA, Ames, IA	Lep.	1	0	1	1	1	1	1	A
EL15	*Ostrinia nubilalis* (Lepidoptera; Cramblidae)	USA, Ames, IA	Lep.	1	0	1	1	1	1	1	A
EL16	*Ostrinia nubilalis* (Lepidoptera; Cramblidae)	USA, Ames, IA	Lep.	0	1	1	1	0	1	1	R
EL17	*Ostrinia nubilalis* (Lepidoptera; Cramblidae)	USA, Ames, IA	Lep.	1	0	1	1	0	1	1	E
EL18	*Ostrinia nubilalis* (Lepidoptera; Cramblidae)	USA, Ames, IA	Lep.	0	1	1	1	0	1	1	R

Table 1. (*cont.*)

Isolate	Host	Geographic origin	Subpop.	ITS1			ITS2				Haplotype
				Hi	Tr	Th	Al	Hh	Si	Th	
EL19	*Ostrinia nubilalis* (Lepidoptera; Cramblidae)	USA, Ames, IA	Lep.	0	1	1	1	0	1	1	R
Bb 150	Unknown (Coleoptera; Elateridae)	France	Col.	1	0	0	1	0	0	0	M
Bb 201	*Diabrotica undecimpunctata* (Coleoptera; Chrysomelidae)	USA, Corvallis, OR	Col.	0	1	1	1	0	0	1	S
Bb 318	Unknown (Coleoptera; Cerambycidae)	USA, RI	Col.	1	0	1	1	0	1	1	F
Bb 721	*Diabrotica* sp. (Coleoptera; Cerambycidae)	Columbia, CIAT, Valle de Cauca	Col.	1	0	1	1	1	0	1	C
Bb 758	*Diabrotica* sp. (Coleoptera; Chysomelidae)	Brazil, CNPAF, Goiania, Goias	Col.	1	0	0	1	0	0	1	L
Bb 783	Unknown (Coleoptera; Tenebrionidae)	Brazil	Col.	1	0	1	0	0	0	1	J
Bb 784	Unknown (Coleoptera; Elateridae)	Brazil, Novo Oriente, Ceara	Col.	1	0	0	1	0	0	0	M
Bb 809	*Diabrotica paranoense* (Coleoptera; Chysomelidae)	Brazil, Goiania, Goias	Col.	1	0	0	1	0	0	1	L
Bb 843	*Chalcodermus* sp. (Coleoptera; Curculionidae)	Brazil, Ponta Pora, Mato Grosso	Col.	1	0	0	1	0	0	0	M
Bb 928	*Diabrotica speciosa* (Coleoptera; Chysomelidae)	Brazil, CNPAF, Goiania, Goias	Col.	1	0	0	1	0	0	1	L
Bb 937	*Diabrotica paranoense* (Coleoptera; Chysomelidae)	Brazil, Ponta Pora, Mato Grosso	Col.	1	0	0	1	0	0	1	L
Bb 938	*Diabrotica paranoense* (Coleoptera; Chysomelidae)	Brazil, Ponta Pora, Mato Grosso	Col.	1	0	0	1	0	0	1	L
Bb 1017	*Dendroctonus rufipennis* (Coleoptera; Scolytidae)	Canada, Hixon, British Colombia	Col.	0	1	0	0	0	0	1	W
Bb 1060	*Diabrotica speciosa* (Coleoptera; Chysomelidae)	Brazil, Londrina, Parana	Col.	1	0	0	1	0	0	1	L
Bb 1155	*Sitona discoideus* (Coleoptera; Chysomelidae)	Moracco, Jramma	Col.	1	0	0	1	0	0	1	L
Bb 1454	*Diabrotica speciosa* (Coleoptera; Chysomelidae)	Brazil, Cascavel, Parana	Col.	1	0	0	0	0	1	1	O
Bb 2010	*Cycloneda sanguinea* (Coleoptera; coccinellidae)	Argentina, Tucuman	Col.	1	0	0	1	0	0	1	L
Bb 2330	*Diabrotica virgifera* (Coleoptera; Chysomelidae)	Mexico, Zapotlanejo, Jalisco	Col.	0	1	1	0	1	0	1	T
Bb 2515	*Diabrotica speciosa* (Coleoptera; Chysomelidae)	Argentina, Blanco Pozo, Tucuman	Col.	1	0	0	1	0	0	1	L
Bb 2520	*Diabrotica speciosa* (Coleoptera; Chysomelidae)	Brazil, Londrina, Parana	Col.	1	0	0	1	0	0	1	L
Bb 2579	*Diabrotica barberi* (Coleoptera; Chysomelidae)	USA, Brookings, SD	Col.	1	0	1	1	1	0	1	C
Bb 2961	*Lagria vilosa* (Coleoptera; Lagriidae)	Brazil, Formosa, Goias	Col.	1	0	0	1	0	0	1	L
Bb 3037	*Anthonomus musculus* (Coleoptera; Curculionidae)	USA, Carver, MA	Col.	0	1	1	0	0	0	0	V
Bb 3112	Unknown (Coleoptera; Staphylinidae)	USA, Lincoln, NE	Col.	0	1	1	1	1	0	1	Q
Bb 3111	*Diabrotica virgifera* (Coleoptera; Chysomelidae)	USA, Mead, NE	Col.	0	1	1	1	1	0	1	Q
Bb 3369	*Diabrotica balteata* (Coleoptera; Chysomelidae)	Mexico, Colima	Col.	1	0	1	1	1	0	1	C
Bb 3386	Unknown (Coleoptera; Chysomelidae)	USA, Yakima, WA	Col.	1	0	0	1	0	0	1	L
Bb 3457	Unknown (Coleoptera; Languriidae)	Costa Rica, Turrialba, Cartago	Col.	1	0	0	0	0	0	1	Q
Bb 6715	*Diabrotica* sp. (Coleoptera; Chysomelidae)	USA, Piper City, IL	Col.	1	0	1	1	1	0	1	C
Bb 152	*Pamphilius betulae* (Hymenoptera; Pamphiliidae)	Commonwealth Independent States	HD	0	1	1	0	0	0	1	U
Bb 1468	Unknown (Hymenoptera; Apoidea)	Brazil, Sao Carlos, Santa Catarina	HD	1	0	1	1	0	0	0	H

Table 1. (*cont.*)

Isolate	Host	Geographic origin	Subpop.	ITS1			ITS2				Haplotype
				Hi	Tr	Th	Al	Hh	Si	Th	
Bb 338	*Neodiprion pini* (Hymenoptera; Diprionidae)	Unknown	HD	1	0	1	1	1	1	0	B
Bb 730	Unknown (Hymenoptera; Vespidae)	Brazil, Costa do Arapapa, Amazonas	HD	1	0	0	1	0	0	0	M
Bb 957	Unknown (Hymenoptera; Megachilidae)	Brazil, CNPAF, Goiania, Goias	HD	1	0	1	1	0	0	0	M
Bb 1960	Unknown (Hymenoptera; Formicidae)	Brazil, Ribeira do Pombal, Bahia	HD	1	0	0	1	0	0	1	L
Bb 1976	Unknown (Hymenoptera; Formicidae)	France, Saint Genys	HD	1	0	0	0	0	1	1	O
Bb 2545	Unknown (Hymenoptera; Sphecidae)	Mexico, San Pedro, Nuevo Leon	HD	1	0	0	1	1	0	1	K
Bb 2869	*B. cubensis* (Hymenoptera; Eurytomidae)	USA, FL	HD	1	0	1	1	0	0	0	H
Bb 1150	Unknown (Diptera; Tachinidae)	France, Senneville	HD	1	0	1	1	1	1	1	A
Bb 1151	*Delia radicum* (Diptera; Anthomyiidae)	Canada, L'Acadie, Quebec	HD	1	0	1	1	0	1	0	F
Bb 1630	*Calliphora* sp. (Diptera; Calliphoridae)	France, Sevres, Yvelines	HD	1	0	1	1	1	1	1	A
Bb 1866	*Delia antiqua* (Diptera)	France, La Miniere	HD	1	0	1	1	1	1	1	A
Bb 2953	*Musca domestica* (Diptera; Muscidae)	USA, Cayuga Co., NY	HD	1	0	0	0	0	0	1	P
Bb 300	*Nysius vinitor* (Hemiptera; Lygaeidae)	Australia, Tamworth, New S. Wales	HHO	1	0	1	1	0	1	0	F
Bb 320	*Mesovelia mulsanti* (Hemiptera; Mesoveliidae)	USA, Gainesville, FL	HHO	1	0	0	1	0	0	1	L
Bb 477	*Zulia entreriana* (Homoptera; Cercopidae)	Brazil, Mato Grosso do Sul	HHO	1	0	0	1	0	0	0	M
Bb 560	*Leptocoris* (Hemiptera; Rhopalidae)	Indonesia, Lambok Island	HHO	1	0	0	1	0	0	1	L
Bb 654	*Nilaparvata lugens* (Homoptera; Delphacidae)	PR China, Huhan, Hupei	HHO	1	0	1	1	1	1	0	B
Bb 708	*Nephotettix bipunctata cinctipeps* (Homoptera; Cicadellidae)	PR China	HHO	1	0	1	1	1	1	1	A
Bb 726	Unknown (Hemiptera; Pentatomidae)	USA, Butte, MT	HHO	1	0	1	1	1	0	1	C
Bb 737	Unknown (Hemiptera; Tingidae)	Brazil, CNPAF, Goiania, Goias	HHO	1	0	0	1	0	0	0	M
Bb 812	*Corythucha ciliata* (Hemiptera; Tingidae)	France, Avignon, Vaucluse	HHO	1	0	0	0	0	0	1	W
Bb 1973	Unknown (Hemiptera; Pentatomidae)	Austria, Burgenland	HHO	1	0	0	1	0	0	1	L
Bb 1988	*Nabis* sp. (Hemiptera; Nabidae)	Austria, Burgenland	HHO	1	0	0	1	0	0	1	L
Bb 2236	*Rhizoecus* sp. (Homoptera; Pseudococcidae)	Unknown	HHO	1	0	0	1	0	0	1	L
Bb 2544	Unknown (Homoptera; Cicadidae)	Mexico, San Pedro, Nuevo Leon	HHO	0	1	0	0	0	0	1	W
Bb 2861	*Diuraphis noxis* (Homoptera; Aphididae)	USA, Parma, ID	HHO	1	0	1	1	1	0	0	D
Bb 3167	*Diuraphis noxia* (Homoptera Aphididae)	Turkey, Sirkeil, Ankara	HHO	0	1	0	0	0	0	0	X
Bb 3312	Unknown (Homoptera; Membracidae)	Mexico, Cuauhtemoc, Colima	HHO	1	0	0	1	0	0	0	M
Bb 153	*Chrysopa* sp. (Neuroptera)	Commonwealth Independent States	HHO	0	1	0	0	0	0	0	X
Bb 3041	*Reticulitermes flavipes* (Isoptera; Rhinotermitidae)	Canada, Toronto, Ontario	HHO	1	0	1	1	1	0	1	C
Bb 3216	*Thrips calcaratus* (Thysanura; Thripidae)	USA, Rusk Co., WI	HHO	0	1	0	0	0	0	1	W

Table 1. (*cont.*)

Isolate	Host	Geographic origin	Subpop.	ITS1 Hi	ITS1 Tr	ITS1 Th	ITS2 Al	ITS2 Hh	ITS2 Si	ITS2 Th	Haplotype
Bb 356	Unknown (Orthoptera; Acrididae)	Australia, New South Wales	HHO	1	0	1	1	1	1	1	A
Bb 796	Unknown (Dermaptera)	Columbia, ICA, Villavicencio, Meta	HHO	1	0	0	1	0	0	0	M
Bb 1959	Unknown (Orthoptera; Acrididae)	Brazil, Ribeira do Pombal, Bahia	HHO	1	0	0	1	0	0	1	L

niartii, and the other *B. amorpha*, *B. caledonica*, and *B. vermiconia*. Of the ten enzymes used, *Mse* I, *Tha* I, and *Tsp* 509 were polymorphic within eleven *B. bassiana* isolates, but 100% linkage between *Mse* I and *Tsp* 509 mutations was shown to exist. Glare & Inwood (1998) also reported two genetically distinct subgroups of *B. bassiana* in New Zealand, that were hypothesized to represent native and introduced European genotypes. Sequence and PCR–RFLP analysis of the *B. brongniartii* ITS region from 28 isolates indicated that 0.70–14.67% and 1.80–16.67% sequence variation occurred within ITS1 and ITS2, respectively (Neuveglise *et al*. 1994). Seven polymorphic *B. brongniartii* restriction sites identified six unique genotypic groups within the species, and uniform *B. brongniartii* genotypes were described within all isolates derived from the white grub *Hoplochelus marginalis*. Within the same study Neuveglise *et al*. (1994) found that two different *B. bassiana* isolate ITS region sequences were 100% identical.

This report describes the characterization of molecular polymorphism among *B. bassiana* isolates. We wished to investigate the level of neutral nucleotide mutation within nuclear rRNA ITS regions of *B. bassiana*. In doing so we wanted to identify unique genetic types, and recognize the evolutionary relationships among isolates infecting various insects.

MATERIALS AND METHODS

Samples

In total, 96 isolates were received from a variety of sources (Table 1). Eighty-five *Beauveria bassiana* isolates with diverse geographical origin and pathogenic capacity were obtained from the ARSEF Collection of Entomopathogenic Fungi maintained by the US Department of Agriculture, Agricultural Research Service, Plant Protection Research Unit, U.S. Plant, Soil, and Nutrition Laboratory, Ithaca, NY (Humber 1992). Isolate Bb6715 was originally isolated from an adult western corn rootworm, *Diabrotica virgifera virgifera* (LeConte), and received from Barbra Mulock (USDA–ARS, Brookings, SD). Isolate Bb726 was obtained from Stephan Jaronski, Myotech Corporation, Butte, MT. Field isolates EL03 and EL12 to EL19 were derived from European corn borer larvae, *Ostrinia nubilalis*, and maintained at the USDA–ARS Corn Insects and Crop Genetics Research Unit (CICGRU), Ames, IA. Isolate Bb1022 was collected by the USDA–ARS CICGRU from a corn plant near Champaign, IL. Isolates were propagated on 10 cm Sauborauds dextrose agar plates at 30 °C. DNA extractions were performed in accordance to that described by Neuveglise *et al*. (1994). Resultant nucleic acid pellets were resuspended in sterile deionized water and stored at −20 ° prior to use.

DNA sequencing and alignment

Oligonucleotide primers, ITS4 and ITS5 (White *et al*. 1990) were used for PCR amplification of a 25 µl reaction that contained 2.5 mM $MgCl_2$, 150 µM dNTPs, 5.0 pmol of each primer, 0.425 U *Taq* polymerase (Promega, Madison, WI) and 50 ng of DNA template. The PTC-100 thermocycler (MJ Research, Watertown, MA) program included an initial denaturation at 94 ° for 3 min, then 40 cycles of 94 ° for 40 s, annealing at 55 ° for 50 s, and 72 ° extension for 50 s. Successful amplification was confirmed by running 5 µl from each reaction on a 2% agarose gel that contained 0.5 µg ml^{-1} ethidium bromide.

Template was prepared from 20-µl of the ITS4 and ITS5 primed PCR product amplified from isolates Bb153, Bb501, Bb726, Bb1022, Bb1149, Bb1155, Bb3113, and Bb3167. Purification was carried out using Qiaquick PCR Purification columns (Qiagen, Valencia, CA) according to directions of the manufacturer. Samples were quantified using UV absorbance at 260 nm on a Molecular Devices SpectraMAX Plus UV spectrophotometer (Molecular Devices, Sunnyvale, CA). Template DNA was submitted to the DNA Sequencing and Synthesis Facility at Iowa State University, Ames, IA at a concentration of 2.5 ng 100-bp^{-1} 1.0 μl^{-1}. Oligonucleotide primers ITS1F (5′-ATTACCGAGTTTTCAACTCCC-3′), and ITS2R (5′-ACCTGATTCGAGGTCAACGTTC-3′) were submitted at 5.0 pmol μl^{-1} for priming of DNA template in two separate sequencing reactions.

DNA sequence data from isolates Bb153, Bb501, Bb726, Bb1022, Bb1149, Bb1155, Bb3113, and Bb3167 was reconstructed from ABI Prism Sequencer 5010 data output of both primer reactions using Contig Express (Informax, San Francisco). Multiple sequence alignment of reconstructed isolate sequences took place using AlignX software (Informax).

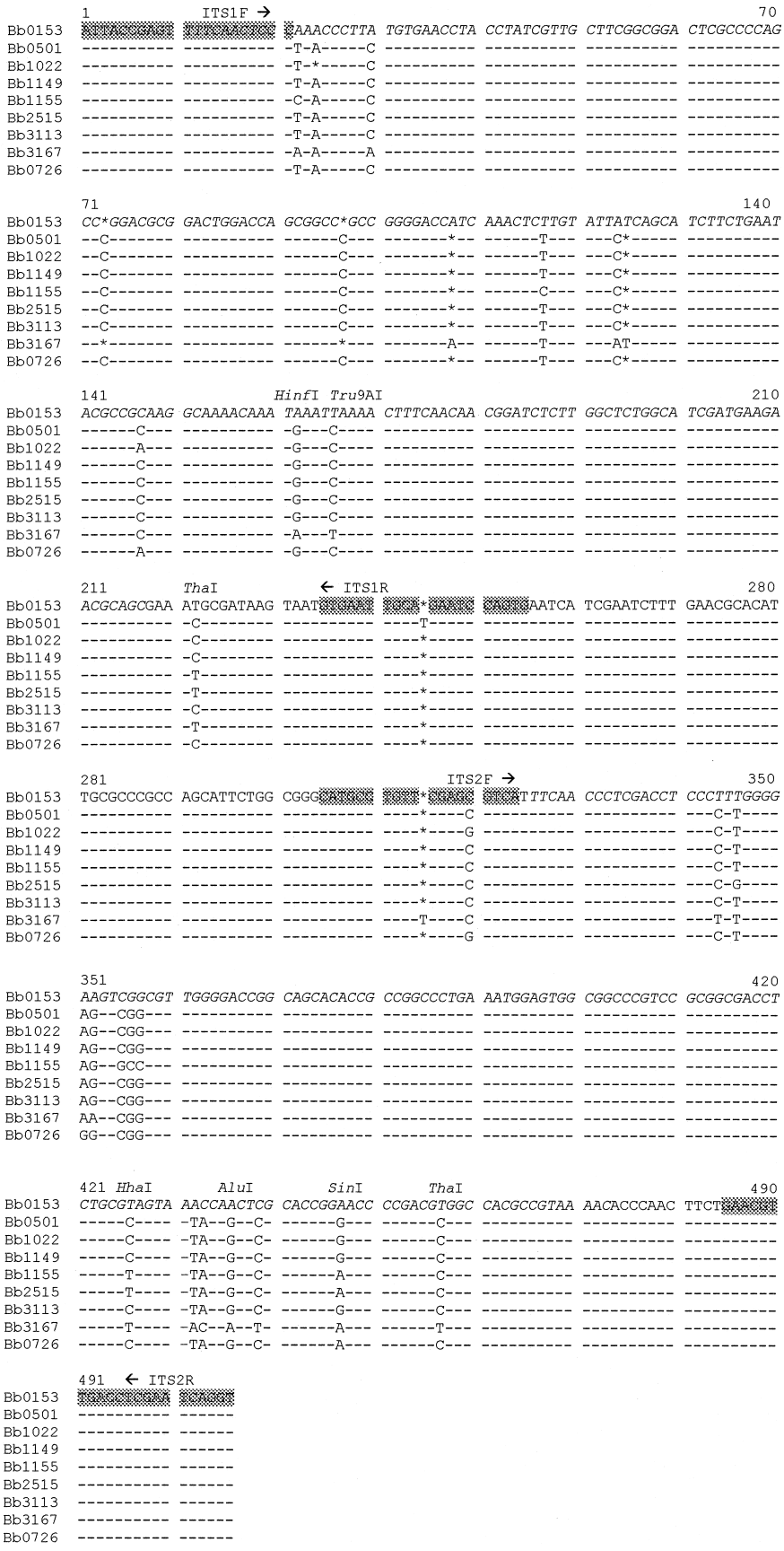

Fig. 1. 506 base pair *Beauveria bassiana* ITS region sequence alignment. ITS1 and ITS2 regions are italicized in Bb0153 sequence, primer binding sites are highlighted and labeled showing primer direction, deletion mutations are indicated by asterisks (*), identical bases are shown as dashes (-), and relevant restriction sites are labeled appropriately.

ITS region mutation detection

Oligonucleotide primers, ITS1F, ITS1R (5′-TCACTGGATTCTGCAATTCAC-3′), ITS2F (5′-CATGCCTGTTCGAGCGTC-3′), and ITS2R were designed using the Primer3 (Rozen & Skaletsky 1998) based upon *B. brongniartii* sequence (Neuveglise *et al*. 1994), and synthesized at Integrated DNA Technologies (Coralville, IA). ITS1F is positioned 22 bp from the 3′ end of primers ITS5 (White *et al*. 1990), and ITS2R is 21 bp from the 3′ end of ITS4 (White *et al*. 1990). The predicted 255 bp ITS1F/ITS1R and 199 bp ITS2F/ITS2R primed PCR products were designed to reduce fragment size such that polyacrylamide gel electrophoresis could be performed and individual PCR–RFLP mutations localized to respective ITS regions. Both ITS1F/ITS1R (ITS1 fragment) and ITS2F/ITS2R (ITS1 fragment) primer pairs were PCR amplified in a 25 μl reaction containing 2.5 mM $MgCl_2$, 150 μM dNTPs, 3.0 pmol of each primer, 0.425 U *Taq* polymerase (Promega) and 25 ng of template DNA. The PTC-100 thermocycler (MJ Research) program included an initial denaturation at 94 ° for 3 min, followed by 35 cycles of 94 ° for 30 s, 57 ° for 40 s, and 72 ° for 20 s. Amplified ITS1 fragments were digested with *Hinf* I, *Tru* 9AI (Promega), and *Tha* I (Life Technologies, Rockville, MD) according to directions of the supplier. ITS2 PCR products were digested with *Alu* I, *Hha* I, *Sin* I (Promega), and *Tha* I (Life Technologies). All PCR–RFLP assays were carried out in individual reactions that contained 3.0 μl of PCR product, 2 μl 10X Buffer, 0.2 μl of 10 mg/μl BSA, and 0.5 U enzyme in a total reaction volume of 20 μl. After incubation at 37 ° or 60 ° for 8–14 h, restriction products were separated on a 20 cm 6% polyacrylamide (19:1 acrylamide: bisacrylamide) gel in 1X TBE buffer at 160 V for 4.5 h. Restriction fragments were visualized using SYBR Green I stain (Molecular Probes, Eugene, OR), and image capture took place on a Fotodyne FOTO/Analyst Investigator PC-FOTO/Eclipse Electronic Documentation System (Fotodyne, Hartland, WI). DNA fragment size estimations were made from the digital images using Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD).

Data analysis

Phylogenetic relationships among PCR–RFLP haplotypes were estimated by Wagner parsimony methods (Eck & Dayhoff 1966) after 1000 bootstrap resampling steps, where calculations were performed using PHYLIP (Felsenstein 1989). Analysis of molecular variance (AMOVA) and θ (Fst) (Weir & Cockerham 1984, Excoffier, Smouse & Quattro 1992, Weir 1996) calculations were generated from PCR–RFLP data using Arlequin software (Schnieder 1997). Arlequin input defined eight groups according to the insect from which the isolates were derived (Humber 1992). Groups were placed into four subpopulations; subpopulation *Lepidoptera* (Lep.), *Coleoptera* (Col.), *Hymenoptera/Diptera* (HD), and *Hemiptera/Homoptera* and others (HHO) (Table 1). Subpopulation *Lepidoptera* (Lep.) consisted of two groups; group 1, *Ostrinia nubilalis*, and group 2, other *Lepidoptera*, excluding *O. nubilalis*, and subpopulation *Coleoptera* (Col.), contained group 3: *Diabrotica* spp., and group 4, other *Coleoptera* excluding *Diabrotica* spp. Subpopulation *Hymenoptera/Diptera* (HD), included group 5, *Hymenoptera*, and group 6, Diptera, and subpopulation *Hemiptera/Homoptera* and others (HHO), consisted of group 7, *Hemiptera/Homoptera*, and group 8 all other insect orders.

Table 2. Restriction endonucleose fragment sizes observed after separation PAGE on a 0.1 × 20 cm 6% polyacrylamide 19:1 (acrylamide:bisacrylamide) 1× TBE buffered gel at 160 V for 4.5 h. Size estimates are in base pairs (bp) and were made from DNA sequence data. Allele designations, 1 or 0, also were used in Table 1.

	Fragments (bp)	
	Allelotype 1	Allelotype 0
ITS1		
Hinf I	103, 92, 60	195, 60
Tru 9AI	166, 89	255
Tha I	154, 79, 32	176, 79
ITS2		
Hha I	118, 81	199
Alu I	129, 70	199
Sin I	79, 61, 59	138, 61
Tha I	149, 50	199

Table 3. AMOVA table comparing *Beauveria bassiana* isolates from eight groups within four subpopulations (subpops.). (A) Subpopulation *Lepidoptera* (Lep.) which consisted of two groups; group 1, *Ostrinia nubilalis*; group 2, other *Lepidoptera*, excluding *O. nubilalis*. (B) Subpopulation *Coleoptera* (Col.) which contained group 3, *Diabrotica* spp., group 4, and other *Coleoptera* excluding *Diabrotica* spp. (C) Subpopulation *Hymenoptera/Diptera* (HD) which included group 5, *Hymenoptera*, and group 6, *Diptera*. And (D) subpopulation *Hemiptera/Homoptera* and others (HHO) with group 7, *Hemiptera/Homoptera* and group 8 all other insect orders.

Source of variation	df	Sum of squares	Variance component	% of variation
Among groups	3	3.863	0.02570 Va	5.72
Among subpops. within groups	4	2.654	0.02378 Vb	5.16
Within subpops.	88	35.233	1.40037 Vc	89.12
Total	95	41.750	7.07995	100.00
Fixation indices				
Fsc (F_{IS})		0.05473		
Fst		0.10880		
Fct (F_{IT})		0.05720		

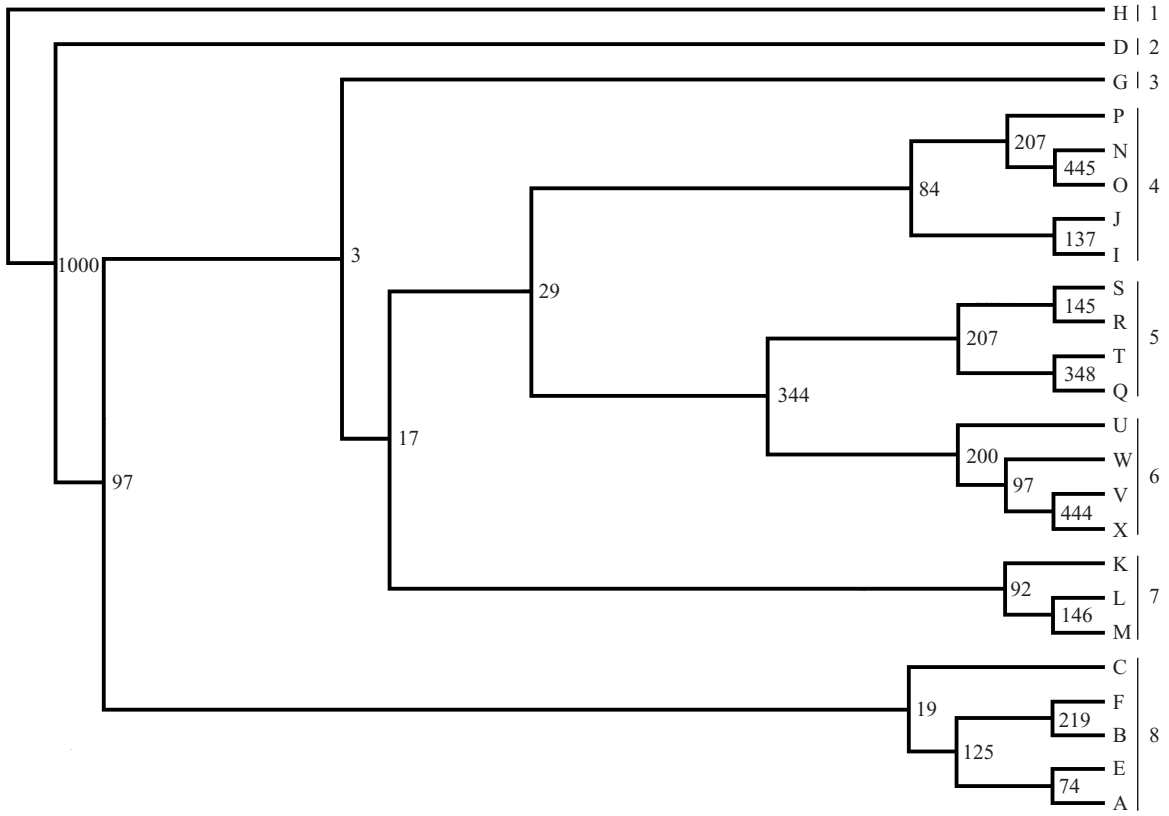

Fig. 2. Consensus phylogeny of *Beauveria bassiana* ITS region PCR-RFLP haplotypes. Wagner parsimony, using MIX followed by CONSENSE programs in the PHYLIP package after 1000 bootstrap resampling steps, was performed.

RESULTS

PCR and DNA sequence alignment

The PCR amplified ITS region of *Beauveria bassiana* was approximately 525-bp when primers ITS4 and ITS5 were used (data not shown). Products were obtained from all isolate DNA samples tested (Table 1), and no length differences were detected. Sequence data were generated from nine *B. bassiana* isolates, and deposited in GenBank (Benson *et al.* 2000): Bb 153 (AF322924), Bb 501 (AF322925), Bb 726 (AF322926), Bb1022 (AF322927), Bb1149 (AF322928), Bb1155 (AF322929), Bb2515 (AF322930), Bb3113 (AF322931), and Bb3167 (AF322932). Multiple sequence alignment identified a 506 nucleotide consensus sequence (Fig. 1) with slight length discrepancies observed between isolates. The number of aligned nucleotides from Bb1022 totaled 501, whereas Bb726, Bb1149, Bb1155, Bb2515, and Bb3113 fragment lengths were 502 nucleotides, and Bb153, Bb501, and Bb3167 were 504. Internal transcribed spacer regions 1 and 2, and the 5.8s rRNA gene were identified in all DNA sequences via comparison to GenBank accession AB027382. Thirty *B. bassiana* intraspecies point mutations were identified, of which 83.3% (25 of 30) resided within the ITS regions. Discounting deletions, *B. bassiana* ITS1 and ITS2 sequences were 0.0–7.1%, and 0.0–9.2% different, respectively. AlignX software identified nucleotide polymorphism at positions in *Alu* I, *Hinf* I, *Hha* I, *Sin* I, *Tru* 9AI, and two *Tha* I restriction endonuclease recognition sequences.

Mutation detection

Digestion of ITS1F/ITS1R PCR products with *Hinf* I, *Tha* I, and *Tru* 9AI, and ITS2F/ITS2R with *Alu* I, *Hha* I, *Sin* I, and *Tha* I, indicated that molecular differences were present at locations predicted from DNA sequence alignments (Fig. 1). Twenty-four haplotypes, A to X, were identified from 96 *Beauveria bassiana* isolates (Table 1). Restriction fragment patterns (Table 2) were identical to those predicted from DNA sequence data, except that fragments smaller than 40 bp typically were not visible (data not shown). During analysis, haplotypes were identified as either allelotype 0 or 1 (Table 2).

Data analysis

AMOVA results generated from PCR–RFLP data indicated that 89.12% of the total genetic variation were present within subpopulations defined by similar insect host preference (Humber 1992), and the fixation index (Fst, θ), 0.1088, suggested that little genetic differentiation had occurred between the same subpopulations (Table 3). A consensus phylogeny was generated from PCR–RFLP data using Wagner parsimony methods (Eck & Dayhoff 1966) after 1000

bootstrap resampling steps and showed the presence of eight clusters that included the 24 ITS region haplotypes, three of which consisted of single genetic types (Fig. 2). No apparent population structure was observed, in that isolates from either *Ostrinia nubilalis* or *Diabrotica* spp. were placed in the same or adjacent phylogenetic clusters (Fig. 2).

DISCUSSION

Differences in nucleotide sequence and in PCR–RFLP pattern from *Beauveria bassiana* internal transcribed spacer regions 1 and 2 were characterized. Multiple sequence alignment of nine *B. bassiana* isolate 5.8S rRNA gene and ITS region data indicated that 0.00–6.62% difference has evolved between isolates. From ITS1 and ITS2 regions, 0.0–7.1%, and 0.0–9.2% sequence variation was respectively shown, and represented a level greater than that observed by Shih *et al.* (1995). The twenty-four ITS region haplotypes identified from 96 *B. bassiana* isolates increased 12-fold the number of haplotypes reported previously by Glare & Inwood (1998), but was less than the 39 genetic groups identified by St Leger *et al.* (1992) using four isozyme markers. The level of ITS region variation corroborated findings by St Leger *et al.* (1992) who suggested, due to the overall degree of genetic diversity observed, *B. bassiana* had maintained a large effective population size over a long period of time. St Leger *et al.* (1992) also stated that gene diversity and genetic distances were affected by the frequency of recombination among fungal strains (Leung & Williams 1986, Zambino & Harrington 1989), and the speed by which reproductive isolation had developed (Ayala, 1979). *Beauveria bassiana* has shown an absence of a sexual phase, and possesses vegetative compatibility groups (VCGs) (Paccola & Meirelles 1991) that serve as barriers to heterokaryon formation (Couteaudier & Viaud 1997) and genetic exchange by recombination prior to reestablishment of the haploid state. Couteaudier & Viaud (1997) indicated that genetic exchange did not occur between coexisting isolates of different VCGs, showed that genetic variation was present between VCGs when telomeric RFLPs were used, suggested an absence of genetic transfer in recent evolutionary time, and proposed that isolates from *O. nubilalis* are clonal as a result of heterokaryon incompatibility.

Based on ITS region PCR–RFLP differences, a fixation index (Fst, θ) of 0.10880 suggested that low level genetic divergence of *B. bassiana* has occurred between subpopulations based on original insect host defined by the ARSEF catalog of strains (Humber 1992). Additionally, AMOVA indicated that 89.12% of the observed molecular variance was present between isolates within each subpopulation, and 5.16% of the total variation occurred between subpopulations. From analysis of PCR–RFLP data we concluded that a weak association was present between isolate haplotype and insect preference. Genetic investigations also have reported little correlation between insect host range and genotype of isolates from *Aspergillus flavis* (St Leger *et al.* 2000), or *Metarhizium* (Cobb & Clarkson 1993, Bidochka *et al.* 1994). Alternatively, Maurer *et al.* (1997) reported unique homogenous subpopulations of *B. bassinia* derived from the pyralids, *Ostrinia* and *Diaryctria*, and the curulionid beetle *Sitona*.

Isolates recovered from *O. nubilalis* have been identified as belonging to a distinct group (Couteaudier & Viaud 1997), or as genetically homogeneous (Viaud *et al.* 1996). Three isolates that were originally derived from *Ostrinia nubilalis* were separated into two groups distinguished by a polymorphic glutathione reductase (St Leger *et al.* 1992). Maurer *et al.* (1997) proposed that host insects were the predominant factor in some population structures of *B. bassiana*, and *O. nubilalis* appeared to be selective against isolates that were not derived from *Ostrinia*. Eighteen isolates derived corn *O. nubilalis* were analyzed by PCR–RFLP of the ITS region, and were placed in six haplotype groups (Table 1). Eleven isolates were on the same phylogenetic branch, as haplotypes A and E, whereas the seven remaining isolates were classified as haplotype M, N, P, or X, and located on separate phylogenetic branches (Fig. 2). Fifteen of 20 isolates that were defined as haplotype A infected *Lepidoptera*, of which 10 were obtained from *O. nubilalis*. Although not statistically significant, the genetic similarities among geographically disperse samples that were isolated from *O. nubilalis* remain intriguing, but may have failed to indicate a strong selective pressure imposed by the host (Maurer *et al.* 1997). *Beauveria bassiana* isolated from *Coleoptera* have been hypothesized to display weak host specificity, or be cross-infective (Glare & Inwood 1998). Similar data has been collected within this study, in that six haplotypes (C, L, O, Q, S, and T) infected *Diabrotica* species. Six haplotypes also were found among isolates that were derived from *Ostrinia*, and suggested that isolates that infected *Ostrinia* have not been exposed to stronger selection forces than isolates that originally infected *Diabrotica* species.

Three of four isolates obtained from North American *Diabrotica* species, *D. berberi*, *D. undecimpunctata*, and *D. virgifera* (haplotype C, S, and Q or T, respectively), were placed on the same branch of the phylogenetic tree. Isolates derived from South American species, *D. speciosa*, and *D. paranoense*, were identified as either haplotype C, L, or O, of which haplotype C was the only one shared with isolates infective toward the North American species *D. berberi*. In a rRNA PCR–RFLP study, Glare & Inwood (1998) demonstrated the importance of geographical origin within the context of observed haplotypes and population structure of *B. bassiana*. Correlation between strain and geographic origin was found when endogenous New Zealand and European isolates, and New Zealand and South American isolates were compared (Glare & Inwood 1998). Poprawski *et al.* (1988) found isozyme

monomorphism within isolates infecting *Sitona* species collected in proximity, but those from different geographical locations were genetically variable.

Four ITS region PCR–RFLP haplotypes were identified among ten isolates recovered from *O. nubilalis* larvae near Ames (IA, USA), and suggested that geography might not play a strong role in genetic diversity, or is case dependent. Alternatively, the evolutionary history of the chromosomal location analyzed and the level of mutation detected through molecular assay may also influence the level of diversity observed. Internal transcribed spacer regions have shown a high rate of genetic divergence (White *et al*. 1990), and the seven PCR–RFLP markers may provide increased resolution. The variation observed among *B. bassiana* ITS regions marker are likely due to selectively neutral spontaneous mutation (Kimura 1983). Rapid evolution of genetic variants from monokaryotic culture has been shown to result in a virulence change in isolates (Samson, Evans & Latge 1988), and may occur more rapidly than ITS region mutation detected by PCR–RFLP assays. Therefore, changes that occurred at virulence-associated loci may have been the driving force of genetic diversity among *B. bassiana* isolates.

Comparatively, random mutation at neutral loci may have evolved more slowly, and some selective virulence switching could have taken place among genetic types that possessed ITS regions that were identical by descent. Thus, the species *B. bassiana* could represent an opportunistic insect pathogen, where selective virulence switching (Samson *et al*. 1988) may have facilitated rapid adaptation to available nutrient sources.

Further studies are needed to investigate the relationship among different *B. bassiana* ecotypes. Additional genetic markers are needed to identify individual isolates as unique genetic types, resolve monophyletic groups within the phylogenetic tree, and to investigate correlation between insect host preference and *B. bassiana* haplotype. The development of additional *B. bassiana* genetic markers has begun. The new markers will be applied to the identification of additional *B. bassiana* haplotypes, and investigation of relationships between isolate ecotypes defined by insect host preference.

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