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Identification of Molecular Variants in Mitochondrial DNAs of Members of the Genera *Beauveria*, *Verticillium*, *Paecilomyces*, *Tolypocladium*, and *Metarhizium*

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A set of five mitochondrial (mt) probes derived from a strain of *Beauveria bassiana* was used to evaluate the similarity of mtDNAs from 15 additional isolates of this fungus and five genera of other entomopathogenic fungi. The probes and genes encoded for (shown in parentheses) were pBbmtE2 (*NAD1*, *ATP6*), pBbmtE3 (*ATP6*, small rRNA [*srRNA*]), pBbmtE4 (*srRNA*, *CO3*, *NAD6*), pBbSE1 (*NAD6*, *tRNA*$^{Val, Ile, Ser, Trp, Pro}$, large rRNA [*lrRNA*]), and pBbXS1 (*lrRNA*). The probes produced identical hybridization patterns in *Eco*RI-digested DNA from nearly all isolates of *B. bassiana* and *Beauveria caledonica*. Similar patterns were also observed with *Beauveria densa*. The isolates of *B. caledonica* and *B. densa* DNAs could be differentiated from each other and from *B. bassiana* on the basis of a *Hin*dIII digestion and probing with pBbmtE3. Probe pBbmtE2 produced either a 5.0-kb or a 4.1-kb band in all of the *B. bassiana* isolates. This observation was used to categorize the mtDNA of *B. bassiana* into two types, designated A and B. Hybridization of the five probes produced distinct banding patterns in *Beauveria brongniartii*, *Tolypocladium cylindrosporum*, *Tolypocladium nivea*, *Metarhizium anisopliae*, *Verticillium lecanii*, and *Paecilomyces farinosus*. Hybridizations carried out with multiple probes simultaneously present produced unique patterns which characterized the *B. bassiana* group from all other fungi tested. These results are discussed in terms of how mtDNA polymorphisms in *B. bassiana* may relate to natural population structures, mt transmission in deuteromycetes, and the use of mtDNA polymorphisms in structural analysis of mtDNA.

Beauveria bassiana is a widely used mycoinsecticide of significant commercial interest for control of many plant-injurious insects (14, 16). To realize its considerable economic potential, implementation of a standardized approach for its identification is required. However, classification of deuteromycetous entomopathogenic fungi is still most often based upon classical taxonomic and morphological characteristics. This involves the propagation of the fungus under a strictly defined set of nutritional and environmental conditions or the use of standardized analytical methods requiring experienced mycologists. In the case of *Beauveria* spp., such classification has led to its grouping on the basis of differing interpretations of taxonomic criteria (5, 33). Additionally, the placement of the species within this genus is subject to variability when classification is made by morphological and biochemical criteria. For example, chemotaxonomic approaches have suggested that *Beauveria densa* and *Beauveria caledonica* are distinct classes within a highly variant *B. bassiana* population and thus constitute subspecies (18, 27). Molecular biological approaches have been utilized to address the uncertainty created by existing taxonomic methods because these techniques are not subject to cultural influences. In this regard, there was evidence for the separation of the genus *Beauveria* from that of *Tolypocladium* on the basis of rRNA sequence comparisons (22). Previously, this conclusion was argued on the basis of chemotaxonomic analysis (18) but was rejected on the basis of morphological criteria (33). Restriction fragment length polymorphism (RFLP) analysis has been used in our laboratory to differentiate *Beauveria* species (15). More recently, a series of DNA probes which is specific for *B. bassiana* and capable of detecting genetic variations that can be exploited for strain identification (11) has been developed. Finally, random amplified polymorphic DNA analysis has been used to separate varieties of the fungal entomopathogens *Hirsutella longicolla* (28) and *Metarhizium anisopliae* (7).

Mutational changes in the length of mitochondrial (mt) DNA and/or restriction enzyme patterns in filamentous fungi are well documented (29). In many cases, there is enough variability to treat the resultant banding patterns as taxonomic characters for cladistic and phenetic analyses and to resolve phylogenetic relationships (17, 26, 30). In addition to their utility in taxonomy, mtDNA RFLPs are becoming increasingly important for the identification of other genetic loci seemingly unrelated to known mt functions. Particular patterns could be correlated with pathogenicity, mating type, and geographical distribution in *Cochliobolus heterostrophus* (9), host preference in mycorrhizial fungi (6), and aggressiveness in *Ophiostoma ulmi* (13). The identification of mt markers in entomopathogenic fungi, whether they be genetic or physical, has had a significant impact on the study of organelle transmission and reproductive isolation in sexual fungi (10, 23).

The mt genome of *B. bassiana* has been subjected to extensive physical analysis via cloning and restriction enzyme mapping (20). Sequencing of approximately 30% of the mtDNA has revealed 11 genes, including structural RNA and tRNA as well as protein-encoding genes (12, 19). Here we evaluate the utility of mtDNA-derived probes to provide mtDNA RFLPs that identify mtDNA variations within isolates of *B. bassiana* and between three other *Beauveria* species as well as *Tolypocladium nivea*, *Tolypocladium*

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TABLE 1. *B. bassiana* GK2016 mtDNA probes

Probe	Genes known to be present
pBbmtE2	*NAD1*, *ATP6* (5′)
pBbmtE3	*ATP6* (3′), *srRNA* (5′)
pBbmtE4	*srRNA* (3′), *CO3*, *NAD6* (5′)
pBbSE1	*NAD6* (3′), $tRNA^{Val, Ile, Ser, Trp, Pro}$, *lrRNA* (5′)
pBbXS1	*lrRNA* (3′)

cylindrosporum, *M. anisopliae*, *Verticillium lecanii*, and *Paecilomyces farinosus*.

MATERIALS AND METHODS

Fungal isolates and DNA probes. The DNA probes used in this study were derived from the mtDNA of *B. bassiana* GK2016 (Bioinsecticide Research Laboratory, University of Saskatchewan). The map of the mtDNA for this strain has been provided (19). A list of the probes used and the genes they are known to encode is shown in Table 1. All fungal isolates used in this study and their sources are listed in Table 2. The fungi were propagated and stored by the procedure of Pfeifer and Khachatourians (20).

Molecular techniques. All DNA manipulations were carried out by the method of Sambrook et al. (25), unless otherwise noted. Fungal DNA was isolated by using the method described by Pfeifer and Khachatourians (21). Plasmid DNA was isolated by the method of Birnboim and Doly (1). Three micrograms of total genomic DNA from each of the fungal isolates was digested with the restriction enzyme *Eco*RI and electrophoretically separated in 0.75% (wt/vol) agarose–TBE (0.09 M Tris, 0.09 M boric acid, 0.02 M EDTA) gels. The DNA was transferred to GeneScreen Plus nylon membranes (Dupont, Boston, Mass.) by the capillary blotting method or with a semidry transfer apparatus (Bio-Rad, Mississauga, Ontario, Canada). The membranes were hybridized with radioisotope-labelled, nick-translated plasmids (24). Stringency conditions for screening consisted of 2× SSC (20× SSC is 175.3 g of sodium chloride and 88.2 g of sodium citrate per liter) and 0.5% sodium dodecyl sulfate at 55°C in the wash procedure. The membranes were autorad-

TABLE 2. Hybridization of *B. bassiana* GK2016 mt probes to isolates of *Beauveria* spp. and other fungi

Isolate	Source	Size of hybridization band(s) (kb) for probe pBbmt				
		E2	E3	E4	SE1	XS1
B. bassiana						
GK2016	Lab isolate[a]	5.0	1.8	1.8	20.0	20.0
ATCC 44860	Soil, Georgia[b]	5.0	1.8	1.8	20.0	20.0
DAOM 144746	Soil, Alberta Canada[c]	5.0	1.8	1.8	20.0	20.0
DAOM 195005	Spruce budworm, Quebec, Canada	5.0	1.8	1.8	20.0	20.0
DAOM 210569	Beetle, British Columbia, Canada	4.1	1.8	1.8	20.0	20.0
USSR 2274	NA[d, e]	5.0	1.8	1.8	2.8	20.0, 2.8
USSR 2533	NA	4.1	1.8	1.8	20.0	20.0
ARSEF 2860	*Schizophis graminum*, Idaho	5.0	1.8	1.8	20.0	20.0
ARSEF 2880	*Schizophis graminum*, Idaho	5.0	1.8	1.8	20.0	20.0
ARSEF 2881	*Schizophis graminum*, Idaho	4.1	1.8	1.8	20.0	20.0
ARSEF 2883	*Schizophis graminum*, Idaho	5.0	1.8	1.8	20.0	20.0
ARSEF 2861	*Diuraphis noxia*, Idaho	5.0	1.8	1.8	20.0	20.0
ARSEF 2864	*Diuraphis noxia*, Idaho	5.0	1.8	1.8	20.0	20.0
ARSEF 2879	*Diuraphis noxia*, Idaho	4.1	1.8	1.8	20.0	20.0
ARSEF 2882	*Diuraphis noxia*, Idaho	5.0	1.8	1.8	20.0	20.0
B. brongniartii ATCC 9452	Mummified larvae, Indonesia	3.6, 3.0	3.6	3.6	4.5	19.0
B. caledonica DAOM 191855	NA	5.0	1.8	1.8	20.0	20.0
B. densa DAOM 57904	Decaying tree, Canada	5.0	2.2	2.2	20.0	20.0
T. nivea ATCC 18981	NA	3.4, 0.51	20.0		20.0	20.0
T. cylindrospora ATCC 56519	Soil, Czechoslovakia	7.8, 0.42	6.4	6.4	6.4, 2.7	7.8, 2.7
M. anisopliae						
SL 297	NA[f]		7.5	19.0	19.0	19.0, 0.47
SL 549	NA		19.0	19.0	19.0	19.0, 0.47
SL 2165	NA			19.0	19.0	19.0, 0.47
V. lecanii ATCC 46578	NA	5.0, 10.3	1.8	1.8, 1.7	20, 10.3	20, 10.3
P. farinosus ATCC 1360	NA	5.5	1.8	1.7	4.5, 1.7	4.5

[a] Obtained from R. G. Lidstone, Department of Applied Microbiology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.
[b] Obtained from the American Type Culture Collection, Baltimore, Md.
[c] Obtained from the Canadian Collection of Fungus Cultures, Ottawa, Ontario.
[d] NA, not available.
[e] Strains USSR 2274 and USSR 2533 were obtained as strains UFC 423 and ASFC 424 from the ALL Soviet Collection Center for Microorganisms, Moscowoblast, Russia, through Vitaley Sencenko.
[f] Obtained from R. St. Leger, Boyce Thompson Institute, Ithaca, N.Y.

iographed on Kodak X-ray film at −20°C for 1 to several days.

RESULTS

DNA probe hybridization. The results of individual DNA probe hybridizations are provided in Table 2. The DNA probes used in this study, which represent approximately one-half of the 28.5-kb mt genome of our laboratory strain GK2016, have been partially or completely sequenced and analyzed (19). The mt small rRNA (*srRNA*) and large rRNA (*lrRNA*) genes of *B. bassiana* were previously shown to be highly conserved when compared with those of other filamentous fungi (19). These genes are located on the pBbmtE3-pBbmtE4 and pBbmtSE1-pBbmtXS1 plasmids, respectively. Hybridization with either of the *srRNA* gene probes resulted in identical 1.8-kb DNA bands for all of the *B. bassiana* strains examined (Fig. 1A and B). Strain GK2016 is known to possess a 1.8-kb *Eco*RI doublet in the mt genome with the E3 and E4 fragments encoding the 5′ and 3′ portions of the mt *srRNA* gene, respectively. Present results indicate that this doublet is also present in all 15 *B. bassiana* strains examined.

When the *lrRNA* gene-encoding probes were utilized, a single high-molecular-weight band of approximately 20 kb was observed for all of the *B. bassiana* isolates. The only exception was strain USSR 2274, which produced a 2.8-kb band when probed with pBbmtSE1, which encodes the *lrRNA* 5′ region, and a 20-kb and a minor 2.8-kb band when probed with pBbmtXS1, which encodes the 3′ region of the mt *lrRNA* gene (Fig. 1C and D). Hybridizations using the pBbmtE2 probe produced either a 4.1-kb (4 strains) or a 5.0-kb (11 strains) hybridization band depending upon the strain tested (Fig. 1E). On the basis of the pBbmtE2 hybridization patterns, we have grouped the *B. bassiana* populations into two categories. The more prevalent category possessing the 5.0-kb band has been designated type A, and the other exhibiting the 4.1-kb band has been designated type B. There was no apparent relationship between geographical locale or host insect source from which the fungus was obtained and the prevalence of a particular mt type (Table 2).

Hybridization of individual probes to *Eco*RI-digested DNA from other *Beauveria* species or other entomopathogenic fungi produced either no bands, single bands, or multiple bands (Table 2). *B. densa* ATCC 57904 possessed 20.0- and 5.0-kb bands similar to those of *B. bassiana* when probed with either pBbmtSE1 and pBbmtXS1 or pBbmtE2 probes, respectively. However, when probed with the *srRNA*-encoding plasmids, a 2.2-kb band, presumably a doublet, was present. In addition, *B. caledonica* possesses 20-, 5.0-, and 1.8-kb *Eco*RI hybridization bands identical to those exhibited by the *B. bassiana* mtDNA of type A. Subsequent analysis using *Hin*dIII-restricted DNA probed with pBbmtE3 revealed that the mtDNAs of *B. densa* and *B. caledonica* could be differentiated from one another as well as from that of *B. bassiana* (Fig. 2). mtDNAs of four *B. bassiana* isolates, including two type A (GK2016 and ATCC 44860) and two type B (DAOM 210569 and ARSEF 2881), examined in this manner demonstrated the presence of identical 2.3- and 2.6-kb hybridization bands. *B. densa* produced a single 5.5-kb band, whereas *B. caledonica* exhibited two bands of 6.0 and 6.5 kb. Sequence analysis of the E3 fragment encoding the 5′ region of the mt *srRNA* gene revealed that a *Hin*dIII restriction site was present in the noncoding region between the terminus of the mt *ATP6* gene and the start of the *srRNA* gene.

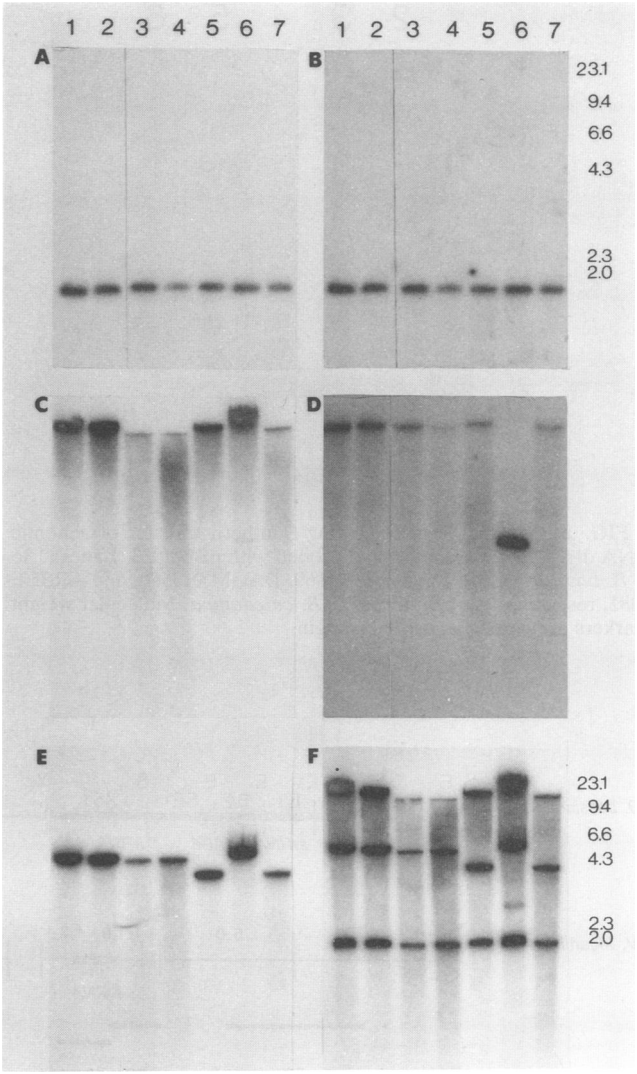

FIG. 1. Autoradiograph showing Southern transfer of *B. bassiana* genomic DNA digested with *Eco*RI and hybridized to mt probes. Lanes: 1 to 7, GK2016, ATCC 44860, DAOM 144746, DAOM 195005, DAOM 210569, USSR 2274, and USSR 2533, respectively. The probes used were pBbmtE3 (A), pBbmtE4 (B), pBbmtXS1 (C), pBbmtSE1 (D), pBbmtE2 (E), and multiprobe (F). Molecular weight markers for panels A to E are shown in the right margin adjacent to panel B. Panel F molecular weight markers are shown in the right margin of that panel.

Hybridization patterns generated by the *B. bassiana*-derived probes were used to derive partial restriction enzyme maps of the mt genomes of *V. lecanii*, *T. cylindrosporum*, and *P. farinosus*. Because the *B. bassiana srRNA* and *lrRNA* genes are located on two different gene probes, overlapping hybridization patterns could be used to define the physical location of these genes relative to *B. bassiana* on the maps of the other fungi (Fig. 3).

RFLP. We hypothesized that the simultaneous use of the entire set of mt probes in hybridization assays would provide further evidence of their utility for the identification of *B. bassiana* isolates. All of the *B. bassiana* isolates possessed

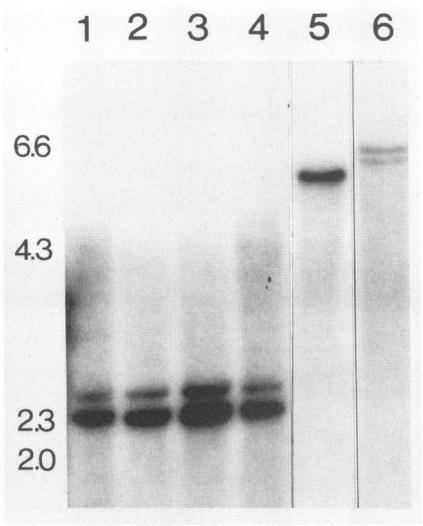

FIG. 2. Autoradiograph showing Southern transfer of genomic DNA digested with *Hin*dIII and probed with pBbmtE3. Lanes: 1 to 4, *B. bassiana* GK2016, ATCC 44860, DAOM 210569, and ARSEF 2881, respectively; 5, *B. densa*; 6, *B. caledonica*. Molecular weight markers are shown in the left margin.

three *Eco*RI hybridization bands of 20.0, 5.0 or 4.1, and 1.8 kb (Fig. 1F), with the exception of strain USSR 2274, as expected from the individual probing experiments (Fig. 1; Table 2). The other fungal species, excluding *B. densa* and *B. caledonica*, also exhibited the expected banding patterns (Fig. 4), which were markedly different from that of *B. bassiana*, thus ameliorating the system. As observed in the individual probing experiments, hybridization of the *B. bassiana*-based mt probes to all three *M. anisopliae* isolates was extremely weak under the conditions used, whereas hybridization to the other fungal species was comparatively stronger. In this case, there was a correspondence between probe hybridization data in that the other fungal species examined were characterized by morphologically congruent, white, velvety colonies, whereas the colonies of *M. anisopliae* were green to brown in appearance.

DISCUSSION

The mtDNA offers an excellent system for the study of fungal evolutionary genetics. Fungal mtDNA possesses unique characteristics, including small size, absence of methylation, ease in purification, high copy number, and simple restriction enzyme patterns, which make its analysis ideal for many taxonomic purposes (3). mtDNA is highly susceptible to size reductions and alterations which present themselves as polymorphisms. Evidence indicates that, in

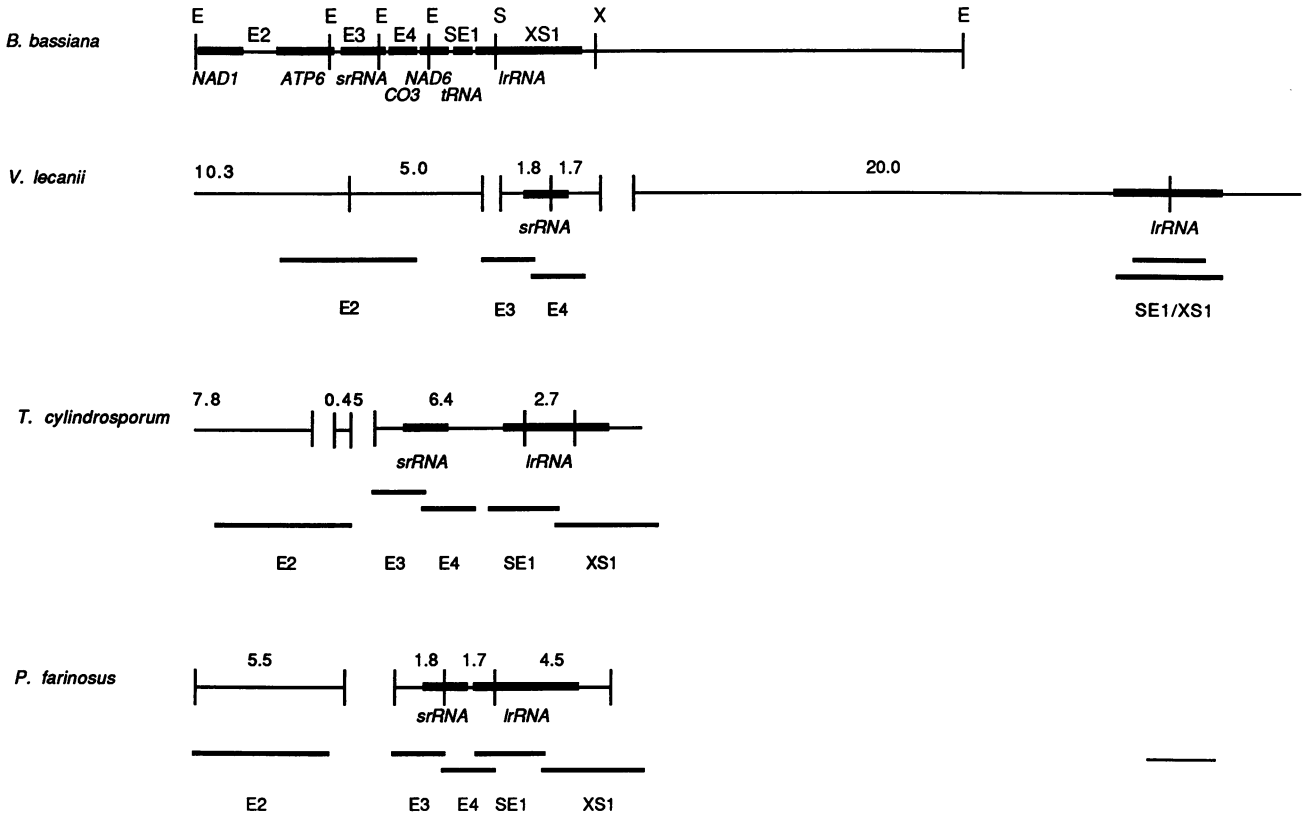

FIG. 3. Complete map of the mt genome of *B. bassiana* in relation to partially mapped *Eco*RI regions of other entomopathogenic fungal genomes. For ease of comparison, all maps were displayed linearly by using the E2 fragment as the primary point of reference. Open ends on maps for *V. lecanii* and *P. farinosus* are contiguous. Restriction enzyme sites, positions of *lrRNA*, *srRNA*, NADH dehydrogenase subunit 1 (*NAD1*), *NAD6*, ATPase subunit 6 (*ATP6*), cytochrome oxidase subunit 3 (*CO3*), and *tRNA* cluster of genes, and the locations of probe hybridizations derived from *B. bassiana* mtDNA (indicated by heavy lines) are shown. The numbers refer to DNA size in kilobases. Abbreviations: E, *Eco*RI; S, *Sal*I; X, *Xho*I. Bar, 2.5 kb.

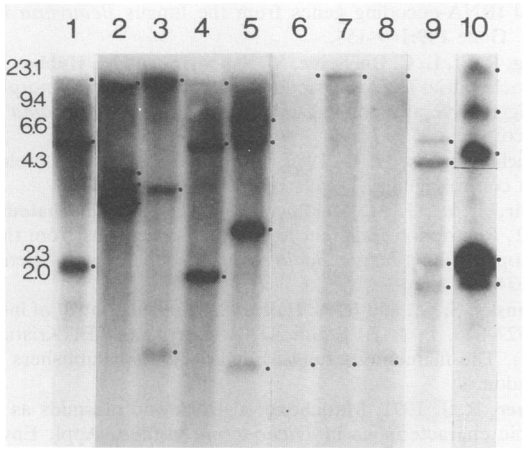

FIG. 4. Autoradiograph showing Southern transfer of fungal genomic DNA digested with *Eco*RI and hybridized to multiple mt probes, namely, pBbmtE2, pBbmtE3, pBbmtE4, pBbmtSE1, and pBbmtXS1. Lanes: 1, *B. densa* DAOM 57904; 2, *B. brongniartii* ATCC 9542; 3, *T. nivea* ATCC 18981; 4, *B. caledonica* DAOM 191855; 5, *T. cylindrosporum* ATCC 56519; 6 to 8, *M. anisopliae* SL 297, SL 549, and SL 2165; 9, *P. farinosus* ATCC 1360; 10, *V. lecanii* ATCC 46578. Molecular weight markers are indicated in the left margin. Dots in the right margin of each lane indicate the positions of hybridization bands.

vertebrates, mtDNA evolves 5 to 10 times faster than the nuclear DNA (32). Fungal mt *srRNA* genes were found to evolve 16 times faster than those of their nuclear counterparts; however, this was highly dependent upon the branch examined (2). In lower vertebrates and invertebrates, the rate of mtDNA evolution varies among the A+T-rich regions, protein, and tRNA genes (4). The mt genome has not been implicated as having involvement in entomopathogenicity, making it an ideal marker for the examination of genetic diversity within unselected gene populations. This conclusion is most clearly indicated by the isolation of both type A and type B mtDNA-containing strains from aphids in Idaho as well as isolation of the less frequently occurring type B from both Canada and countries of the former Soviet Union. In no case did we observe both type A and type B mtDNA within the same strain. This would be indicative of either the complete lack of mechanisms for mt mixing, such as anastomosis, or the expression of vegetative incompatibility resulting in a clonal population structure to reflect the resultant mtDNA.

We have shown the highly conserved nature of the *B. bassiana* mt genome, even within isolates from highly diverse locales. This finding, in conjunction with the extensive differences with other fungi, can be used for its identification. The presence of a unique *Hin*dIII site in the E3 fragment of *B. bassiana* mtDNA but not in that of other closely related species likely reflects the lack of selective pressure to maintain the DNA sequence in a noncoding region of the mt genome. Conversly, the *Eco*RI sites which define the E1 to E4 fragments are located within or directly adjacent to the coding regions of structural RNA or protein-encoding genes and are conserved. However, despite the high degree of conservation, the observed variations may be exploited so that mtDNA restriction fragment patterns may resolve phylogenetic relationships between closely related species (30) or subspecies (17, 31). The use of restriction fragment patterns in combination with cloned DNA probes should greatly enhance the taxonomic placement of any fungal isolate. mtDNA probes which can either identify a given species or differentiate between isolates may be developed. This is evidenced in the larger mt genomes of certain fungi, for example *Candida* spp. (8). Here, complex restriction enzyme patterns with sufficient variation were observed so that epidemiological studies based solely upon mtDNA analysis were possible. In spite of the drawback of a limited sample size, we show that the mt genomes of *B. densa* and *B. caledonica* which are closely related to that of *B. bassiana* could be resolved by restriction enzyme sites within noncoding regions of the genome.

Previous taxonomic evaluations of *Beauveria* spp. have provided conflicting arguments as to the number of true species within this genus. Morphological and biochemical parameters supported a *B. bassiana* subspecies designation for *B. densa* (18). Additionally, genetic diversity assessments of isozymes indicated that *B. caledonica*, rather than a separate species, may be a rare diploid strain (27). Our studies (11) revealed that *B. bassiana*-specific nuclear DNA-based probes recognized all of the *B. bassiana* isolates described in this study but failed to hybridize to either *B. densa* or *B. caledonica* under high-stringency conditions. However, *B. bassiana*-DNA probes, whether species specific or not, produced hybridization patterns identical for *B. densa* and *B. bassiana* under lower stringency conditions (11). These observations, the similarity of the mtDNAs from *B. bassiana*, *B. densa*, and *B. caledonica*, and the high degree of diversity between *B. bassiana* and *Beauveria brongniartii* support the assertion that *B. densa* and *B. caledonica* are subspecies of *B. bassiana*. While this assertion remains to be proven, there is the likelihood that *B. densa* and *B. caledonica* are sister species to *B. bassiana*, sharing ancestral and/or transmitted mtDNA.

We have shown two distinct mtDNA varieties, types A and B, within the *B. bassiana* isolates. More importantly, the presence of a particular mtDNA type does not correlate with geographic locale or host species. On the basis of current knowledge of mtDNA transmission, two hypotheses are put forward. The first is that the two varieties of mtDNA diverged from an ancestral source and were distributed worldwide. This premise depends on either one of two assumptions. The first assumption is that the mt genomes provide a selective advantage to the ecology of the fungus for its entomopathogenic interaction with its host(s). However, this is not plausible given that two different mtDNA types could be isolated from the same host and geographic origin. The second assumption is that distinct mtDNA populations arise from mutational events by which certain varieties are maintained because of mt incompatibility. Any subsequent variations would be the result of additional mutational events and then new clonal development or selection within the population. An alternative hypothesis to the ones above is that the mtDNAs interconvert and that competition within a given ecological setting or the expression of rejection responses produces a predominately clonal derivative of the heteroplasmon (10). Recent observations of anastomosis in basidiomycetes have revealed that while nuclei freely mix between non-self cells, mt express a stringent rejection mechanism and migrate away from the fusion region. In fact, the rejection response can be so absolute that sibling clonal lines, which presumably possess the same mtDNA, also fail to exhange mt. This has helped in the postulate that distinct nuclear-mt relationships are critical for the expression of rejection responses (23). The existence of the two mtDNA types provides a means for testing mt compatability in these fungi. However,

additional genetic or physical markers will be required to examine the notion whether recombination can occur at the molecular level.

We recognize that, in many situations, the integration of chemotaxonomic and molecular biological methods with pairwise sequence comparisons will be required to delineate the true picture of *Beauveria*, a diverse and complex genus. Universal primer sequences for the highly conserved nuclear and mt rRNA genes (3) or random amplified polymorphic DNA methods (7, 28) have been described and may be highly useful in this respect. This will become increasingly important as issues regarding virulence, host specificity, and pathogen tracking become of concern. mt RFLPs currently being exploited for such purposes include host preference of mycorrhizal fungi (6) and the differentiation of aggressive and nonaggressive strains of *O. ulmi*, the causative agent of Dutch elm disease (13). Our discovery of mtDNA polymorphisms in *B. bassiana* will undoubtedly permit us to conduct studies, despite the lack of genetic markers, and analyze the mode of mt transmission in this entomopathogenic fungus.

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