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Nuclear small subunit rRNA group I intron variation among Beauveria spp provide tools for strain identification and evidence of horizontal transfer

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Keywords
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RESEARCH ARTICLE

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Abstract An optional group I intron was characterized at a single insertion point in nuclear small subunit rRNA (nuSSU rRNA) genes of the imperfect entomopathogenic fungi, *Beauveria bassiana* and *B. brongniartii*. Insertion points were conserved among nuSSU rRNA genes from 35 *Beauveria* isolates. PCR-RFLP and DNA sequencing identified 12 group I intron variants and were applied to the identification of strains isolated from insect hosts. Alignment of 383–404-nt subgroup IB3 group I introns indicated that four insertion/deletion (indel) mutations were the main basis of fragment length variation. Phylogeny reconstruction using parsimony and neighbor-joining methods suggested six lineages may be present among nuSSU rRNA group I intron sequences from *Beauveria* and related ascomycete fungi. Terminal node placement of *Beauveria* introns conflicted with previously published phylogenies constructed from gene sequences, suggesting horizontal transfer of group I introns. PCR-RFLP among introns provided a means for the differentiation of *Beauveria* isolates.

Keywords *Beauveria* · Group I intron · Strain identification · Horizontal transfer

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Introduction

Group I introns are mobile genetic elements described from plant, fungal, archaebacterial, and bacteriophage systems, capable of horizontal transfer between lineages (Dujon et al. 1989; Lambowitz and Belfort 1993). Site-specific transposition of group I elements may be facilitated by intron-encoded endonucleases (homing endonucleases) that target intronless alleles by generating double-stranded breaks (Lambowitz 1989; Perlman and Butow 1989). Host viability is retained by self-splicing of introns from pre-rRNA via two consecutive transesterification reactions catalyzed by the ribozyme core (Zaug et al. 1983; Cech 1987). Ribozyme specificity was shown to depend on conserved stem and loop structures (P1–P10; Legualt et al. 1992; Setlik et al. 1993) and metal ion concentration in vitro (Grosshans and Cech 1984; Gampel and Tzagoloff 1987; Jaeger et al. 1991; Colmenarejo and Tinoco 1999). Little primary sequence similarity is present among group I introns, but ten stem-loop structures typically are present (Davies et al. 1982; Michel et al. 1982). Intron secondary structures were verified by probing (Inoue and Cech 1985) and mutagenesis experiments (Couture et al. 1990) and contributed to structural models proposed by Burke (1988), Cech (1988), and Michel and Westhof (1990). Tertiary interactions involve an internal guide sequence (IGS; Davies et al. 1982: Michel et al. 1982) and GNRA tetraloops of stems P2 and P9 (Jaeger et al. 1994).

Fungi of the genus *Beauveria* (Ascomycota: Hypocreales) have been described with respect to entomopathogenic characteristics and applied within agricultural biocontrol programs (Bing and Lewis 1991, 1992; Krueger and Roberts 1997; Mulock and Chandler 2000). Strains of *Beauveria* were identified by PCR-RFLP of nuclear rRNA internal transcribed spacer (ITS) regions (Neuveglise et al. 1994, 1997; Glare and Inwood 1998; Coates et al. 2002), randomly amplified polymorphic DNA-PCR (Bidochka et al. 1994; Maurer et al. 1997), isozyme analysis (Poprawski 1988;

St Leger et al. 1992), and allelic variation at a minisatellite locus, *BbMin1* (Coates et al. 2001). Glare and Inwood (1998) identified seven polymorphic restriction sites among *B. amorpha*, *B. bassiana*, *B. brongniartii*, *B. caledonica,* and *B. vermiconia* and proposed that two distinct groups were present, the first containing *B. bassiana* and *B. brongniartii*, and the second *B. amorpha*, *B. caldonica*, and *B. vermiconia*. Variation at rRNA ITS regions placed two isolates of *B. bassiana* into a single group that was distinguished from 30 *B. brongniartii* isolates divided into six other genetic groups (Neuveglise et al. 1994). PCR-RFLP analysis of rRNA ITS regions identified 24 haplotypes from 96 *B. bassiana* isolates (Coates et al. 2002), and six different haplotypes among four species of *Beauveria* (Glare and Inwood 1998). Phylogenetic analysis of isozyme markers among *Beauveria* spp by St Leger et al. (1992) defined seven clusters, of which four were comprised solely of *B. bassiana* isolates; and single groupings were predicted with *B. brongniartii* and *B. amorpha*, *B. bassiana* and *B. vermiconia*, and *B. bassiana* and *B. caldonica* isolates. Bidochka et al. (1994) proposed that genetic similarity among isolates of *B. bassiana* resulted from clonal propagation or recent speciation. St Leger et al. (1992) and Maurer et al. (1997) implicated insect host preference as a significant determinant of genetic similarity, where strong selective pressures imposed by insect hosts restrict the number of successful genotypes. Supporting data by Viaud et al. (1996) and Couteaudier and Viaud (1997) found genetic homogeneity among isolates from *Ostrinia nubilalis* and implicated vegetative compatibility groups in defining the likelihood of genetic exchange. Two studies by Coates et al. (2001, 2002) indicated a weak association between genotype and insect host preference, which mirrored conclusions from *Aspergillus* (St Leger et al. 2000). Significant genetic variation among haplotypes was defined at a minisatellite locus, *BbMin1*, but a high level of polymorphism was implicated in the generation of homoplasy between strains from different insect hosts (Coates et al. 2002). From ITS region PCR-RFLP haplotype data, Coates et al. (2002) showed a high level of genetic variation which did not correlate with insect host range and suggested that, within each lineage, some *Beauveria* isolates may be opportunistic parasites capable of infecting multiple insect hosts.

Group I introns have been characterized from the nuclear small subunit (nuSSU) rRNA gene of the Ascomycete fungi *Pneumocystis carinii* (Sogin and Edman 1989) and *Nectria galligena* (Crockard et al. 1998). Single nuSSU rRNA group I intron sequences have been identified for *B. bassiana* (Suh et al. 2001; GenBank AF280633) and *B. brongniartii* (Nikoh and Fukatsu 2000; GenBank AB027335), but intron structure and population diversity were not defined. In the following study, sequence divergence of 12 group I intron variants from 35 *Beauveria* isolates are characterized with respect to RFLP pattern. In conjunction with previously published strain identification methods (Coates et al. 2001, 2002), group I intron PCR-RFLP and DNA sequence data were applied to the identification of strains, evaluation of insect host range, and determination of phylogenetic relationships among haplotypes of *Beauveria* and ascomycete fungi from the family Hypocreales.

Materials and methods

Sample preparation

A total of 112 *Beauveria* isolates were evaluated in the present study. Of these, 104 isolates with diverse geographical origin and pathogenic capacity were obtained from the USDA–ARS Collection of Entomopathogenic Fungi (ARSEF), Ithaca, N.Y. (Humber 1992). Sources for *B. bassiana* isolates Bb726, Bb1022, Bb6715, EL03, and EL12–EL19 are described by Coates et al. (2002). Isolate propagation and DNA extraction procedures were as described by Coates et al. (2002).

nuSSU rDNA PCR amplification

PCR amplification of nuSSU rRNA genes from *Beauveria* isolates used oligonucleotide primer pairs NS1 with NS2, NS3 with NS4, NS5 with NS6, and NS7 with NS8 (White et al. 1990). The 12.5-µl PCR reactions used 2.5 mM $MgCl_2$, 150 µM dNTPs, 5 pmol of each primer, 0.425 units of *Taq* polymerase (Promega, Madison, Wis.), and 40–50 ng of template DNA. The PTC-100 thermocycler (MJ Research, Watertown, Mass.) program was 95 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 50 °C for 40 s, and 72 °C for 1 min. Entire PCR product volumes were loaded onto a 15-cm 1× Tris-borate EDTA 0.5 µg ethidium bromide/ml 2% agarose gel. Electrophoresis took place at 90 V for 2 h on a Subcell-GT (Bio-Rad, Hercules, Calif.). Products were visualized under UV illumination and digital image capture took place on a Foto/Analyst Investigator PC-Foto/Eclipse electronic documentation system (Fotodyne, Hartland, Wis.).

Characterization of group I intron DNA sequence variants

PCR products from NS5- and NS6-primed reactions were DNA sequenced. Sequencing templates using isolates Bb3167 (350 bp) and Bb2297/Bb2737 (750 bp) were prepared in separate 50-µl PCR reactions that used primer NS5 with NS6 (reagent volumes scaled 4× from those above). Template purification and sequencing was as described by Coates et al. (2001). Primer NS5-R, 5′-CU GGUGAGUUUCCCCGUGU-3′, was designed with Primer3 software (Rozen and Skaletsky 1998) from isolate Bb2297 and Bb2737 DNA sequence data, and was synthesized at Integrated DNA Technologies (Coralville, Iowa). The NS5-R primer binding site was located 25 bp immediately following the group I intron insertion point (Fig. 1). Primer NS5-R was later used in DNA sequencing reactions, so that more accurate internal nucleotide sequences could be generated. Also, primer NS5-R was designed to be used with NS5 for PCR amplification of a predicted 444-bp fragment that reduced fragment size, so that polyacrylamide gel electrophoresis of group I intron restriction products could be performed and polymorphism be assessed mainly within the insertion element.

Thirty-five *Beauveria* SSU rRNA group I introns were subjected to PCR-RFLP analysis of PCR products primed by oligonucleotides NS5 and NS5-R. Three informative single enzyme digests used restriction endonucleases *Hae*III, *Hha*I, and *Msp*I (Promega). The template for restriction analysis was PCR-amplified in a 50-µl reaction that used 2.5 mM $MgCl_2$, 150 µM dNTPs, 17 pmol each of primers NS5 and NS5-R, 1.2 units of *Taq* polymerase (Promega), and 100 ng of template DNA. The PTC-100 thermocycler (MJ Research) program was 94 °C for 3 min, then 40 cycles of 94 °C for 30 s, 50 °C for 50 s, and 72 °C for 50 s. PCR-RFLP assays and electrophoresis was performed as described by Coates et al. (2001).

Fifteen PCR products were purified and DNA sequenced as described previously, except that oligonucleotides NS5 and NS5-R were used separately to prime two separate sequencing reactions. Full-length intron sequences were reconstructed from overlapping electrophoretic data from NS5 and NS5-R primed sequencing reactions, using Contig Express software (Informax, San Francisco, Calif.). Submissions were made to GenBank (Benson et al. 2000) for introns from isolates Bb151 (AF479737), Bb350 (AF398395), Bb758 (AF479738), Bb783 (AF464941), Bb937 (AF464940), Bb959 (AF464939), Bb1060 (AF479736), Bb1155 (AF464942), Bb1314 (AF398396), Bb2297 (AF398397), Bb2737 (AF398398), Bb6715 (AF398399), Bsp590 (AF398400), Bt617 (AF398401), and Bt1068 (AF398402).

A *Beauveria* SSU group I intron DNA sequence alignment was generated by AlignX software (Informax) with a gap penalty of one. Secondary structure models, proposed by Burke (1988), Cech (1988), and Michel and Westhof (1990), and data of SSU rRNA introns from *P. carinii* (Sogin and Edman 1989) and *Ustilago maydis* (De Wachter et al. 1992; EMBL X62396) were used to manually identify stem and loop regions.

Phylogenetic analysis

Align X software (Informax) was used to construct a 520-nt consensus alignment from 15 *Beauveria* nuSSU rRNA sequences

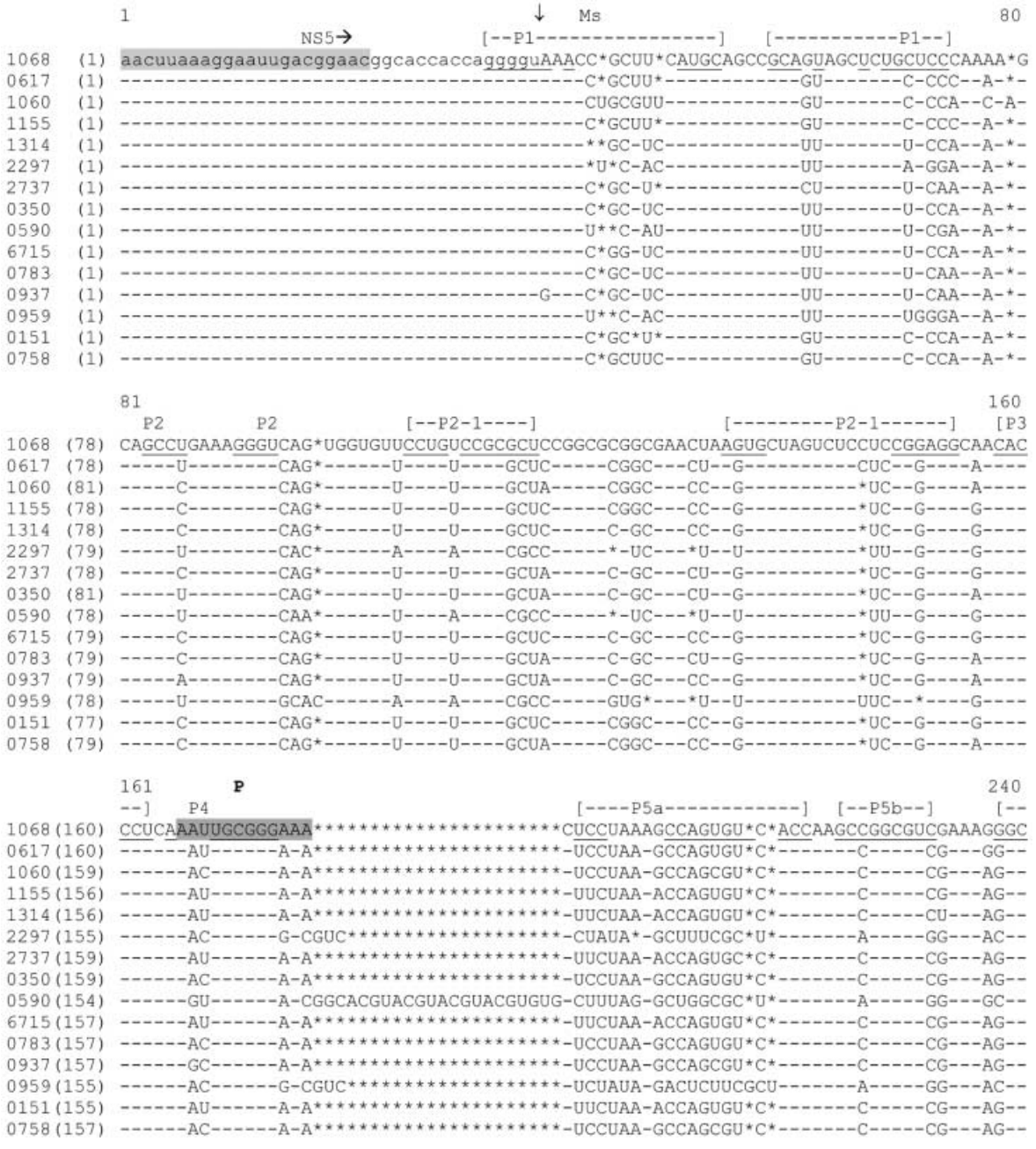

Fig. 1. DNA sequence alignment of 15 *Beauveria* small subunit (SSU) rRNA group I intron variants. The location of the intron insertion is indicated by *an arrow*, deletions are indicated by *stars*, and *dashes* indicate conserved nucleotides identical to top sequence. Secondary structural elements, relevant restriction cleavage sites, NS5 and NS5-R primer-binding sites, and the ribozyme core regions P, Q, R, and S are *indicated above the alignment*. NS5 and NS5-R primer-binding sites and ribozyme core regions P, Q, R, and S are also *highlighted*. *Ha Hae*III, *Hh Hha*I, *Ms Msp*I

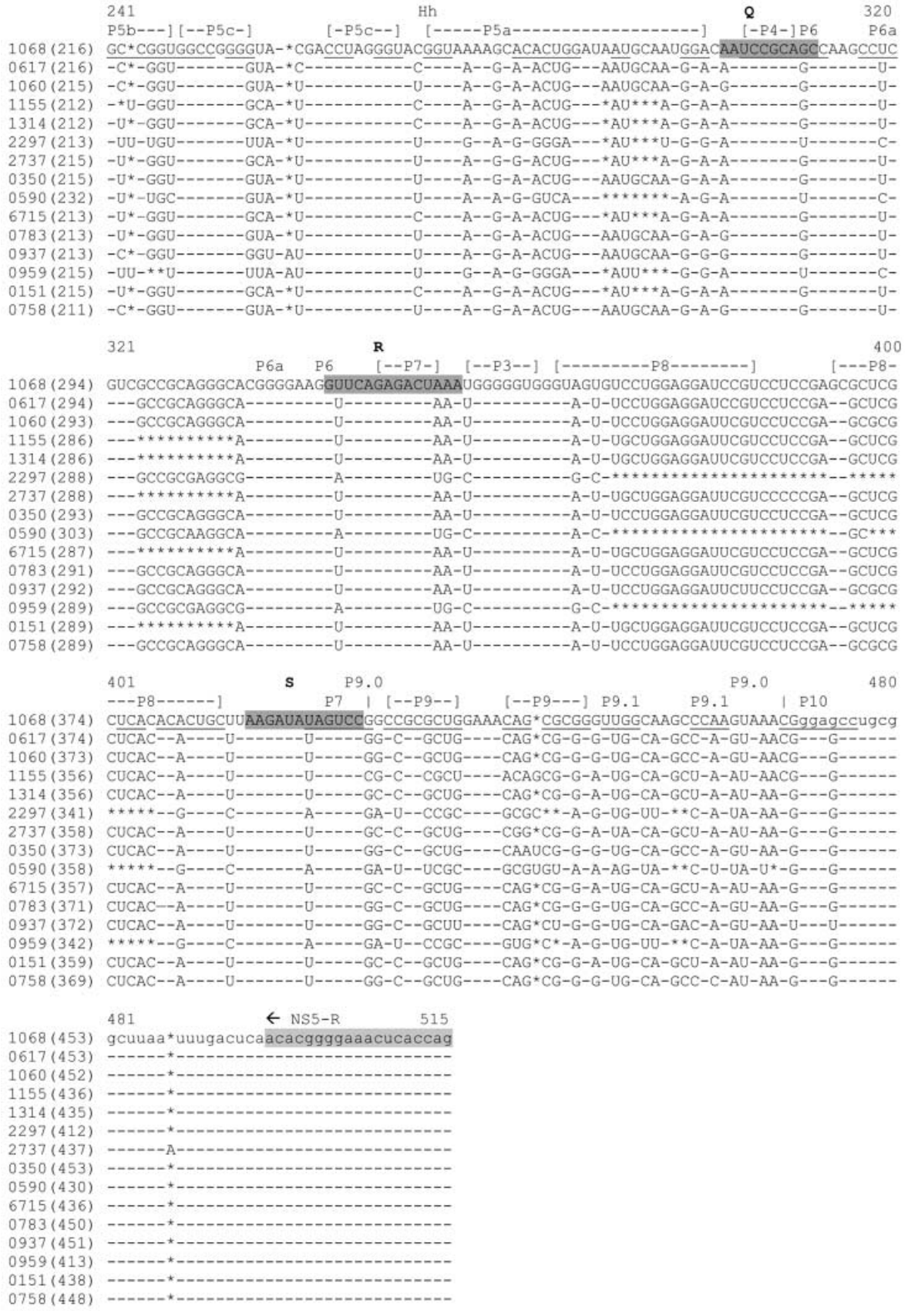

Fig. 1. (Continued)

(Fig. 1) and related sequences from *B. bassiana* (AF280633), *B. brongniartii* (AB027335), *Cordyceps* sp. (AB044630, AB029332), *C. militaris* (AJ009683, AJ007571, AF327392, AJ0027333), *C. pruinosa* (AB044629), *C. pseudomilitari* (AF327394), *C. tuberculata* (AF327401), *Isaria japonica* (AB016607), *Metarhizium anisopliae* (AF363478), and *Paecilomyces tenuipes* (AB027334). Nucleotide frequency and ratio of transitions to transversions (ts:tv) were evaluated using MacClade (Maddison and Maddison 2001). Weighted parsimony and the neighbor-joining distance methods were used to estimate the relationship among group I introns from ascomycete fungi of the family Hypocreales, with the *Pneumocystis carinii* SSU rRNA group I intron (Sogin and Edman 1989) as the outgroup. Parsimony weighting of stem regions to 1 and loops to 0 was indicated on the Seqboot input file (PHYLIP package; Felsenstein 1989), available upon request from the corresponding author. One thousand bootstrap resampling steps were first performed. Parsimony trees were generated using the DNAPars program (Felsenstein 1989) and a strict consensus tree was estimated from all possible phylogenies with CONSENSE (Felsenstein 1989). A heuristic search was used to generate a neighbor-joining tree with PAUP 4.0 (Swofford 2001) with the Jukes–Cantor model of DNA sequence evolution (Jukes and Cantor 1969).

Results and discussion

SSU rRNA group I intron insertion and variation in primary structure

The nuSSU rRNA gene of the imperfect filamentous ascomycete fungi *B. bassiana* and *B. brongniartii* contained an optional group I intron and, respectively, were referred to as *Bb*SSU and *Bt*SSU. Intron-positive *Beauveria* nuSSU rRNA genes showed a 750-bp PCR product when PCR-amplified with oligonucleotide primers NS5 and NS6 (White et al. 1990), whereas a 350-bp fragment was obtained from isolates that lacked the insertion (data not shown). No length polymorphism was observed when PCR products from reactions that used primer pairs NS1 with NS2, NS3 with NS4, or NS7 with NS8 were analyzed (data not shown). Of 112 *Beauveria* isolates, 35 had an intron inserted between primer NS5 and primer NS6 binding sites. The intron was present in three *B. brongniartii* isolates (*Bt*SSU), 31 *B. bassiana* isolates (*Bb*SSU), and one *Beauveria* sp. isolate, Bsp590 (Humber 1992). No introns were discovered at similar positions of a single *B. calidonica* isolate, or four *B. amorpha* isolates.

PCR-RFLP identified 12 *Beauveria* nuSSU rRNA group I intron variants (Table 1). Fragments less than 40 bp typically were not detected (data not shown) and accounted for the failure of fragments to match the total lengths determined from DNA sequence data. The frequency of each intron variant was determined and indicated that intron type *Bb*SSUi1-3 was most prominent among *B. bassiana* isolates (Table 1). Single intron types were described from three *B. brongniartii* isolates (*Bt*SSUi1-1) and isolate Bsp590 (*Bsp*SSUi1-1) that was given an ambiguous species assignment (Humber 1992). Isolates from each group I intron type were DNA

Table 1. *Beauveria* small subunit (SSU) rRNA group I lineages (*Ln*) defined by phylogenetic analysis of DNA sequence data (Figs. 1, 3). Haplotypes were identified by SSU group I intron PCR-RFLP pattern, internal transcribed spacer (ITS) RFLP (Coates et al. 2002), and minisatellite *BbMin1* (Coates et al. 2001) data. *Intron RFLP patterns* from NS5 and NS5-R primed PCR products are given in base pairs. ITS RFLP haplotypes and *BbMin1* repeat number (*RN*) are as defined by Coates et al. (2001, 2002). All isolates are *B. beauveria*, except those marked *Bt* (*B. brongniartii*) and *Bsp* (unspecified species). – Data not available, *ARSEF* USDA–ARS Collection of Entomopathogenic Fungi, *Col.* Coleoptera, *Hemi.* Hemiptera, *Hom.* Homoptera, *Hym.* Hymenoptera, *Lep.* Lepidoptera, *Orth.* Orthoptera

Ln	Intron	SSU rRNA Intron RFLP patterns			ITS RFLP	*BbMin1*	Insect host	ARSEF
		*Msp*I	*Hha*I	*Hae*III		(RN)	(Order)	Isolate(s)
A	BbSSUi1-1	250, 125	250, 120	250, 215	A	8	Lep.	1314[a]
					BA	2	Hemi.	307, Bsp1195
					L	–	Lep.	1564
					A	8	–	1145
A	BbSSUi1-2	250, 125	250, 120	215, 180	L	3	Col.	1155[a]
A	BbSSUi1-3	250, 120	250, 120	250, 215	G	3	Lep.	151[a]
					A	8	Lep.	502, 3113
					A	8	Hom.	708
					A	8	Diptera	1866
					A	4	Diptera	1630
					C	2	Orth.	726
					L	1	Diptera	1973, 1988
					P	4	Diptera	2953
A	BbSSUi1-4	250, 145	250, 120	250, 215	C	2	Col.	6715[a]
A	BbSSUi1-5	250, 145	140, 120	250, 215	A	6	Lep.	2737[a]
					B	2	Hym.	338
					C	2	Col.	721
F	BbSSUi1-6	175, 120	250, 120	250, 215	C	8	Lep.	959[a]
					C	2	Lep.	2297[a]
B	BbSSUi1-7	175, 145	250, 120	215, 180	J	2	Col.	783[a]
	BbSSUi1-8	175, 145	155, 120	215, 180	L	6	Col.	758[a], 809
					L	8	Col.	1060[a]
B	BbSSUi1-9	200, 160	155, 120	215, 180	L	6	Col.	927[a], 928, 938
B	BbSSUi1-10	275	155, 120	215, 180	BA	8	–	350[a]
					L	3	Orth.	1959
B	BtSSUi1-1	175, 120	160, 120	215, 180	BB	8	Col.	Bt617[a]
					BA	8	Col.	Bt1068[a]
					AP	–	–	Bt1871
F	BspSSUi1-1	220, 215	225, 125	200, 125	O	6	Lep.	Bsp 590[a]

[a] DNA sequence data used in alignment

sequenced and used to construct a 515-nt consensus sequence, with individual introns ranging over 366–406 nt (Fig. 1). The alignment contained 82 nt from the flanking nuSSU rRNA gene sequence and group I intron lengths were 383–404 nt. Four insertion and deletion (indel) mutations were identified from group I intron consensus sequence positions 178–199, 291–297, 324–333, and 372–405. Outside of indel mutations, a total of 108 nt substitutions were identified, with a range of 0–68 (Fig. 1).

A high degree of heterogeneity in *Beauveria* nuSSU rRNA intron sequence was observed. An optional group I intron from the mycorrhizal fungus *Cenococcum geophilum* SSU rRNA (*Cg*SSU) varied from 488 nt to 514 nt between ten isolates (Shinohara and LoBuglio 1996), but *Cg*SSU DNA sequences differed only by 0.4–6.8%. Intron length polymorphism in the marine red alga *Porphyra spiralis* var. *amplifolia* was dependent upon indel mutations in loop region 1, whereas other regions were relatively conserved (Oliviera and Ragan 1994). A high level of DNA sequence diversity among *Bb*SSU introns may suggest an ancient intron acquisition; and time and relaxed functional constraint may have resulted in the observed levels of nucleotide sequence variation.

The rRNA gene insertion point of *Bb*SSU and *Bt*SSU, AGGGGU ∨ GGAGCC, was identical in all 15 *Beauveria* nuSSU rRNA gene sequences and those previously reported from *Beauveria* (Nikoh and Fukatsu 2000; Suh et al. 2001), and *M. anisopliae* (GenBank AF363478). Both *Bb*SSU and *Bt*SSU insertion points were identified at position 1199 of the nuSSU rRNA gene (Nikoh and Fakatsu 2001). Introns *Bb*SSU and *Bt*SSU interrupted the nuSSU rRNA gene at a similar position as those from *Ankistrodesmus stipitatus* (Davila-Aponte et al. 1991) and *U. maydis* (De Wachter et al. 1992). The nuSSU rRNA introns from *C. geophilum* (Shinohara and LoBuglio 1996) and several lichen-forming fungi (Garcias et al. 1995) were inserted at different positions.

Introns *Bb*SSU and *Bt*SSU were present in 35 of 112 *Beauveria* isolates (31.25%) and indicated that insertion was not common. The *Beauveria* genome was previously shown to have few group I introns. The mitochondrial DNA was proposed to lack group I introns (Pfeiffer et al. 1993), which was supported by a reduced size of the molecule (28.5 kb; Pfeifer and Khachatourians 1989) and Southern blot (Neuveglise et al. 1994). The group I intron, *Cg*SSU, was present in 61 of 70 *C. geophilum* isolates (Shinohara and LoBuglio 1996), which suggested insertion frequency may be species-specific and dependent upon the time since acquisition or mobility (Dujon 1989; Lambowitz and Belfort 1993).

Characterization of *Beauveria* nuSSU rRNA group I intron structure

Secondary structure elements (stem and loop structures P1–P9.0, IGS) were identified from aligned *Bb*SSU and *Bt*SSU sequences in accordance with the models of Burke (1988), Cech (1988), and Michel and Westhof (1990; Fig. 1). Potential stem and loop arrangements were constructed for *Bb*SSU from isolate Bb1314, along with tertiary interaction at the IGS (Fig. 2). The predicted structure of the *Beauveria* intron was similar to introns from *C. geophilum* (Shinohara and LoBuglio 1996), and *U. maydis* (De Wachter et al. 1992), in that stems P9.1, P9.2, P5a, P5b and P5c were present. All sequenced introns were defined as belonging to subgroup IB3 (Michel and Westhof 1990), as found previously for a *B. brongniartii* nuSSU group I intron (Nikoh and Fukatsu 2000). Four indel mutations were present in the alignment of *Bb*SSU and *Bt*SSU sequence variants (Fig. 1), one of which resulted in reduction of a functionally important P8 stem (Burke 1988; Guo et al. 1991). Of 105 point mutations, 58 (55.2%) were located in stem regions (Fig. 1), but compensatory sequence alterations typically maintained secondary interactions.

Beauveria nuSSU group I intron catalytic core region elements P, Q, R, and S (Cech 1988) were identified (Figs. 1, 2). Four variant ribozyme core sequences were present in *Bb*SSU and *Bt*SSU introns from isolates Bt1068, Bb350, Bb2297, and Bsp590. Core regions of 48 nt differed by 0–7 nt. A Bt1068 intron-like core region was present from intron sequences of isolates Bb1314, Bb2737, and Bb6715, indicating that similar core regions may be the predominant form in both *Bb*SSU and *Bt*SSU introns and they possibly resemble that of the original invading intron.

Intron phylogeny and mobility by horizontal transfer

Twelve variant *Beauveria* nuSSU rRNA group I intron sequences were placed into a phylogeny with related nuSSU rRNA intron sequences (Fig. 3). A lower level of homoplasy [consistency index (CI) = 0.71, retention index (RI) = 0.65] was encountered from weighted parsimony analysis that emphasized mutations in stem structures, as opposed to unweighted analysis (CI = 0.64, RI = 0.63; data not shown). Six group I intron lineages (A–F) were indicated from weighted parsimony analysis (Fig. 3). Bootstrapped branch support from 17 of 29 divisions were greater than 50% of 1000 iterations and separation of group F from remaining group I introns was most strongly supported (Fig. 3a). A ts:tv ratio of 1.0 and chi-square analysis of nucleotide frequency variance ($\chi^2 = 118.35$, $P = 0.0392$) suggested the appropriateness of the Jukes–Cantor model of DNA sequence evolution. The resulting neighbor-joining tree (Fig. 3b) was similar to that from parsimony analysis (Fig. 3a) with respect to clustering of intron sequences. Phylogenetic positioning of two introns, one from *B. brongniartii* (AB027335) and one *Cordyceps tuberculata* (AF327401) differed between tree reconstruction methods. Weighted parsimony analysis placed 15 *B. bassiana* sequences from the current study into three lineages (A, B, F; marked with * in Fig. 3), of which introns from isolates Bb959, Bb2297, and Bsp590

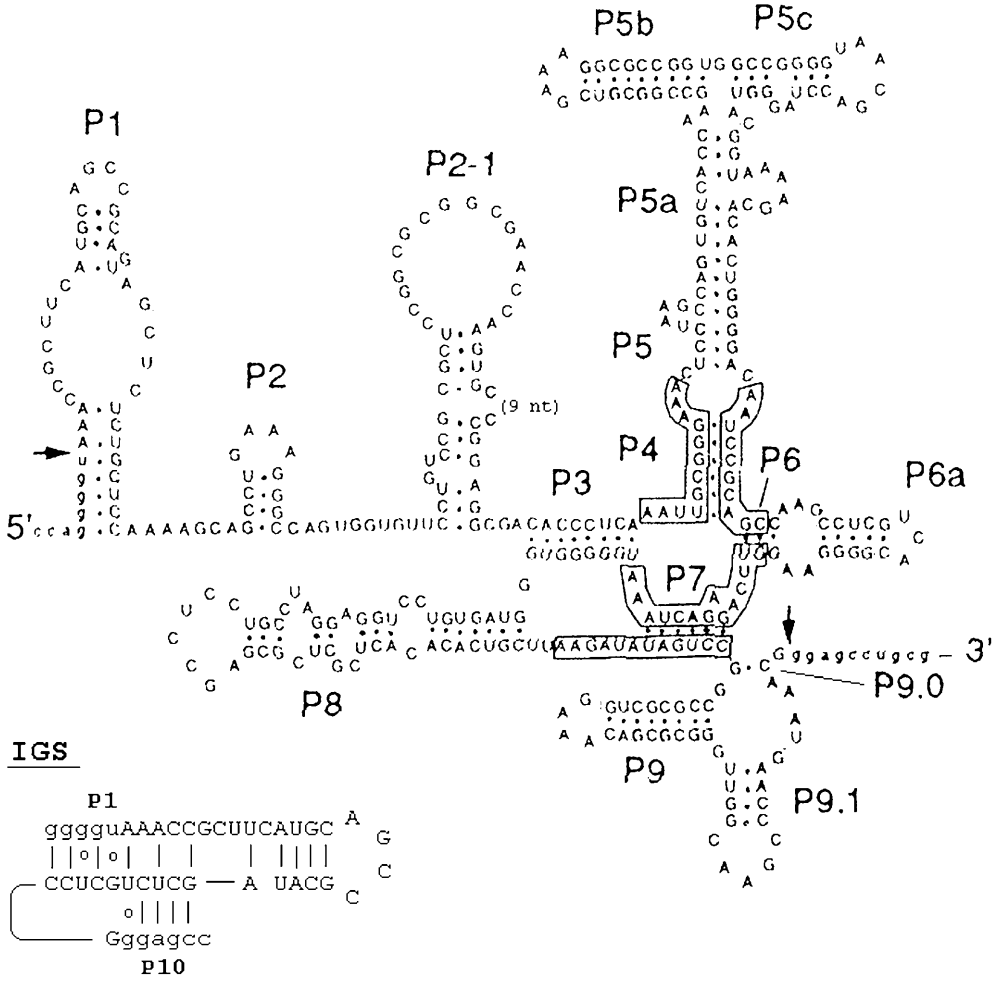

Fig. 2. Isolate Bb1314 SSU rRNA group I intron stem and loop structures as defined by Michel and Westhof (1990). *IGS* Internal guide sequence

(group F) were more closely related to introns from *Cordyceps* sp. (groups D, E) than to introns from other *Beauveria* isolates (groups A, B, C; Fig. 3). Parsimony analysis clustered accession numbers AF280633 (*Bb*SSU) and AB027335 (*Bt*SSU) with introns from *Cordyceps* sp. and *M. anisopliae* (group C; Fig. 3a), but AB027335 was placed closer to *Bt*SSU introns from isolates Bt0617 and Bb1068 in the neighbor-joining tree (Fig. 3b). Identical deletions of a portion of stem P8 were observed from group I introns of Bsp590, Bb0959, and Bb2297 (Fig. 1) and *Cordyceps* sp., *Igenus japonica*, and *Paccilomyces tenuipes*, and previously from *P. cephalum* (Johansen et al. 1992). The P8 deletion appeared to be the basis of the phylogenetic separation of groups E and F (Fig. 3) and suggested that group F introns formed a lineage distinct from other *Beauveria* nuSSU group I introns.

Isolate Bsp590 was described as having morphological characters intermediate to *B. bassiana* and *B. brongniartii* (Humber 1992). DNA sequence analysis indicated BspSSUi1-1 was similar to introns from two *B. bassiana* isolates and was more similar to introns from three other ascomycete fungi than to those from the 12 remaining *Beauveria* introns in groups A and B (Fig. 3). *B. bassiana* and *B. brongniartii* were included in the analysis by Nikoh and Fukatsu (2000), who indicated congruence between parsimony trees constructed from *Cordyceps* spp nuSSU group I introns inserted at position 1199 and rDNA data, except in the placement of *C. militaris* and *C. pruinosa*. A comparison of the nuSSU rRNA group I intron phylogeny with the rRNA gene phylogeny constructed by Nikoh and Fukatsu (2000) and one from ITS region RFLP data (Coates et al. 2002; appropriate designations indicated on Fig. 3a) indicated disparity in the placement of isolates containing introns from group F (Fig. 3). Introns of group F contain two *Beauveria* ITS haplotypes, O and C, that differ by cleaving at four restriction sites. Furthermore, ITS haplotype C has only one restriction-site difference from haplotype A (Coates et al. 2002), positioned in groups A and B of the nuSSU rRNA intron phylogeny. Inconsistencies of *Beauveria* group I intron haplotypes in relation to the rRNA gene tree of *Cordyceps* fungi (Nikoh and Fukatsu 2000) and the *Beauveria* ITS haplotype tree implicates a complex history of horizontal transfer events between *Beauveria* strains and related ascomycete fungi. Further support for horizontal transfer of nuSSU group I introns comes from a similarity of deletion mutations in intron groups E and F, suggesting identity by descent even though from

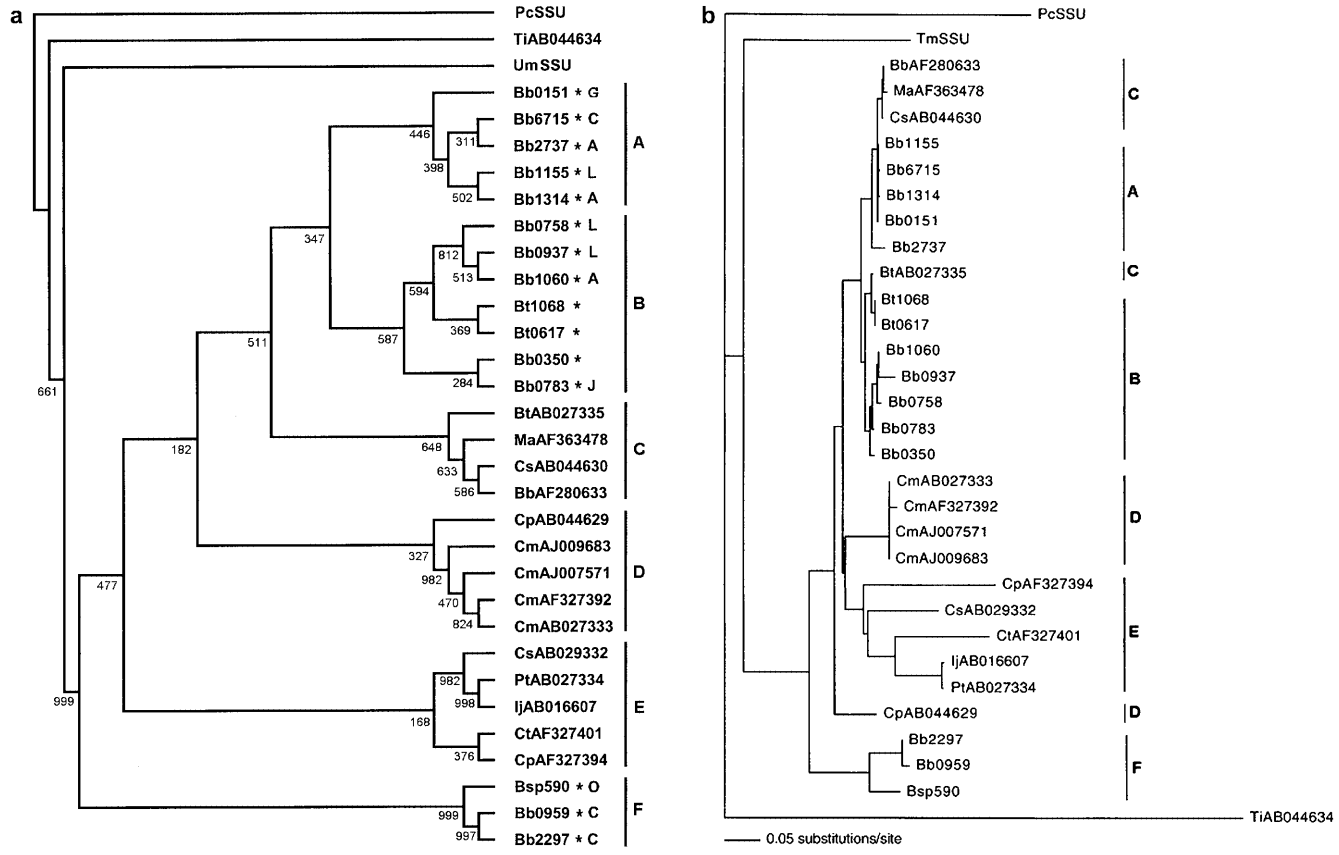

Fig. 3a, b. Phylogenetic analysis of nuclear SSU rRNA group I intron sequences from ascomycete fungi from the family Hypocreales. **a** Weighted parsimony with branch support from 1000 bootstrap iterations (consistency index = 0.71, retention index = 0.65). *Beauveria* group 1 intron sequence data generated in this study are marked with an *asterisk*, followed by internal transcribed spacer region haplotype (A, C, G, L, O), as determined by Coates et al. (2002). **b** Neighbor-joining distance tree using Jukes–Cantor model of DNA sequence evolution. Abbreviations are followed by GenBank accession numbers when appropriate. The group I intron, PcSSU, from *Pneumocystis carinii* (Sogin and Edman 1989) was used for outgroup analysis. *Bb B. bassiana*, *Bt B. brongniartii*, *Cd Cordyceps pseudomilitari*, *Cm C. militaris*, *Cp C. pruinosa*, *Cs Cordyceps* sp., *Ct C. tuberculata*, *Ij Isaria japonica*, *Ma Metarhizium anisopliae*, *Pt Paccilomyces tenuipes*

different species. Strong bootstrap support (99.9%) of the split of group F from other *Beauveria* intron lineages suggests that a unique group I intron lineage (group F) exists and may have been acquired by interspecies horizontal transfer.

Horizontal transfer of a mobile genetic element, the hAT transposon, was implicated by Kempken et al. (1998) as the mechanism by which transfer occurred between *B. rosarienis*, *B. nivea*, and *Tolypocladium* spp. Parasexuality and vegetative compatibility among *B. bassiana* was previously described (Paccola-Meirelles and Azevedo 1991; Couteaudier and Viaud 1997) and may contribute to genetic similarity among stains (Viaud et al. 1996; Couteaudier and Viaud 1997). Genetic transfer between incompatible isolates, strains, or different *Beauveria* spp has not been shown directly, but heterokaryon formation was implicated in the genetic exchange of a transposon between *Beauveria* spp (Kempken et al. 1998) and might explain group I intron switching among ITS region haplotypes (Fig. 3a). Even though anastomoses are unstable in interspecies contacts, horizontal transfer of plasmid DNA was shown to take place between species of *Neurospora* (Collins and Saville 1990; Kempken et al. 1998); and horizontal transfer of the transposable element *Fot*1 between species of *Fusarium* has also been suggested (Daboussi and Langin 1997). Alternatively, evidence has indicated that heterokaryon incompatibility of *Aspergillus niger* strains blocks intraspecies mycovirus transfer (Van Diepeningen et al. 1997). Protoplast fusion experiments on *Aspergillus*, that bypassed heterokaryon incompatibility, also resulted in limited intraspecies genetic transfer (Van Diepeningen et al. 1998).

As found in *Aspergillus*, horizontal group I intron transfer between *Beauveria* strains may be restricted by similar vegetative compatibility barriers, combined with effects of host specificity. St Leger et al. (1992) and Maurer et al. (1997) suggested that insect host range affected genetic similarity among strains, with selection imposed by insect pathogen defense restricting the number of successful genotypes, but this was contradicted by data presented by Coates et al. (2001, 2002) and analogous data from *Aspergillus* (St Leger et al. 2000). Viaud et al. (1996) and Couteaudier and Viaud (1997) suggested that vegetative compatibility groups influenced genetic similarity among isolates and likelihood of genetic exchange, especially those from *Ostrinia*

nubilalis (Lepidoptera: Crambidae). Two of ten isolates that infected *O. nubilalis*, Bb501 and Bb3113, housed the same group I intron type, the same ITS region and *BbMin1* haplotypes. Results indicate that a single *Beauveria* lineage virulent towards *O. nubilalis* was invaded once by a single group I intron type and remaining intronless isolates infecting *O. nubilalis* form a separate lineage.

Effects of isolate host range, defined by insect order, are observable in the distribution of nuSSU group I introns. All intron types of group F, BbSSUi1-6 and BspSSUi1-1, for which evidence of interspecies horizontal transfer has been proposed, were identified from *Beauveria* isolates that infected lepidopteran insects (Table 1). Two ITS region haplotypes, C and O, are present in isolates carrying group F introns. Prior evidence indicated that four mutation steps separated ITS haplotypes C and O; and isolate Bsp590 is not closely related to either isolate Bb0959 or Bb2297 (Coates et al. 2002). Bsp590 is ambiguous in its species placement, using morphological features (Humber 1992), and thus the most parsimonious route of interspecies transfer cannot be determined. Movement equally might have occurred directly, either to Bsp590 and then to Bb959 or Bb2297, or to isolate Bb959 or Bb2297 and then to Bsp590. A single intron type, BbSSUi1-3, was present among five of eight isolates infecting insects from the Order Diptera, although four different ITS and *BbMin1*-defined haplotype groups were present. The single intron within dipteran-infecting strains has a recent common ancestry to introns that invaded some lepidopteran-, homopteran-, and orthopteran-infecting *Beauveria* strains. It likely entered the *Beauveria* lineage only once and has been transferred between strains. The prevalence of group I introns within the dipteran-infecting lineages of *B. bassiana* might be influenced by frequency of heterokaryon formation or time since initial intron invasion. The intron phylogeny contains a branch, group B, that contains seven unique group I intron types, of which six are from isolates that infected coleopteran insects. Four of nine *Beauveria* isolates with introns from group B had ITS region PCR-RFLP haplotype L (Coates et al. 2002), suggesting decreased mobility or direct descent of introns within the branch.

Lineages from intron group A are not as constrained by insect host range, in that the lineage is found among isolates infecting five insect Orders (Table 1). In contrast to intron lineages B and F, lineage A might initially have invaded a promiscuous *Beauveria* strain that had few barriers to heterokaryon formation and readily exchanged genetic information with a large subset of the species *B. bassiana*. Alternatively, seminal intron invasion by the ancestral group A intron may have occurred within an isolate with a loose host range.

Beauveria isolates infecting Coleoptera were described as being very heterogeneous (Glare and Inwood 1998; Coates et al. 2002), which is corroborated by nuSSU group I intron diversity among strains. Additionally, isolates of *Beauveria* that infected lepidopteran insects were diverse in the range of intron lineages, which agreed with the conclusions of Coates et al. (2001, 2002), who indicated a level of genetic diversity equal to that of Coleopteran-infecting strains. Low overall prevalence of nuSSU group I intron insertion might result from a rarity of genetic exchange between *Beauveria* strains, due to low mobility of the group I intron elements, strong vegetative compatibility barriers, or low frequency of heterokaryon formation.

Variability among *Bb*SSU DNA sequences has allowed the development of genetic markers for the identification of strains. Twelve PCR-RFLP haplotypes were distinguished from among 35 isolates having the intron inserted at position 1199 of the nuSSU rRNA gene, of which ten haplotypes were present among 35 *Beauveria* isolates (Table 1). In conjunction with genotyping based on ITS region PCR-RFLP (Coates et al. 2002) and the *BbMin1* minisatellite (Coates et al. 2001), 26 haplotypes were characterized (Table 1). The level of polymorphism among *Bb*SSU agreed with previous conclusions that *B. bassiana* was a heterogeneous species complex (Magnai et al. 1989; St Leger et al. 1992; Coates et al. 2001). Multiple horizontal transfer events likely occurred in the phylogenetic histories of nuclear nuSSU rRNA group I introns from *B. bassiana* and related ascomycete fungi. Due to horizontal transfer events, incorrect species phylogeny reconstructions can be obtained using intron sequences alone, yet evaluation of introns provides both a means to detect horizontal transfer via comparison to species trees and other *Beauveria* genes and a means to estimate the frequency of genetic transfer between stains of this imperfect fungus. The frequency of nuSSU rRNA group I intron insertion and comparison of phylogenies based on different nucleotide regions allowed a qualitative estimation of levels of genetic exchange among strains of *Beauveria* and between species of the family Hypocreales. A low frequency of intron insertion into the nuSSU rRNA gene, compared with other fungi (Shinohara and LoBuglio 1996), may be interpreted as showing the presence of strong vegetative barriers, assuming the frequency of intron insertion is inversely proportional to the strength of barriers to genetic exchange (propensity toward clonal propagation). Sympatric existence of clonal *Beauveria* lineages, proposed by St Leger et al. (1992), Bidochka et al. (1994), Maurer et al. (1997), and Urtz and Rice (1997), might be supported by the exclusive presence of an intron lineage (group B) from isolates that infect coleopteran insects, the presence of a single intron type, BbSSUi1-3, in all dipteran-infecting isolates, and the uniqueness of intron group F to isolates infecting Lepidoptera. When additionally considering that intron type BbSSUi1-3 is dispersed among isolates infecting insects from several insect orders and the evidence that intron types BbSSUi1-6 and BspSSUi1-1 (group F) were horizontally transferred from outside the genus *Beauveria*, the coleopteran-specific intron group B is the only set where clonal inheritance can be predicted, which contradicts prior evidence of high genetic diver-

sity within that lineage (St Leger et al. 1992; Maurer et al. 1997; Glare and Inwood 1998; Coates et al. 2002). Individual introns in group B likely evolved via mutation over time from a single progenitor intron that invaded the ancestral isolate that evolved the potential to infect coleopteran insects. *Beauveria* introns from group A (Fig. 3) might have passed frequently between different *Beauveria* lineages by invasion into a promiscuous strain with low vegetative compatibility barriers. The correlation between intron lineage B in coleopteran-infecting isolates and intron type BbSSUi1-3 with dipteran-infecting isolates might result from the frequency of isolate contact. If multiple *Beauveria* lineages, regardless of whether or not they are identical by descent, have a greater propensity to share a common microenvironment, the insect host, then frequency of chance contact might increase as could the potential for heterokaryon formation or incidental horizontal transfer of group I introns. Although no direct evidence exists, co-infection of the same insect or increased circumstantial cohabitation of insect microenvironments might increase the probability of hyphal contact between two strains and result in a greater frequency of genetic exchange. There likely exists a complex web of nuSSU rRNA group I intron lineages among isolates of *Beauveria*, characterized by varying frequencies of horizontal transfer between strains and between species. Further investigation is required with respect to group I intron variation and the frequency of transfer between *Beauveria* lineages and the frequency and efficiency of group I intron movement by horizontal transfer within and between common environments.

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