EVALUATION OF NUCLEAR AND MITOCHONDRIAL RIBOSOMAL PHYLOGENETIC MARKERS FOR THE TAXONOMIC CLASSIFICATION OF ENTOMOPATHOGENIC FUNGI: A COMPARATIVE STUDY BASED ON ISOLATES FROM UZBEKISTAN

Anaida G. Guzalova[1#], *Kakhramon Ergashev*[2,3#], *Erkin N. Abdullaev*[2], *Zafar F. Izmailov*[3], *Andreas Leclerque*[4,*]

[1]Institute of Microbiology, Academy of Sciences of Uzbekistan, Tashkent, Republic of Uzbekistan
[2]Department of Zoology, Samarqand State University, Samarqand, Republic of Uzbekistan
[3]Department of Genetics, Breading, and Theory of Evolution, Samarqand State University, Samarqand, Republic of Uzbekistan
[4]Molecular Biology Centre (ZMBH), University of Heidelberg, Heidelberg, Germany

[#]Both first authors contributed equally to this study
*correspondance address:
Julius Kühn Institute (JKI) – Federal Research Institute for Cultivated Plants
Institute for Biological Control
Heinrichstraße 243, 64287 Darmstadt, Germany
Tel.: +49-6151-407229
Fax: +49-6151-407290
E-mail: andreas.leclerque@jki.bund.de or leclerque@hotmail.com

Abstract: Five ribosomal phylogenetic markers – an internal fragment of the mitochondrial small rDNA, an internal fragment of the nuclear 18S rDNA, the complete nuclear 5.8S rDNA and both internal transcribed spacer (ITS) elements of the nuclear rDNA cluster - were compared for their respective suitability to provide the basis for a rapid and reliable taxonomic screening of filamentous fungi isolated from insects, with a particular focus on a generic and specific classification of entomopathogenic hyphomycetes belonging to the form-genera *Beauveria*, *Metarhizium*, *Paecilomyces* and *Lecanicillium*. While a low density of phylogenetically useful information made both nuclear coding sequences unlikely candidates for the purpose in question, the mitochondrial marker proved appropriate for strain assignments at the genus level; both ITS elements were found to allow for a generic as well as specific classification. The markers were subsequently used to classify entomopathogenic fungal strains isolated from insects in Uzbekistan that turned out to belong to the species *Beauveria bassiana* and *Metarhizium anisopliae* as well as to the genera *Aspergillus*, *Fusarium* and *Trichoderma*.

Key words: phylogeny, taxonomy, *Beauveria*, *Metarhizium*

INTRODUCTION

The form genera *Metarhizium*, *Beauveria*, *Verticillium* clade B (*Lecanicillium* gen. nov.) and *Paecilomyces* comprise numerous facultative insect pathogens that are of considerable interest with respect to the biological control of pest insects. Several lines of evidence (Shimazu et al. 1988; Fukatsu et al. 1997) suggest that these deuteromycetes are closely related to entomopathogenic ascomycetes of the genus *Cordyceps* (*Pyrenomycetes*, *Clavicipitales*). Nevertheless, as no teleomorphs are obtained, morphological classification relies entirely on the relatively simple anamorphic structures. Recently, the application of molecular phylogenetic studies based on nuclear rDNA sequences (Zare et al. 2000; Sung et al. 2001) have led to a reorganization of the form-genus *Verticillium*, its subdivision into four distinct clades, termed A through D, and the introduction of new genera with the name *Verticillium* now being reserved for strains falling under clade A (Gams & Zare 2001). In the following, we still use the name *Verticillium* in its old extension; to take into account the taxonomic reorganization the respective clade letter is indicated in brackets. Interestingly, these clade distinctions coincide largely with differences in host range, with entomopathogenic and mycoparasitic isolates clustering in *Verticillium* (B).

In view of an application of imperfect fungi as insect biocontrol agents, reliable classification criteria are the more required as other fungi frequently isolated from the same host, e.g. *Aspergillus*

flavus, are pathogenic not only to insects, but also to higher animals, including humans (St. Leger et al. 2000).

Departing from the assumption of an evolutionary early „RNA world" (Crick 1968; Gilbert 1986), the sequences of rDNAs (i.e. genes encoding ribosomal RNAs) have become prominent molecular phylogenetic markers (Pace et al. 1986; Olsen & Woese 1993; Taylor 1995). In the eukaryotic cell, rDNAs occur in the nuclear as well as in the mitochondrial genome. In fungi, nuclear rDNAs are organized in large repetitive clusters of a complex rDNA transcription unit containing the 18S, the 5.8S and the 28S rRNA genes. Within these clusters, transcription units are separated from each other by so-called (non-transcribed) intergenic spacer (IGS) elements. Individual ribosomal RNA subunits are generated from a 35S rRNA precursor by a series of posttranscriptional splicing events that eliminate two internal transcribed spacer (ITS) sequences that separate the central 5.8S from the upstream 18S and the downstream 28S rRNA (Woolford & Warner 1991). The two genes encoding the small and large mitochondrial rRNA are generally organized as two distinct transcriptional units in fungi.

Both nuclear (Rakotonirainy et al. 1991; Bidochka et al. 1999) and mitochondrial (Li et al. 1994) rDNA sequences have been used as molecular markers in the phylogeny of entomopathogenic and related deuteromycetes. As mitochondrial genomes are thought to be subject to several fold higher rates of mutation (Brown et al. 1979; Vawter & Brown 1986), sequence comparisons for both types of rRNA encoding sequences are assumed to be useful tools for phylogenetic investigations at different taxonomic levels. In addition to the highly conserved rRNA encoding sequences, nuclear rDNAs contain several types of presumably more variable sequences as, on the one hand, the abovementioned IGS and ITS elements that have both been widely used in phylogenetic studies applying RFLP or AFLP **(**Neuvéglise et al. 1994; Aquino de Muro et al. 2003**)** as well as DNA sequencing approaches (Shih et al. 1995; Coates et al. 2002a). On the other hand, the nuclear genes encoding both the 18S (Nikoh & Fukatsu 2000; Suh et al. 2001) and the 28S rRNA (Neuvéglise & Brygoo 1994) have been reported to contain introns in numerous fungal strains. Comparison of intron sequences from both rDNAs has been shown to be a useful tool of phylogenetic analyses with the important drawback that only very few isolates contain the complete set of introns (Neuvéglise et al. 1997; Coates et al. 2002b; Wang et al. 2003).

Within the wider framework of a research project dealing with the isolation of fungal entomopathogens from agricultural pests, namely the Colorado potato beetle, *Leptinotarsa decemlineata*, in Central Asia, our concern with the study presented here was to identify molecular markers that permit to reliably and positively assign new fungal isolates at the genus or species level to one of the aforementioned form-taxa. Aiming towards a method suitable for the taxonomic screening of high numbers of isolates and taking practical constraints into account, markers in question should be shorter than 1kb to facilitate PCR amplification with non-matching or degenerate primers and nevertheless contain enough variable, phylogenetically informative positions to render classifications insensitive to a low level of nucleotide misincorporations during amplification or erroneous sequencing. Here we report on the evaluation of five genetic markers – a fragment of the mitochondrial small rDNA, a fragment of the nuclear 18S rDNA, the complete nuclear 5.8S rDNA and both internal transcribed spacer (ITS) elements of the nuclear rDNA cluster – for this very purpose. Fungal standard strains of well supported taxonomy were employed to test each marker's suitability; if suitable, the respective marker was used to classify entomopathogenic fungal isolates from Uzbekistan thereby corroborating or rejecting their morphology based previous classification.

MATERIAL AND METHOD

Fungal strains

The thirteen fungal strains from Uzbekistan used in this study (Table 2) were isolated from moribund or dead mycosed insects found in the field. Single spore-derived colonies were selected on Czapek medium containing varying concentrations of dodine and the dye Bengal Rose (Goettel & Inglis 1997). The morphology of sporulating pure cultures as revealed by light-microscopy was taken as the criterion for preliminary taxonomic classification.

The following fungal strains, received from the USDA-ARS Collection of Entomopathogenic Fungi, Ithaca, New York, USA, and the fungal strain collection of the All-Russian Institute of Plant

Protection (VIZR) in Sankt-Peterburg, Russia, were included in our investigation as internal standards: *Beauveria bassiana* VIZR#13, *Beauveria bassiana* ARSEF#252, *Beauveria bassiana* ARSEF#1106, *Metarhizium anisopliae* var. *anisopliae* ARSEF#2575, *Paecilomyces fumosoroseus* ARSEF#4700. Furthermore, sequences already available in the GenBank database were referred to (see Appendix, Tables A1-A3).

Bioassays

To check if the fungi isolated from insect cadavers were themselves entomopathogens and not just saprophytes, bioassays using the Colorado potato beetle, *Leptinotarsa decemlineata*, and the wheat shield bug, *Eurygaster integriceps*, were carried out. Twenty adult insects were placed on young potato and wheat plants, respectively, that were subsequently sprayed with 5 ml of a suspension of 1.5 x 10^9 conidia/ml in 0.02 % Tween 20; controls were sprayed with 0.02 % Tween only. For each fungal isolate, the bioassay was performed with five plants and repeated twice. Mortalities were recorded for up to 15 days after application of the conidial suspension.

DNA isolation

Fungal total DNA was isolated from ground mycelium and purified by passage over an AXG NucleoBond column (Macherey & Nagel). For that purpose, 400 ml of liquid YPD medium (0.2 % Bacto yeast extract, 1 % Bacto peptone, 2 % glucose monohydrate) containing 60 µg/ml ampicillin were inoculated with 4 ml of a one week old, generally fully sporulated liquid culture of the respective strain and incubated at 25 °C, 150 rpm, for approximately 24 h. Mycelia were harvested by vacuum filtration over three layers of Miracloth (Calbiochem), washed with water, dried briefly, frozen in liquid nitrogen and ground to a fine powder. Without allowing to thaw, the latter was suspended in 20 ml 1x lysis buffer (20 mM Tris-Cl at pH=8.0, 25 mM EDTA, 250 mM NaCl, 1 % SDS) containing 100 mg/ml Proteinase K and incubated several hours at 65 °C. After separating from remaining cellular debris by centrifugation at 4000 rpm and room temperature for 5 min and extracting proteinaceous contaminants with equal volumes of phenol and chloroform, DNA was recovered from the lysate by adsorption to and elution from an AXG500 NucleoBond column according to the manufacturer's standard protocol, precipitation with 0.7 volumes of isopropanol, and dissolution in TE buffer (pH=8.0). The quality of the DNA preparation was controlled by 1 % agarose gel electrophoresis and staining with ethidium bromide as well as photometrically by determination of the OD_{260}/OD_{280} ratio.

Amplification of Markers by PCR and Sequencing

PCR reactions were performed using the Taq Polymerase (Promega) at 1unit per 150 µl in reaction mixtures containing 1.5 mM $MgCl_2$, 250 µM of each deoxyribonucleotide (dNTP), 1 µM of each oligonucleotide primer, and 3 ng/µl of genomic DNA template. For each marker, a 150 µl reaction mixture containing all components was prepared on ice; three times 45 µl were aliquoted to 200 µl thin-walled PCR tubes. Independent amplification from the three identical reaction mixtures was carried out in a T3 thermocycler (Biometra) using the following cycling parameters: initial denaturation at 94 °C for 10 min, 25 cycles of denaturation at 94 °C for 1 min, annealing at 50-60 °C for 1 min and elongation at 72 °C for 1 min, followed by a final 5 min elongation step at 72 °C.

The sequences of the primers used in the different amplification reactions are given in Table 1. The universal primer pair ns7/ns8 (White et al. 1990) amplifies an approximately 370bp long internal fragment of the nuclear 18S rDNA. PCR reactions containing primers its1 and its4 (White et al. 1990) are designed to amplify a nuclear DNA segment of an expected size of 570bp comprising the 5.8S rRNA encoding gene flanked by both internal transcribed spacer (ITS) sequences. Finally, primers nms1 and nms2 (Li et al. 1994) together with nms3 and nms4 form a nested primer set with nms1 and nms3 being collinear to each other as are nms2 and nms4, with nms3 and nms4 located downstream of nms1 and nms2, respectively.

In order to check for fragment size and possible side products, 3 µl of each PCR reaction were separated electrophoretically on a 1 % agarose gel stained with ethidium bromide. For sequencing, PCR products were ligated into cloning vector pCR-XL-TOPO and transformed into electrocompetent *Escherichia coli* TOP10 cells (available with the TOPO-XL PCR Cloning Kit, Invitrogen) according to the manufacturer's instructions. Plasmid DNA was isolated from overnight cultures of recombinant

clones using the NucleoSpin Kit (Macherey & Nagel). Purified plasmids were digested in parallel with restriction endonucleases EcoRI and NsiI, both cutting plasmid pCR-XL-TOPO on either side of the site of insertion. The size of inserted DNA fragments was controlled by conventional agarose gel electrophoresis of these digests; expectedly sized insertions were sequenced on both strands, using universal primers M13F and M13R.

Sequences have been submitted to the GenBank database under accession numbers AY755520 to AY755537 for the mitochondrial rDNA fragments, AY755538 to AY755554 for the 18S rDNA fragments, and AY755502 to AY755519 for the 5.8S rDNA regions including ITS elements.

Sequence data analysis

Performing PCR reactions in triplicate and sequencing each reaction product on both DNA strands, for each marker a six fold set of sequence data was obtained per fungal isolate that was subsequently condensed into a single consensus sequence. These consensus sequences were used for comparisons among different fungal strains. This clumsy approach was followed to rule out sequence alterations that might have occurred during amplification or incorrect sequencing.

Consensus sequences were generated from primary sequence data using the EditSeq and MegAlign programs of the LaserGene package and compared in ClustalX (Thompson et al. 1999; Chenna et al. 2003). Phylogenetic analyses including searches for maximum parsimony trees, confidence limit assessments based on the bootstrap resampling algorithm (Felsenstein 1985), and construction of sequence distance matrices were carried out using PAUP* 4.0 beta 10 (Swofford 2002). Throughout the paper, sequence distance values are stated as percentage of non-identical unweighed characters. The TreeView program was used for the basic graphic representations of cladograms that were further refined in Canvas 7.

RESULTS

Morphological classification and entomopathogenicity of fungal isolates from Uzbekistan

Table 2 contains the description of isolates UZB#1-16, listing the host, the results of a taxonomic classification based on purely morphological characteristics as well as an estimate of the pathogenicity for the respective host assessed as described in the Materials and Methods section.

Phylogenetic analysis of mitochondrial small rDNA sequences

Initial attempts to amplify an internal fragment of the mitochondrial small rRNA encoding gene from total genomic DNA preparations using primers nms1 and nms2 turned out not to give very reliable results. Therefore, primers nms3 and nms4 that hybridize to a location immediately downstream of the supposed nms1 and nms2 priming sites, respectively, were created and further PCR reactions run in the presence of the complete nested set of four primers. In all reactions, at least one of the possible PCR products of the expected fragment lengths of 630bp (nms1/nms2), 600bp (nms1/nms4 and nms3/nms2) or 570bp (nms3/nms4) was generated. For sequence comparison, the whole stretch of DNA between the two inner primers of the nested set, nms3 and nms4, was considered.

Of the 643 characters considered in the phylogenetic analysis of this fragment of mitochondrial rDNA, only 35 % are parsimony-informative (Table 10). Consistent with this rather moderate density of character information, the salient feature of the consensus tree (Figure 1) derived from the results of a heuristic (TBR branch swapping) parsimony analysis of the sequence data is a well established (100 % bootstrap support) dichotomy above the genus level. With the *Aspergillus flavus* sequence behaving expectedly as an outgroup, the mitochondrial rDNAs of fungal standard strains are grouped into two distinct clades, comprising, on the one hand, the genera *Beauveria, Paecilomyces,* and *Verticillium* (B) and, on the other hand, the genera *Metarhizium, Fusarium,* and *Trichoderma*. For sake of terminological simplicity, both clades will in the following be referred to as „BPV clade" and „MFT clade", respectively.

For the fungal standard strains included into the phylogenetic tree, Table 3 compares the *inter*generic sequence distances *within these clades* ranging from 4.9 % to 7.9 % for the BPV and from

6.1 % to 15.4 % for the MFT clade, with values for *inter*generic comparisons *across clade borders* ranging of 14.7 % to 23.6 %, and *infra*generic sequence distances varying widely for different genera.

Furthermore, when the isolates from Uzbekistan given in Table 2 were integrated into this background, each of them showed a significantly higher degree of sequence identity to one of the aforementioned genera than to the remaining (Table 4), the only exception being the presumed *Fusarium* strains UZB#9 and UZB#10.

Phylogenetic analysis of the internal transcribed spacer (ITS) sequences of nuclear rDNA cluster

Using primers its1 and its4, DNA fragments somewhat less than 600bp in length were amplified from genomic fungal DNA preparations as described above; repeated trials to amplify the 5.8S rDNA region from isolate UZB#7 were unsuccessful. For phylogenetic analysis the consensus sequences generated from the primary sequence data were divided into three segments: the central 158bp corresponding to the 5.8S rRNA encoding gene and the two flanking DNA stretches of slightly variable length that were identified as internal transcribed spacer ITS1 and ITS2 by recognition of the 3´ and 5´ end of the neighboring 18S and 28S rDNAs, respectively.

Expectedly, the percentage of parsimony-informative characters in the non-coding ITS elements is almost twice higher (Table 10) than in the mitochondrial rRNA gene. This is consistent with the much finer filigree structure of both maximum parsimony trees generated by heuristic (TBR) searches on the basis of ITS1 and ITS2 sequence comparisons (Figure 2 & 3). Rooted by the respective sequence from *Aspergillus flavus* both trees confirm with a 100 % bootstrap support the existence of one of the two suprageneric clades already identified on the basis of mitochondrial rDNA sequence comparisons, the "BPV clade". Its further subdivision (93 % bootstrap support) into a seemingly monophyletic *Beauveria* clade and a *Paecilomyces/Verticillium* (B) clade in the ITS1 tree is not confirmed by the analysis of ITS2 sequences. In contrast to the BPV clade, the MFT clade of both trees shows a considerable tendency to disintegrate; for the ITS2 tree the situation is further complicated by the unclear position of a phytopathogenic *Verticillium* (A) standard strain that flip-flops in different heuristic tree searches between the positions of an MFT clade ingroup or an additional outgroup. In both trees, *Trichoderma* standard strains form a distinct and stably structured clade; the same is not true neither for *Metarhizium* nor *Fusarium*. Interestingly, while *Verticillium* (B) strains cluster together as part of the BPV clade, strains from the *Verticillium* clades A, C and D comprising plant, nematode, and nematode cyst pathogens tend to group loosely with *Metarhizium* and *Fusarium* strains.

The disparity between the BPV and MFT clades apparent from both ITS based cladograms is reflected by an analysis of intra- and intergeneric ITS sequence distances (Table 5 for ITS1; analogous ITS2 data not shown). While intergeneric ITS1 sequence distances *within the BPV clade* range from 10.7 % to 16.7 %, those *within a supposed MFT clade* (32.2 % - 47.9 %) fall in the same range as intergeneric differences *across BPV and MFT clade borders* (28.2 % – 47.0 %). *Infra*generic ITS1 and ITS2 sequence differences range from very low values to almost intergeneric levels for *Metarhizium* and *Trichoderma*. A consistent ITS sequence based strain classification at the genus level might thus seem problematic in these cases. However, an analysis of inter- and infra*specific* sequence distances (Table 6) indicates that – for the species represented in this study – ITS sequences of e.g. *Metarhizium anisopliae* and *M. flavoviride* strains diverge to a significantly higher degree if compared *across* species boundaries than *within*, this difference being much smaller in the case of *Beauveria bassiana* and *B. brongniartii*. Hence, a specific classification of isolates could be possible where a generic is not.

When isolates UZB#1 through UZB#15 are integrated into the framework of intergeneric sequence distances put up by the comparison of standard strains, all isolates show a much higher degree of sequence identity with strains from one particular genus (data not shown), the effect being more pronounced than in the case of the mitochondrial rDNA marker. Table 7 shows the result of going further into the details of the interspecific relationships within the most closely related genus for each Uzbek isolate. For all isolates, the results obtained with both ITS markers for the most closely related species among those considered are consistent, with the expected discrepancies in the sequence distance value gap between the proposed assignments and its alternatives, e.g. relatively close values in the alternative of an assignment to either *B. bassiana* or *B. brongniartii* compared to a wide sequence distance gap in the case of the two *Metarhizium* species considered.

Phylogenetic analysis of nuclear 5.8S rDNA sequences

With only 19 out of the 158 nucleotides that make up the complete 5.8S rDNA sequence contributing to its phylogenetic analysis as parsimony-informative characters (Table 10) and a parsimony consensus tree length of not more than 35 character variations, the marker leaves little space for subtle discriminations at or below the genus level. This is evident in the 5.8S rDNA based phylogenetic tree (Figure 4) by the high number of both zero length branches and collapsed nodes as well as by the fact that the distinction between *Verticillium* clades B and C vanishes if 5.8S rDNA sequences alone are taken into consideration. The little pronounced discrepancies between inter- and infrageneric sequence distances deducible from Table 8 shed doubt on the possibilities of a generic strain classification based on the 5.8S marker alone. This is exemplified by the respective values for isolates UZB#1 through UZB#16 given in Table 9, with e.g. the 5.8S rDNA sequences from isolates UZB#9 and UZB#10 displaying a similarly high degree of identity to sequences from both *Fusarium* and *Metarhizium* strains. At the species level, the 5.8S rDNA marker almost completely lacks power of resolution, with e.g. the 5.8S rRNAs from the four *Trichoderma* species considered being 100 % identical (data not shown).

Phylogenetic analysis of nuclear 18S rDNA sequences

Primers ns7 and ns8 were used to amplify an internal fragment of approximately 370bp of the nuclear gene encoding the 18S rRNA as described in the Materials and Methods section. Amplification reactions worked well with all strains despite the fact that the ns7 priming site of the nuclear rDNA of *Beauveria bassiana* (Suh et al. 2001) and *B. brongniartii* (Nikoh & Fukatsu 2000) is frequently disrupted by an intron.

The character content information found for the amplified 18S rDNA fragment is very similar to that presented above for the 5.8S rDNA gene, with only 11 % of the 333 nucleotides forming the DNA stretch between primers ns7 and ns8 being parsimony-informative (Table 10). This scarcity of information is clearly reflected in the rather short and undifferentiated maximum parsimony tree constructed from ns7/ns8-fragment sequences (Figure 5). The resolution between inter- and infrageneric as well as inter- and infraspecific sequence distance values (data not shown) is still less obvious than in the case of the 5.8S rDNA sequences.

Discussion

The aim of the study presented here was to evaluate phylogenetic markers for the classification of fungal strains isolated from insects, focussing on a reliable assignment to the main form-genera of entomopathogenic hyphomycetes and the subdividing form-species. Robustness of classification was taken to be an important feature to allow for the taxonomic screening of numerous isolates in an experimental set-up basically consisting in a single amplification reaction followed by sequencing the PCR product once on both strands. By commitment to such an approach that sets limits for the length of marker sequence to be considered and is prone to a low level of non-systematic errors in marker sequence determination, a sufficient density of phylogenetically useful information becomes a key evaluation parameter.

Table 10 shows that in agreement with the presumed selection pressure they are subject to the five markers evaluated for the respective purpose differ markedly in this characteristic. While the investigated coding sequences of the nuclear rDNA cluster show the least (11 - 12 %) and the non-coding ITS elements the highest (around 60 %) character information content, an intermediate value (35 %) has been determined for the mitochondrial small rDNA fragment. Consequently, the sequences can be considered useful as phylogenetic markers for the classification at different taxonomic levels.

For the investigated 5.8S and 18S nuclear rDNA sequences, the differences between intergeneric and infrageneric sequence distances were found to be too small to permit a well founded generic classification of entomopathogenic deuteromycetes. The 0.6 % gap, for example, that separates the sequence distance value of a *Beauveria–Beauveria* from that of a *Beauveria–Verticillium* (B) 5.8S rDNA sequence comparison (Table 8), corresponds to a single basepair exchange in the sequence of 158 nucleotides altering app. 5 % of the total phylogenetic information the marker offers.

A single nucleotide misincorporation during PCR amplification of the 5.8S marker from a new isolate would therefore suffice to cause the latter’s assignment to the wrong genus.

Although the 18S rDNA marker has been used for generic classifications of fungi if a larger part of its sequence - possibly including highly variable intron sequences - is considered, the characteristics of the fragment investigated here are still less favorable than that of the 5.8S marker.

While 18S rDNA intron sequences will presumably perform much better in this respect, they were not considered here for the reasons stated in the introduction section of this paper. Both the nuclear 5.8S rDNA and the ns7/ns8-fragment of the nuclear 18S rDNA are therefore judged inappropriate for the type of investigation in question. Within the framework of this study, the 5.8S rDNA marker's well known suitability for molecular taxonomic investigations *above* the genus level is clearly indicated by the unequivocal respective assignment of isolate UZB#16 to the outgroup in Figure 4 and Table 9 (last line).

In contrast, when nms3/nms4-fragments of the mitochondrial small rDNA are compared, intergeneric sequence distance values are at least 5 % higher than their infrageneric counterparts for several genera, e.g. *Beauveria* or *Trichoderma* (Table 3), a difference corresponding to a simultaneous change in over 30 positions of the sequence of 643 nucleotides, that should be sufficiently large to lay the basis for a reliable generic classification. Unfortunately, for other genera there is no gap at all separating infra- from intergeneric sequence distances. Thus, on the one hand, the distances between the mitochondrial small rDNA sequences of strains from both main species of the genus *Beauveria, B. bassiana* and *B. brongniartii*, seem to be at least four fold smaller than the values for any respective intergeneric relationship involving *Beauveria*; on the other hand, the top end sequence distance values found within the genus *Fusarium* are not lower than the smallest values determined from intergeneric comparisons with *Fusarium* being one of the *relata*.

Obviously, this reflects a discrepancy in the compactness of different genera with respect to their species-structure. In the following we will refer to this difference by the notion of apparently "homogeneous" and "heterogeneous" genera, defining this concept as with respect to a particular molecular marker; the higher the degree of homogeneity of a genus, the more reliable the respective generic classification of fungal isolates by means of the marker in question. Hereby it goes without saying that this apparent homogeneity of a genus might be changed considerably by the inclusion of new species and that – due to the relatively small number of nms3/nms4-fragment sequences available for comparison - our study can by no means intend to draw general conclusions concerning the marker-specific homogeneity of the genera in question.

Our intention with the introduction of this terminology is merely to point out how the aforementioned problem with the *generic* classification in the case of heterogeneous genera might - perhaps against intuition - be solved stepping forward to an immediate *specific* classification within the latter. For example, the mitochondrial rDNA fragments from isolates UZB#9, UZB#10, and UZB#14 show both sequence distances of below 1 % relative to *Fusarium solani* and of 10 % or higher compared to *F. fujikuroi* and *F. oxysporum* (Table 4) and could thus - with reservation as to the inclusion of marker sequences from further *Fusarium* species - be assigned to this first species. In contrast, the mitochondrial rDNA marker does not seem to provide any basis of a corresponding specific classification of *Beauveria* strains, distinguishing *B. bassiana* from *B. brongniartii*. If one follows the further suggestion that the above-defined heterogeneity of genera as *Fusarium* might be a consequence of an underlying polyphyletic structure that is likely to be resolved by future taxonomic revisions splitting the genus into different monophyletic clades, the proposed "specific" classification within apparently heterogeneous genera might in the end turn out to be essentially generic.

Compared to the mitochondrial rDNA marker, the difference between intergeneric and infrageneric sequence distances is still larger for both internal transcribed spacer elements of the nuclear rDNA cluster, being in the range of 10 % or more (Table 5). Therefore, erroneous assignment to the next closely related genus would require the simultaneous change of more than 20 nucleotides of the marker sequence. A problem in the strain assignment to genera as *Fusarium* or *Metarhizium* that are apparently heterogeneous with respect to the ITS markers, might be solvable by the approach discussed above for the mitochondrial rDNA marker, i.e. by skipping the genus level and proceeding to a specific classification that might turn out to become a generic one due to future taxonomic revisions. Furthermore, and in contrast to the mitochondrial rDNA marker, the distinctions drawn using the ITS markers are sufficiently filigree to allow for the species-level classification in genera that do *not* show a respective marked heterogeneity. Thus, the difference between inter- and

infraspecific ITS sequence distances permits the differential assignment of strains to either *B. bassiana* or *B. brongniartii*.

Our phylogenetic analyses based on comparisons of the ITS sequences and the nms3/nms4-fragment of the mitochondrial small rDNA coincide in supporting a close phylogenetic relationship of the form-genera *Beauveria, Paecilomyces* and *Verticillium* (B), forming what has been termed above the "BPV clade". In contrast, the form-genus *Metarhizium* that has been longtime believed to be correctly placed in the immediate phylogenetic vicinity of these, is found to be obviously much more closely related to *Fusarium* and the mycoparasite *Trichoderma*.

Interestingly, this remains even true if the revised taxonomy of the (former) genus *Verticillium* with its correspondence of clades and host ranges is referred to as an external standard: while fungal isolates belonging to *Verticillium* clade B that comprises entomo- and mycoparasitic strains, form a subdomain of the BPV clade, plant and nematode pathogens from *Verticillium* clades A, C, and D - instead of, as one might expect, forming an outgroup relative to the BPV clade together with the insect pathogen *Metarhzium* and the mycoparasite *Trichoderma* – intercalate into the *Metarhizium,* but not into the *Trichoderma* clades of both ITS based maximum parsimony trees, thereby giving further support to a polyphyletic view of the genus *Metarhizium*.

In all but one cases, the taxonomic classification of the entomopathogenic fungal isolates from Uzbekistan based on the mitochondrial small rDNA marker, both ITS elements and – with the reservations stated above – the 5.8S rDNA led to a conclusive result confirming the previous morphological classification (Table 11). Isolates UZB#9 and UZB#10 were identified as *Fusarium* species with highest sequence identity among all sequences accessible through the GenBank database to the internal transcribed spacers of a *F. solani* strain. On the same basis, isolate UZB#12, identified as a *Trichoderma* species, is closest to strains of *T. harzianum*. The discovery of this entomopathogenic *Trichoderma* strain is consistent with the relatively close phylogenetic proximity of the genera *Trichoderma* and *Metarhizium;* it might be an interesting question to address if host switches from insect to fungus or *vice versa* can be induced in isolates from both genera. For isolate UZB#14, the original classification as a *Paecilomyces* sp. could neither be confirmed by molecular means nor be replaced by conclusive assignment to another genus as the results obtained with nuclear and mitochondrial rDNA sequences contradict each other in assigning this strain to *Beauveria* (*bassiana*) and to the genus *Fusarium*, respectively. As we feel that the possibility of a simple contamination of the culture has been ruled out by additional rounds of purification by single spore isolation, the clarification of this point will require further investigation. Isolate UZB#16, previously classified to be an entomopathogenic *Aspergillus* strain, is assigned to the outgroup in phylogenetic analysis of all five molecular markers. Both ITS sequences from this isolate are found to be 100% identical to those of *Aspergillus oryzae* strains, with relatively small sequence distances to *A. flavus* (0.6 % for both markers) and considerably higher values for other *Aspergillus* species, e.g. *A. nidulans* (24.3 % and 15.9 % for ITS1 and ITS2, respectively).

Finally, the nuclear rDNA internal transcribed spacer sequences of one of the standard strains included into our study, the Colombian isolate *Beauveria brongniartii* #9301, display 2.6 % (ITS1) and 3.1 % (ITS2) of sequence distance from other *B. brongniartii* strains, but only 0.0 % - 0.6 % and 0.0 % - 1.9 %, respectively, relative to *B. bassiana*. Therefore, we would like to invite to consider a reclassification of this isolate as a *Beauveria bassiana* strain.

List of abbreviations

AFLP Amplified Fragment Length Polymorphism
EDTA Ethylenediamin-tetraacetate
IGS Intergenic Spacer
ITS Internal Transcribed Spacer
dNTP Deoxynucleosidetriphosphate
PCR Polymerase Chain Reaction
rDNA Ribosomal RNA encoding DNA sequence
RFLP Restriction Fragment Length Polymorphism
SDS Sodium Dodecylsulfate

Acknowledgements

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Table 1

Description of the oligonucleotide primers used in the marker amplification reactions

PRIMER	Sequence
NS7	5´-GAGGCAATAACAGGTCTGTGATGC
ns8	5´-TCCGCAGGTTCACCTACGGA
its1	5´-TCCGTAGGTGAACCTGCGG
its4	5´-TCCTCCGCTTATTGATATGC
nms1	5´-CAGCAGTGAGGAATATTGGTCAATG
nms2	5´-GCGGATCATCGAATTAAATAACAT
nms3	5´-CTGAACTGGCAACTTGGAGAAGTG
nms4	5´-ACTGGTGTCAGAAACGGTCTAGTG

Table 2

Fungal strains from Uzbekistan investigated in this study, their previous taxonomic classification based on morphology criteria, and a rough estimate of their pathogenicity for the respective host using a descriptive scale ranging from non-pathogenic (-) to highly pathogenic (+++). Isolates are referred to as "UZB#…" throughout this study; the respective acronyms assigned to for registration in the Microorganism Collection of the Academy of Sciences of Uzbekistan (for UZB#1-14) and the strain collection of the Samarqand State University (UZB#15-16) are given in brackets in the first column. For strain requests address to Dr. A.G. Guzalova.

Isolate	Original host	Morphological classification	Pathogenicity
UZB #1 (ALG)	*Leptinotarsa decemlineata*	*Beauveria bassiana*	+++
UZB #2 (ALGK)	*Eurygaster integriceps*	*Beauveria bassiana*	++
UZB #3 (B3)	*Leptinotarsa decemlineata*	*Beauveria bassiana*	+++
UZB #4 (B4)	*Leptinotarsa decemlineata*	*Beauveria bassiana*	++
UZB #5 (B5)	*Leptinotarsa decemlineata*	*Beauveria bassiana*	+++
UZB #6 (M99)	*Leptinotarsa decemlineata*	*Metarhizium anisopliae*	+++
UZB #7 (M7)	*Leptinotarsa decemlineata*	*Metarhizium anisopliae*	++
UZB #8 (M8)	*Leptinotarsa decemlineata*	*Metarhizium anisopliae*	++
UZB #9 (F9)	*Leptinotarsa decemlineata*	**Fusarium *sp.***	+
UZB #10 (F10)	*Leptinotarsa decemlineata*	*Fusarium* sp.	+
UZB #12 (T12)	*Leptinotarsa decemlineata*	*Trichoderma* sp	++
UZB #14 (B14)	*Leptinotarsa decemlineata*	*Paecilomyces* sp.	++
UZB #15 (Bb0015)	*Leptinotarsa decemlineata*	*Beauveria bassiana*	+++
UZB #16 (Af0016)	*Leptinotarsa decemlineata*	*Aspergillus* sp.	++

Table 3

Ranges of infra- and intergeneric sequence distances (in %) for the amplified portion of mitochondrial rRNA genes from standard strains. Infracladistic relationships with respect to the distinction of an MTF- and a BVP clade (see text) are in bold face. Infrageneric sequence distances form the diagonal. In the first column, genus designations from the first row are represented by its initial letter.

	Aspergillus	***Paecilomyces***	***Verticillium***	***Beauveria***	**Fusarium**	***Trichoderma***	**Metarhizium**
M	22.5	14.7 – 16.0	16.9	18.0 – 18.9	**11.2 – 15.4**	**6.1**	0.0
T	21.6 – 21.8	15.4 – 16.6	16.9 – 17.1	18.7 – 19.9	**9.2 – 13.6**	1.5	
F	22.4 – 23.6	15.6 – 19.1	17.3 – 20.7	18.0 – 23.6	5.4 – 10.0		
B	25.9 – 26.2	**6.1 – 7.2**	**6.6 – 7.9**	0.4 – 1.3			
V	25.6	**4.9 – 5.3**	-				
P	24.6 – 25.6	4.4					
A	-						

Table 4

Ranges of mitochondrial small rDNA nms3/nms4-fragment sequence distances (in %) between fungal isolates from Uzbekistan and standard strains (clustered by genus). For several isolates, values are given in separate for *Beauveria bassiana / B. brongniartii* or *Fusarium solani / F. oxysporum, F.fujikuroi.* Closest sequence proximities are indicated in bold face.

isolate	*Asp.*	*Beauveria*	*Paecilom.*	*Vert. (B)*	*Fusarium*	*Metar.*	*Trich.*
UZB #1	26.2	**0.2-1.0 / 1.9**	6.3-6.4	6.8	18.4-22.5	18.2	18.9-19.1
UZB #2	25.7	**0.2-1.0 / 1.3**	6.4-6.6	7.1	18.2-22.4	17.7	18.7-18.9
UZB #3	26.2	**0.2-1.0 / 1.9**	6.4	6.8	18.5-22.6	18.2	18.9-19.1
UZB #4	26.0	**0.0-0.8 / 1.7**	6.1-6.2	6.6	18.2-22.3	18.0	18.7-18.9
UZB #5	25.7	**0.2-1.0 / 1.3**	6.4-6.6	7.1	18.2-22.4	17.7	18.7-18.9
UZB #6	23.1	18.6-19.6	15.4-16.7	17.5	11.8-15.9	**0.6**	6.7
UZB #7	22.5	18.0-18.9	14.8-16.0	16.9	11.2-15.4	**0.0**	6.1
UZB #8	22.9	18.2-19.1	15.0-16.4	17.1	11.6-15.7	**0.4**	6.5
UZB #9	22.2	18.0-19.4	15.4-16.6	17.1	**0.0 / 9.8**	11.1	9.1
UZB #10	22.2	18.0-19.4	15.4-16.6	17.1	**0.0 / 9.8**	11.1	9.1
UZB #12	22.6	19.5-20.5	16.2-17.2	17.8	9.4-14.0	6.7	**2.1-2.3**
UZB #14	22.6	18.4-19.7	15.8-16.8	17.5	**0.4 / 11,7-12.5**	12.9	11.2
UZB #15	26.0	**0.0-0.8 / 1.7**	6.1-6.2	6.6	18.2-22.4	18.0	18.7-18.9
UZB #16	**0.2**	26.2-26.4	24.9-25.8	25.8	22.6-23.8	22.7	21.8-22.0

Table 5

Ranges of infra- and intergeneric sequence distances (in %) for the ITS1 elements from standard strains. Infracladistic relationships with respect to the distinction of an MTF- and a BVP clade (see text) are in bold face. Infrageneric sequence distances form the diagonal. Strain *Beauveria brongniartii* #9301 was not considered. In the first column, genus designations from the first row are represented by its initial letter.

	Aspergillus	***Paecilomyces***	***Verticillium* (B*)***	***Beauveria***	**Fusarium**	***Trichoderma***	**Metarhizium**
M	48.2-52.7	37.1-41.3	37.8-41.6	32.9-39.1	**32.2-45.9**	**39.5-46.6**	0.0-29.9
T	50.3-58.7	38.9-47.0	35.5-45.6	34.9-45.6	**37.3-47.9**	0.5-23.5	
F	41.1-44.6	34.8-40.4	32.9-35.4	28.2-32.3	21.1		
B	35.7-37.7	**13.3-16.7**	**10.7-13.2**	0.0-3.2			
V	38.1-38.8	**12.4-13.5**	1.1-3.3				
P	39.3-40.1	0.0-5.5					
A	-						

Table 6

Ranges of infra- and interspecific sequence distances (in %) for both ITS elements from standard strains; dashes separate corresponding values for ITS1 / ITS2. Strain *B. brongniartii* #9301 was not considered.

	B. brongniartii	*B. bassiana*
B. bassiana	2.6-3.2 / 2.5-3.7	0.0-0.6 / 0.0-1.9
B. brongniartii	0.0 / 0.0	
	M. flavoviride	*M. anisopliae*
M. anisopliae	27.8-29.9 / 23.0-25.8	0.0-1.5 / 1.1-2.8
M. flavoviride	6.2 / 8.4	

Table 7

Ranges of internal transcribed spacer (ITS) sequence distances (in %) between fungal isolates from Uzbekistan and standard strains (clustered by species). The morphological classification of isolates is given in brackets following the designation. Dashes separate corresponding values for ITS1 / ITS2. Closest sequence proximities are indicated in bold face. Strain *Beauveria brongniartii* #9301 was not considered.

Isolate	*B. bassiana*	*B. brongniartii*
UZB #1	**0.6-1.2 / 0.0-1.9**	3.2 / 3.1
UZB #2	**0.0-0.6 / 0.6-1.2**	2.6 / 3.1
UZB #3	**0.0-0.6 / 0.0-1.9**	2.6 / 3.1
UZB #4	**0.0-0.6 / 0.6-1.2**	2.6 / 3.1
UZB #5	**0.0-0.6 / 0.0-1.9**	2.6 / 3.1
UZB#14	**0.0-0.6 / 0.0-1.9**	2.6 / 3.1
UZB#15	**0.6-1.2 / 0.0-1.9**	3.2 / 3.1
	M. anisopliae	*M. flavoviride*
UZB #6	**0.0-1.5 / 0.0-1.7**	27.8-29.8 / 23.0-25.2
UZB #8	**0.0-1.5 / 0.0-1.7**	28.0-30.0 / 23.0-25.2
	F. oxysporum	*F. solani*
UZB #9	21.1 / 24.6	**0.0 / 0.6**
UZB#10	21.1 / 24.6	**0.0 / 0.6**

	T. harzianum	*T atro-/viride*	***T. reesei***
UZB#12	**0.0-1.0 /0.0-2.9**	15.6-16.1 /8.2-9.4	15.5 / 5.6

Table 8

Ranges of infra- and intergeneric sequence distances (in %) for the nuclear 5.8S rRNA gene from standard strains. Infrageneric sequence distances form the diagonal. Strain *Beauveria brongniartii* #9301 was not considered. In the first column, genus designations from the first row are represented by its initial letter

	Aspergillus	***Paecilomyces***	***Verticillium(B)***	***Beauveria***	**Fusarium**	***Trichoderma***	**Metarhizium**
M	7.6-8.9	2.5-3.2	1.3-1.9	2.5-3.8	0.6-1.9	1.3-1.9	0.0-1.3
T	7.6	2.5	1.3	2.5-3.2	1.3-1.9	0.0	
F	8.9	2.5-3.2	1.3-1.9	1.9-3.2	1.9		
B	10.1-10.8	2.5-3.2	1.3-1.9	0.0-0.6			
V	8.9	1.3	0.0				
P	8.9	0.0					
A	-						

Table 9

Ranges of 5.8S rDNA sequence distances (in %) between fungal isolates from Uzbekistan and standard strains (clustered by genus). Closest sequence proximities are indicated in bold face. Strain *Beauveria brongniartii* #9301 was not considered

	Aspergillus	***Beauveria***	***Paecilomyces***	***Verticillium(B)***	***Fusarium***	***Metarhizium***	***Trichoderma***
UZB #1	10.1	**0.0-0.6**	2.5	1.3	1.9-2.5	2.5-3.2	2.5
UZB #2	10.8	**0.0-0.6**	3.2	1.9	2.5-3.2	3.2-3.8	3.2
UZB #3	10.1	**0.0-0.6**	2.5	1.3	1.9-2.5	2.5-3.2	2.5
UZB #4	10.8	**0.0-0.6**	3.2	1.9	2.5-3.2	3.2-3.8	3.2
UZB #5	10.1	**0.0-0.6**	2.5	1.3	1.9-2.5	2.5-3.2	2.5
UZB #6	8.9	3.2-3.8	3.2	1.9	1.3-1.9	**0.0-1.3**	1.9
UZB #8	8.9	3.2-3.8	3.2	1.9	1.3-1.9	**0.0-1.3**	1.9
UZB #9	8.9	1.9-2.5	3.2	1.9	**0.0-1.9**	**0.6-1.3**	1.9
UZB #10	8.9	1.9-2.5	3.2	1.9	**0.0-1.9**	**0.6-1.3**	1.9
UZB #12	7.6	2.5-3.2	2.5	1.3	1.3-1.9	1.3-1.9	**0.0**
UZB #14	10.1	**0.0-0.6**	2.5	1.3	1.9-2.5	2.5-3.2	2.5
UZB #15	10.1	**0.0-0.6**	2.5	1.3	1.9-2.5	2.5-3.2	2.5
UZB #16	**0.0**	10.1-10.8	8.9	8.9	8.9	7.6-8.9	7.6

Table 10

Character information content for the five different phylogenetic markers under study. Characters were classified as either constant or variable, with the latter further subdivided into parsimony-informative and -uninformative characters

Sites	**Mitochondrial small rDNA fragment**	**Nuclear 5.8S rDNA**	**Internal Transcribed Spacer 1**	**Internal Transcribed Spacer 2**	**nuclear 18S rDNA fragment**
total (=100%)	643	158	261	225	333
constant	366 (57%)	131 (83%)	80 (30%)	58 (26%)	289 (87%)
uninformative	50 (8%)	8 (5%)	20 (8%)	37 (16%)	7 (2%)
informative	227 (35%)	19 (12%)	161 (62%)	130 (58%)	37 (11%)

Table 11

Comparison of morphology and molecular marker based taxonomic classification proposals for entomopathogenic fungal isolates UZB#1 through UZB#16

Isolate	**Taxonomic classification according to**				
	morphology only	**mitochondrial small rDNA fragment (nms3/nms4)**	**Internal Transcribed Spacer 1**	**Internal Transcribed Spacer 2**	**nuclear 5.8S rDNA**
UZB#1	*Beauveria bassiana*	*Beauveria* sp.	*Beauveria bassiana*	*Beauveria bassiana*	(*Beauveria* sp.)
UZB#2	*Beauveria bassiana*	**Beauveria *sp.***	*Beauveria bassiana*	*Beauveria bassiana*	(*Beauveria* sp.)
UZB#3	*Beauveria bassiana*	*Beauveria* sp.	*Beauveria bassiana*	*Beauveria bassiana*	(*Beauveria* sp.)
UZB#4	*Beauveria bassiana*	*Beauveria* sp.	*Beauveria bassiana*	*Beauveria bassiana*	(*Beauveria* sp.)
UZB#5	*Beauveria bassiana*	*Beauveria* sp.	*Beauveria bassiana*	*Beauveria bassiana*	(*Beauveria* sp.)
UZB#6	*Metarhizium anisopliae*	*Metarhizium* sp.	*Metarhizium anisopliae*	*Metarhizium anisopliae*	(*Metarhizium* sp.)
UZB#7	*Metarhizium anisopliae*	*Metarhizium* sp.	-	-	-
UZB#8	*Metarhizium anisopliae*	*Metarhizium* sp.	*Metarhizium anisopliae*	*Metarhizium anisopliae*	(*Metarhizium* sp.)
UZB#9	**Fusarium *sp*.**	*Fusarium* sp.	*Fusarium (solani)*	*Fusarium (solani)*	(*Fusarium* sp. or *Metarhizium* sp.)
UZB#10	**Fusarium *sp.***	*Fusarium* sp.	*Fusarium (solani)*	*Fusarium (solani)*	(*Fusarium* sp. or *Metarhizium* sp.)
UZB#12	*Trichoderma* sp	*Trichoderma* sp.	*Trichoderma (harzianum)*	*Trichoderma (harzianum)*	(*Trichoderma* sp.)
UZB#14	*Paecilomyces* sp.	*Fusarium* sp.	*Beauveria bassiana*	*Beauveria bassiana*	(*Beauveria* sp.)
UZB#15	*Beauveria bassiana*	*Beauveria sp.*	*Beauveria bassiana*	*Beauveria bassiana*	(*Beauveria* sp.)
UZB#16	*Aspergillus* sp.	*Aspergillus* sp.	*Aspergillus flavus*	*Aspergillus flavus*	(*Aspergillus* sp.)

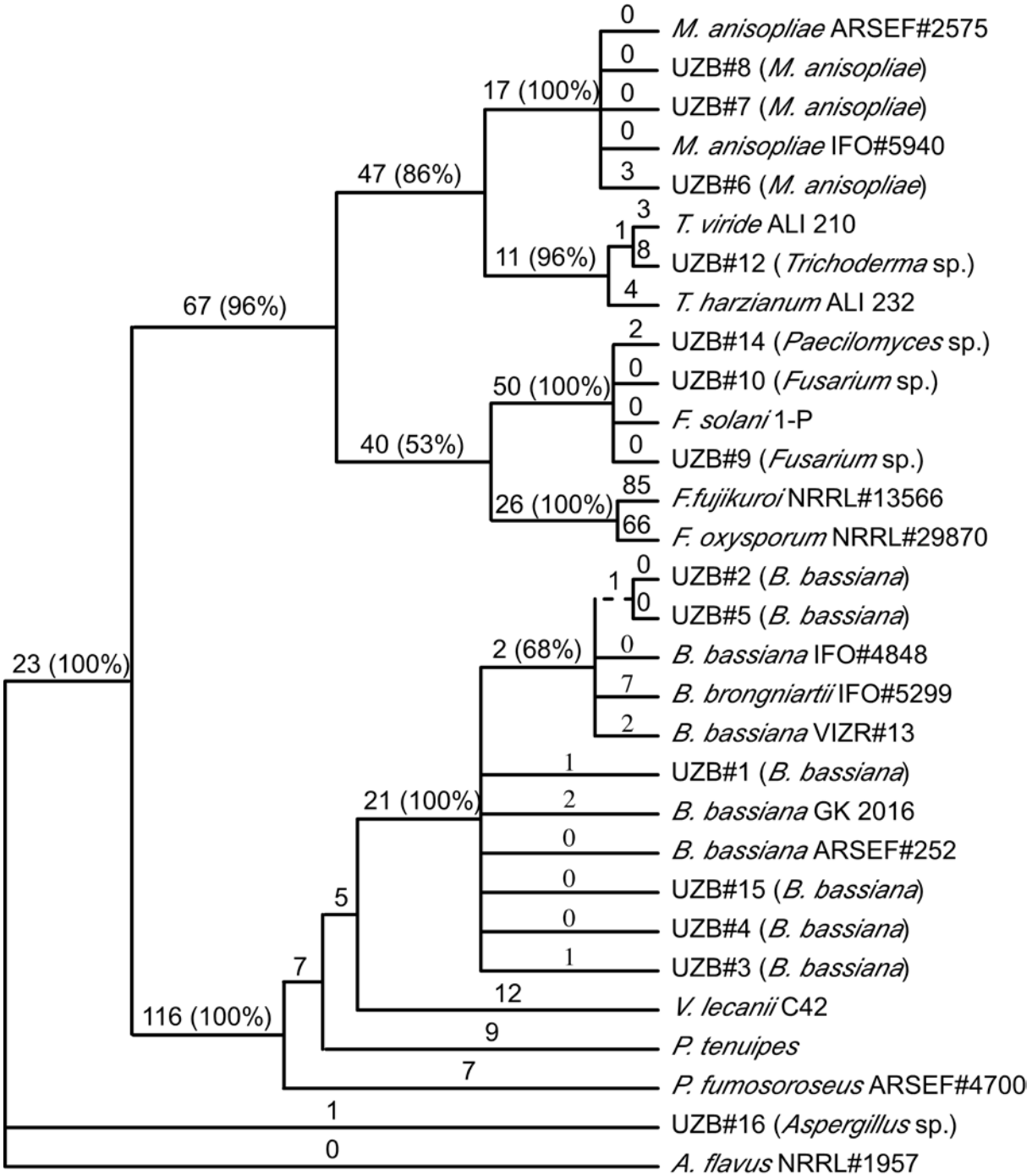

Figure 1

Maximum parsimony tree based on a comparison of mitochondrial rRNA gene sequences. The cladogram shown is the 50% majority rule consensus of 1087 most parsimonious trees (tree length 438, CI=0.8676, HI=0.1324, RI=0.9557) generated in a heuristic search using the TBR branch swapping algorithm. Dashed lines indicate branches that collapse in the respective strict consensus tree. Branch lengths are indicated on top of branches, with the bootstrap support values following in brackets. 1000 bootstrap replications were performed with the same search settings. The mitochondrial rDNA sequence from Aspergillus flavus was used to root the tree.

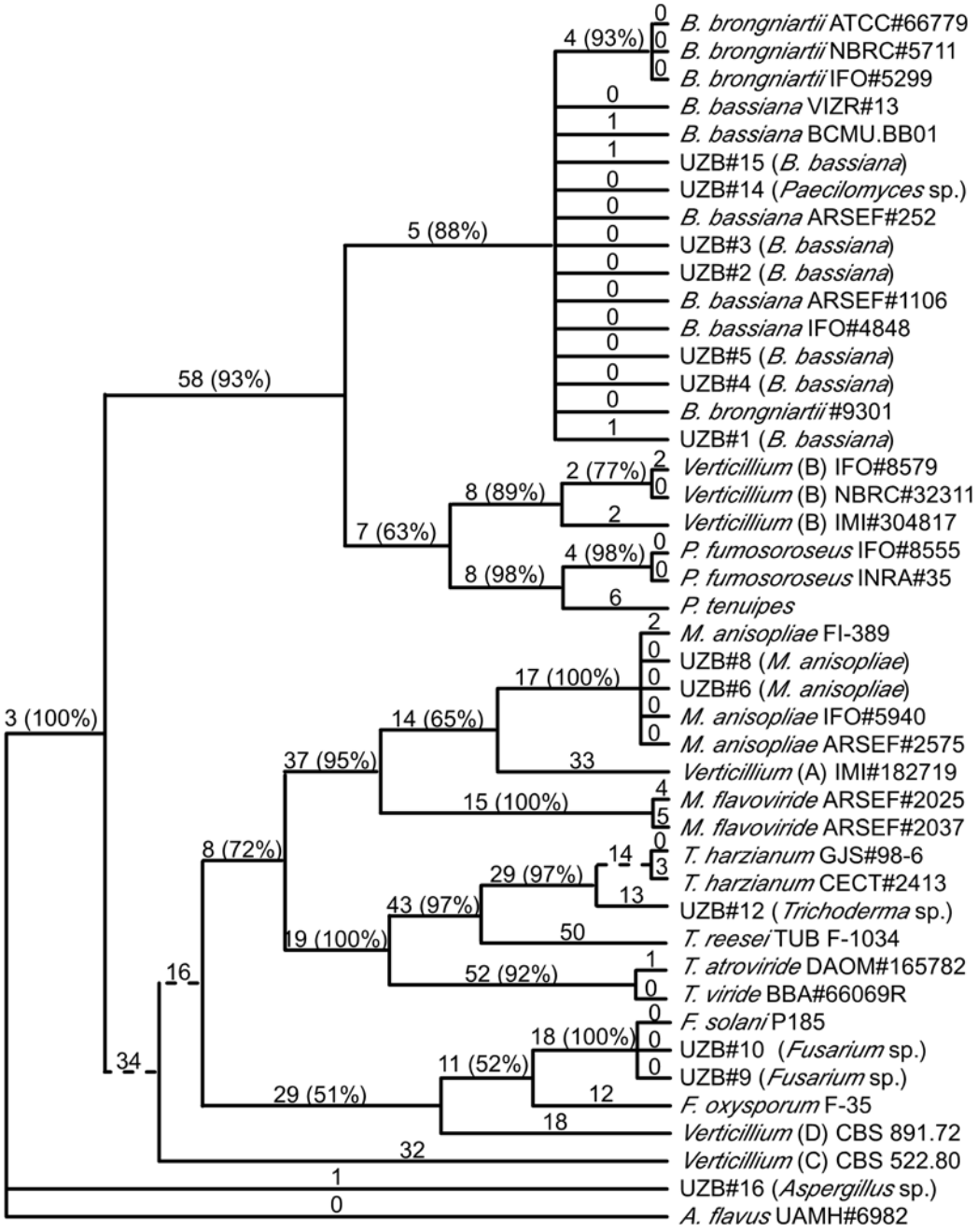

Figure 2

Maximum parsimony tree based on a comparison of ITS1 sequences. The cladogram shown is the 50% majority rule consensus of 2248 most parsimonious trees (tree length 476, CI=0.6828, HI=0.3172, RI=0.8692) generated in a heuristic search using the TBR branch swapping algorithm. Dashed lines indicate branches that collapse in the corresponding strict consensus tree. Branch lengths are indicated on top of branches, with the bootstrap support values in brackets. 1000 bootstrap replications were performed with the same search settings, generating a total of 611 329 trees. The ITS1 sequence from *Aspergillus flavus* was used to root the tree.

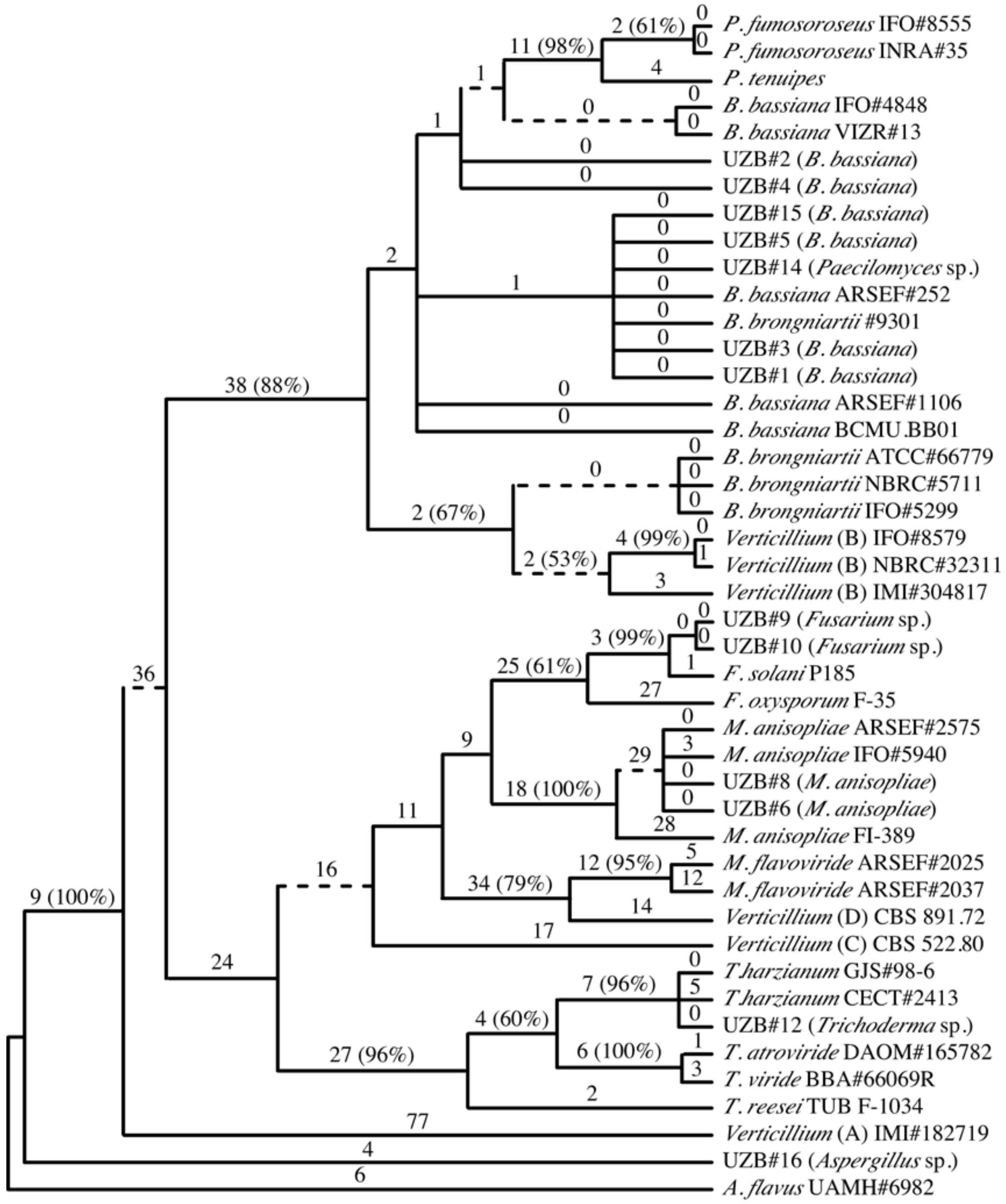

Figure 3

Maximum parsimony tree based on a comparison of ITS2 sequences. The cladogram shown is the 50% majority rule consensus of 31 most parsimonious trees (tree length 397, CI=0.6675, HI=0.3325, RI=0.8567) generated in a heuristic search using the TBR branch swapping algorithm. Dashed lines indicate branches that collapse in the respective strict consensus tree. Branch lengths are indicated on top of branches, with the bootstrap support values given in brackets. 1000 bootstrap replications were performed with the same search settings, generating a total of 20 126 trees. The ITS2 sequence from *Aspergillus flavus* was used to root the tree.

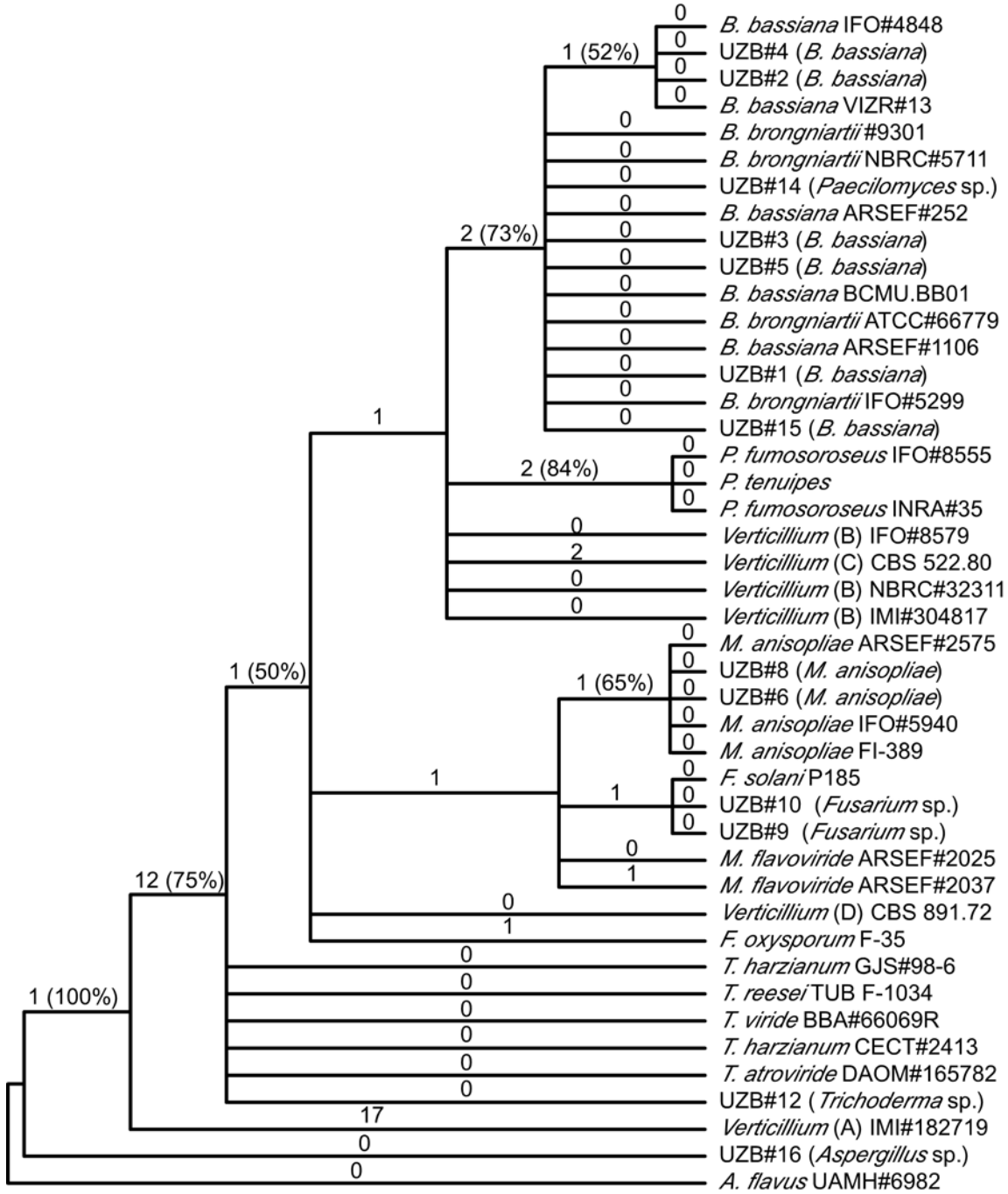

Figure 4

Maximum parsimony tree based on a comparison of fungal 5.8S rRNA gene sequences. The cladogram shown is the strict consensus of the 2 most parsimonious trees (tree length 35, CI=0.8571, HI=0.1429, RI=0.9500) found in a heuristic search using the TBR branch swapping algorithm. Branch lengths are indicated on top of branches, with the bootstrap support values given in brackets. 1000 bootstrap replications were performed with the same search settings and generated a total of 5126 trees. The 5.8S rDNA sequence from *Aspergillus flavus* was used as outgroup.

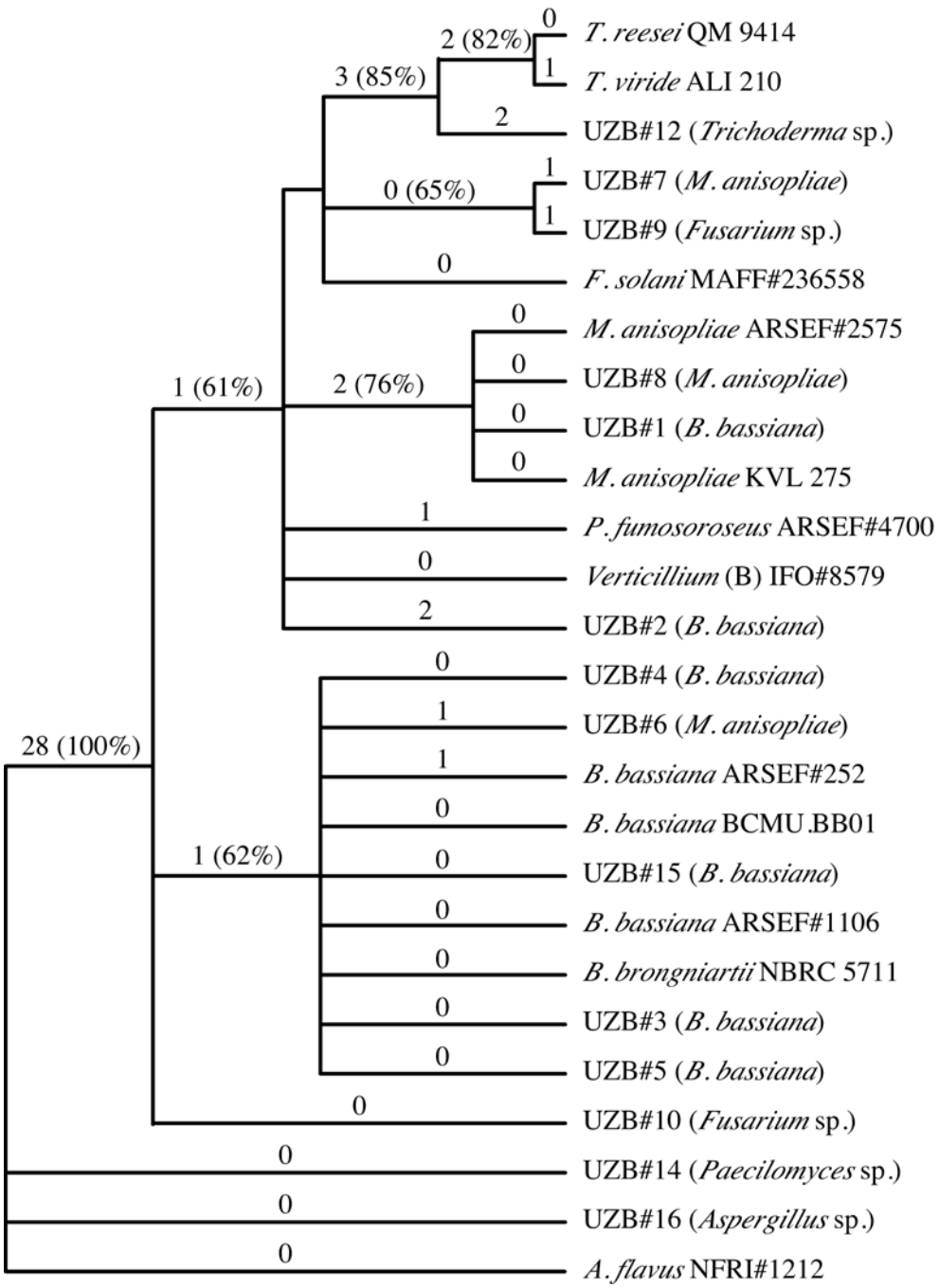

Figure 5

Maximum parsimony tree based on a comparison of nuclear 18S rRNA gene sequences. The cladogram shown is the strict consensus of the 2 most parsimonious trees (tree length 48, CI=0.9375, HI=0.0625, RI=0.9712) generated in a heuristic search using the TBR branch swapping algorithm. Branch lengths are indicated on top of branches, with the bootstrap support values following in brackets. 1000 bootstrap replications were performed with the same search settings, generating 3569 trees. The 18S rDNA fragment sequence from *Aspergillus flavus* was used as outgroup.

Appendix

Table A1

Mitochondrial small rDNA sequences from the GenBank database referred to in this study. For an explanation of strain collection acronyms see legend of Table A2.

Strain designation	Accession number	Reference
Aspergillus flavus NRRL #1957	U 29214	
Beauveria bassiana IFO #4848	AB 027360	Nikoh & Fukatsu (2000)
Beauveria bassiana GK 2016	U 91338	Hegedus et al. (1998)
Beauveria brongniartii IFO #5299	AB 027359	Nikoh & Fukatsu (2000)
Fusarium fujikuroi NRRL #13566	U34499	O'Donnell et al. (1998)
Fusarium oxysporum NRRL #29870	AF 362203	
Fusarium solani 1-P	AF125026	
Metarhizium anisopliae IFO #5940	AB 027361	Nikoh & Fukatsu (2000)
Paecilomyces tenuipes	AB 027358	Nikoh & Fukatsu (2000)
Trichoderma harzianum ALI 232	AY291265	Zeng et al. (2003)
Trichoderma viride ALI 210	AY291257	Zeng et al. (2003)
Verticillium lecanii C 42	AF 487277	

Table A2

Nuclear 5.8S rDNA and internal transcribed spacer (ITS) sequences from the GenBank database referred to in this study. ALI: Laboratory Collection of A.Lestander, Umea University, Sweden; ARSEF: USDA-ARS Collection of Entomopathogenic Fungi, USA; ATCC: American Type Culture Collection, USA; BBA: Biologische Bundesanstalt für Land- und Forstwirtschaft, Germany; BCMU: Collection of the Meijo University, Japan; CBS: Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; CECT: Coleccion Espanola de Cultivos Tipo, Valencia, Spain; DAOM: Department of Agriculture, Ottawa, Canada; GJS: Laboratory Collection of G.J.Samuels, USDA-ARS, Beltsville, USA; GK: Laboratory Collection of G.G.Khachatourians, University of Saskatchewan, Canada; IFO: Institute for Fermentation, Osaka, Japan; IMI: International Mycology Institute, UK; KVL: Royal Veterinary and Agricultural University, Denmark; MAFF Genebank of the Ministry of Agriculture, Forestry, and Fisheries, Japan; NBRC: NITE Biological Resource Center, Japan; NFRI National Food Research Institute, Tsukuba, Japan; NRRL: USDA-ARS Culture Collection, National Center for Agricultural Utilization Research, Peoria, USA; QM: U.S. Army Natick Laboratories; TUB: Collection of the Technical University of Budapest, Hungary; UAMH: University of Alberta, Mold Herbarium and Culture Collection, Edmonton, Canada.

Strain designation	Accession number	Reference
Aspergillus flavus UAMH #6982	AY 521473	
Beauveria bassiana IFO #4848	AB 027382	Nikoh & Fukatsu (2000)
Beauveria bassiana BCMU.BB01	AB079609	Yokoyama et al. (2002)
Beauveria brongniartii #9301	AY 334545	
Beauveria brongniartii ATCC #66779	AY 245628	
Beauveria brongniartii NBRC #5711	AB 106649	
Beauveria brongniartii IFO #5299	AB 027381	Nikoh & Fukatsu (2000)
Fusarium oxysporum F-35	AY 555719	
Fusarium solani P185	AF132801	Harrington et al. (2000)
Metarhizium anisopliae FI-389	AF 137061	Driver et al. (1999)
Metarhizium anisopliae IFO #5940	AB 027383	Nikoh & Fukatsu (2000)
Metarhizium flavoviride ARSEF #2025	AF 138269	Driver et al. (1999)
Metarhizium flavoviride ARSEF #2037	AF 138271	Driver et al. (1999)
Paecilomyces fumosoroseus IFO #8555	AB 083036	
Paecilomyces fumosoroseus INRA #35	AF 461745	
Paecilomyces tenuipes	AB 027380	Nikoh & Fukatsu (2000)
Trichoderma atroviride DAOM #165782	Z48818	
Trichoderma harzianum GJS #98-6	AF469189	Chaverri et al. (2003)
Trichoderma harzianum CECT#2413	AF278790	
Trichoderma reesei TUB F-1034	AF486007	Kubicek et al. (2003)
Trichoderma viride BBA#66069R	AJ230681	Lieckfeldt et al. (1999)
Verticillium balanoides CBS 522.80	AJ292413	Zare et al. (2000)
Verticillium gonnoides *CBS 891.72*	AJ292409	Zare et al. (2000)
Verticillium lecanii IFO #8579	AB 079127	
Verticillium lecanii IMI #304817	AJ 292383	Zare et al. (2000)
Verticillium lecanii NBRC #32311	AB 111495	
Verticillium luteo-album IMI #182719	AJ292420	Zare et al. (2000)

Table A3

Nuclear 18S rDNA sequences from the GenBank database referred to in this study. For an explanation of strain collection acronyms see legend of Table A2.

Strain designation	Accession number	Reference
Aspergillus flavus NFRI #1212	D63696	Nikkuni et al. (1996)
Beauveria bassiana BCMU.BB01	AB079609	
Beauveria brongniartii NBRC 5711	AB106649	
Fusarium solani MAFF#236558	AF150486	Suga et al. (2000)
Metarhizium anisopliae KVL 275	AF487274	Pantou et al. (2003)
Trichoderma reesei QM 9414	AF548102	Wu et al. (2003)
Trichoderma viride ALI 210	AF548104	Wu et al. (2003)
Verticillium lecanii IFO #8579	AB 079127	

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