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EXPERIMENTAL ARTICLES

Investigations into the Taxonomy of the Mushroom Pathogen *Verticillium fungicola* and its Relatives Based on Sequence Analysis of Nitrate Reductase and ITS Regions

R. C. Amey[a,c], A. Athey-Pollard[a], P. R. Mills[b], G. D. Foster[a], and A. Bailey[a,1]

[a] *School of Biological Sciences, University of Bristol, Woodland Rd, Bristol BS8 1UG, UK*

[b] *Warwick-HRI, University of Warwick, Wellesbourne, Warwickshire CV35 9EF, UK*

[c] *Faculty of Applied Sciences, University of the West of England, BS16 1QY Bristol*

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Abstract—The full sequence of the nitrate reductase gene was obtained from a type isolate of *Verticillium fungicola* var. *fungicola* and used for phylogenetic analysis against other ascomycete fungi. Sequencing obtained 2749 bp of coding region, 668 bp of 5' flanking sequence and 731 bp of 3' flanking sequence. *In silico* analysis indicated that the coding region contains a single intron and translates into an 893 amino acid protein, with BLAST analysis identifying five conserved nitrate reductase domains within the protein. The 5' flanking sequence contains numerous conserved sites putatively involved in binding nitrogen regulatory proteins, indicating that the regulation of the gene is likely to be subject to the same regulation as that of model fungi such as *Aspergillus nidulans*. The central portion of this gene was amplified and sequenced from a number of *V. fungicola* isolates and related fungi and the resulting phylogenies compared to those obtained from analysis of the rDNA internal transcribed spacer regions for these fungi. Both nitrate reductase and ITS analyses provide additional evidence that reinforces previous findings that suggest the mushroom pathogenic *Verticillium* species are more related to other chitinolytic fungi such as the insect pathogens *Verticillium lecanii* and *Beauveria bassiana* than to the plant pathogenic *Verticillia*.

Key words: *Agaricus bisporus*, mushroom pathogen, nitrate reductase, taxonomy, *Verticillium fungicola.*

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The fungus *Verticillium fungicola* (Preuss) Hassebrauk var. *fungicola* has the ability to cause a devastating disease within mushroom farms, being a severe pathogen of *Agaricus bisporus*. The most commonly observed symptoms are brown lesions on the cap of the mushroom which, whilst not drastically reducing the crop levels per se, result in a considerable reduction in marketable yield for growers. Earlier infections of the mushroom beds can result in more severe symptoms such as stipe blowout, when the stipe of the mushroom disintegrates, or dry bubble, the production of undifferentiated masses of tissue instead of fruiting bodies.

Originally described as *Verticillium malthousei* [1], the pathogen was later named *V. fungicola* [2]. *Verticillium* is a form genus and its members characteristically have verticillate whorls of phialides arranged on branching conidiophores. However, within the *Verticillium* group the pathogens have many different host types, and thus these criteria alone are insufficient for taxonomic studies.

In a number of recent studies it has become increasingly apparent that *V. fungicola* and its close relatives are likely to be more closely related to other chitin-degrading fungi such as insect pathogens like *Beauveria bassiana*, than to the other plant pathogenic verticillia. Carder and Barbara [3] show that *V. fungicola* and *V. psalliotae* (another mushroom pathogen) are only distantly related to the plant pathogenic Verticillium species such as *V. dahliae*, and at commencement of this study it was suggested that *V. fungicola* and similar species are in fact more closely related to species such as *B. bassiana* and *B. brongniartii* [4]. Bidochka et al. [5] included two isolates of *V. fungicola* within their analysis of chitinolytic Verticillia and this showed that *V. fungicola* clustered with other *Verticillium* species such as *V. chlamydosporium* and *V. leptobactrum* on the basis of ITS and 5S rDNA sequence. It will be particularly important to understand the relationships within the chitin-degrading group of species since some of the insect pathogenic fungi are being evaluated as potential biocontrol agents. In the event of a disease outbreak it is important to be able to rapidly differentiate between such beneficial fungi and the detrimental

[1] Corresponding author; e-mail: Andy.Bailey@bris.ac.uk

mushroom pathogens. Current control strategies for *V. fungicola* include good hygiene within production areas and also the use of the ergosterol-14 demethylase inhibiting fungicide prochloraz or the β-tubulin inhibitor fungicide carbendazim [6]. Concerns over the use of these fungicides are arising due to the emergence of resistant mushroom pathogens [7, 8], so if new control methods are to be devised for *V. fungicola* it would be useful to have a clearer understanding of how it is related to other similar fungi.

Bonnen and Hopkins [9] showed that the *V. fungicola* complex could be divided into four groups on the basis of RAPD analysis, but that the recent fungicide resistant isolates displayed little variation, suggesting a somewhat homogeneous population. Collopy et al. [10] investigated a number of *V. fungicola* isolates by ITS sequencing and demonstrated that all of their USA derived isolates were monophyletic with respect to ITS sequence, suggesting a single dominant isolate was responsible for disease outbreaks within the USA. However, the European isolates show some divergence of sequence within the ITS regions and were distinct from the USA grouping. They suggested that analysis of additional genetic loci was needed to further study the relatedness of *V. fungicola* to the entomopathogenic fungi. Indeed, it is commonly believed that the preferred method for measuring phylogenetic relationships is by comparing the DNA sequences of protein-coding regions [11]. The entomopathogenic *Verticillium* species and their relatives have been further studied by Zare and Gams [12]; however, the revisions suggested in the taxonomy of these species have not yet been widely accepted.

It is important to determine the evolutionary relationship of *V. fungicola* with other fungal pathogens, not only to determine how the pathogen has evolved and developed over time, but also to obtain indications of what may be important for the pathogen during the infection process. Therefore, the aim of this study was to perform analysis of both the protein-encoding nitrate reductase gene and also the non-coding internal transcribed spacer 1 (ITS1) region of the ribosomal rDNA. The nitrate reductase gene was chosen since this gene has already been cloned and sequenced from a number of fungi (including insect pathogens) so is another useful gene for taxonomic studies. In a study of nineteen nitrate reductase genes from fungi, algae and higher plants, Zhou and Kleinhofs [13] showed *niaD* genes to display substantial sequence similarity, making them useful as classification tools. The gene family has a wide array of deviation in their intron number [13] and GC content at the third codon position. The genes therefore exhibit a high degree of conservation, leading to closely related organisms having highly analogous DNA base sequences. In fact, phylogenetic trees based on *niaD* sequence data closely correlate with dendrograms created using alternative molecular studies and systematics [13]. An investigation using two independent regions for taxonomic analysis should give additional strength to the validity of these results. Nitrate reductase genes have proven useful in development of transformation studies in other fungi [14], so they could be of use in a similar procedure in *V. fungicola*. It has also been used to investigate vegetative incompatibility groups in fungi and may be a useful target for a transposon trap, where spontaneous nitrate reductase mutants selected on the basis of chlorate resistance have often been shown to be generated due to an insertion element incorporated into the nitrate reductase gene.

MATERIALS AND METHODS

All isolates were obtained from Warwick-HRI and were maintained on Potato Dextrose Agar (PDA). For isolate details see the table.

DNA extraction. DNA was extracted from fungal isolates essentially as described by Keon and Hargreaves [15].

Production, cloning and analysis of PCR products. Amplification of the ITS region was performed using primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS2 (GCTGCGTTCTTCATCGATGC) with amplification conditions of 9°C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 4 min. An internal region of the nitrate reductase gene was amplified using degenerate primers NR3 (CTNGGNATGATGAAYAAYCCNTGGTT) and NR4 (GTNCCNATRTGRTARTCNGGCATCAT), with amplification conditions of 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 48°C for 30 s, 72°C for 30 s and a final extension of 72°C for 4 min.

Amplification products were electrophoresed through Agarose–TAE gels, excised, purified and cloned into the pGEM-T Easy vector (Promega). Sequencing was usually performed by Lark (Saffron Walden, UK) on Wizard (Promega) purified plasmid templates, using templates derived from two independent PCR reactions, with a clone from a third reaction being used in cases of discrepancy in the first two products. Forward and reverse sequencing primers were used in all cases and the consensus sequence produced using Sequencher software (GeneCodes Corporation).

Phylogenetic analysis. Phylogenetic analysis was performed using Clustal X to compile alignments. All analyses are the result of bootstrapped analysis (1000 times) with trees being derived from the consensus alignment produced from the parsimony programs DNAPARS or PROTPARS of the Phylip package version 3.6 [16] for DNA and protein analysis respectively.

Isolation of the nitrate reductase gene. Library screening and phage manipulation were performed according to Sambrook et al. [17] using the lambda BlueStar-based library (Invitrogen) of *V. fungicola* isolate 150–1 constructed by Amey et al. [18]. Hybridisation-positive phages were isolated and auto-excised

List of species and isolates, country of origin, host, symptom severity on *Agaricus bisporus* and accession numbers for ITS and nitrate reductase sequences where applicable used in this study

Isolate*	Country of origin	Host	Symptom severity on *Agaricus*	ITS accession, No.	NR accession, No.
Verticillium fungicola					
CBS 440-34	Unknown	Agaricus	Severe	**AY943962**	**AY945323**
5-5	New Zealand	Agaricus	No details	–	**AY945324**
5-6	New Zealand	Agaricus	No details	–	**AY945325**
8-1	Spain	Agaricus	High virulence	–	**AY945317**
21.3	Spain	Agaricus	Mild	**AY943963**	–
88.1	USA	Agaricus	Low/medium virulence	–	**AY945318**
115-1	UK	Agaricus	Cap spotting	–	**AY945314**
124.1	Ireland	Agaricus	Severe	**AY943967**	–
149-9	UK	Agaricus	No details		**AY945315**
150.1	UK	Agaricus	Medium	**AY943966**	**AY945308**
182	UK	Agaricus	Medium	**AY943965**	**AY945316**
201A	UK	Agaricus	Dry Bubble	**AY943964**	–
486	Malta	Agaricus	No details	**AY943961**	–
V. lecanii 1-72	No details	Aphid	N/A	**AY943960**	**AY945319**
V. psalliotae 247 A	UK	Agaricus	Mild spotting	–	**AY945321**
V. psalliotae 176-8	Japan	Agaricus	No details	–	**AY945320**
V. psalliotae 5-8	New Zealand	Agaricus	No details	–	**AY945322**
V. dahliae 120.85	UK	Strawberry	N/A	–	**AY945313**
B. bassiana CBS393	Unknown	Insect	N/A	**AY943959**	**AY945311**
B. bassiana CBS394	Unknown	Insect	N/A	**AY943969**	**AY945310**
B. brongniartii CBS 232.85	Unknown	Insect	N/A	**AY943970**	**AY945309**
B. brongniartii CBS 319.89	Unknown	Insect	N/A	**AY943968**	**AY945312**

Note: * CBS, Centraalbureau voor Schimmelcultures, Baarn, the Netherlands.

according to the manufacturer's recommended methods. Inserts were sequenced using custom-designed oligonucleotides with automated sequencing reactions (Lark) and the sequence assembled using Sequencher software (Genecodes corp). Screens for nitrate reductase mutants were made on the basis of selection for chlorate resistance following the methods of Cove [19].

RESULTS AND DISCUSSION

Isolation of the Nitrate Reductase Gene from Verticillium fungicol

Primers NR1 and NR2 were used to amplify a central portion of the nitrate reductase gene from *V. fungicola* var *fungicola* isolate 150-1, resulting in a PCR product of approximately 500 bp. This product was cloned into the vector pGEM-T Easy and sequenced. BLAST comparisons with the NCBI database indicated that this amplicon was derived from the nitrate reductase gene and showed that the closest match within the database was that of *Beauveria bassiana*, with 76% identity at the DNA sequence level. The fragment was used to screen a lambda BlueStar based genomic library [18] resulting in the isolation of three positive phages as determined by PCR (data not shown). Positive phages were purified by additional rounds of screening, converted to plasmids by cre-mediated autoexcision and the inserts sequenced by primer walking. In total a region of 4148 bp was sequenced, comprising 2749 bp of coding region along with 668 bp of 5' flanking sequence and 731 bp of 3' untranslated region. This sequence has been deposited within the databases and is available as accession **AY184495**. Sequence analysis suggests that this encodes a protein of 893 amino acids, which is similar to those encoded by other fungi which range from 864 amino acids for *Penicillium chrysogenum* [20] to 982 amino acids for *N. crassa* [21]. This predicted protein shows 83% sequence identity to that of *B. bassiana*. The coding region is interrupted by a single intron between bp positions 2443 to 2509 by comparison to the consensus sequences identified by Gurr et al. [22]. Up to six introns have been observed in other fungal species [23]. The position of the *VfNr1* intron relates to the position

Intron:	I	II	III	IV	V	VI	
Aspergillus nidulans [31]	+	+	+	+	+	+	I
Aspergillus niger [32]	+	+	+	+	+	+	
Aspergillus orizae [30]	+	+	+	+	+	+	
Penicillium chrysogenum [20]	+	+	+	+	+	+	
Leptosphaeria maculans [33]	–	+	+	+	–	+	II
Staginospora nodorum [34]	–	+	+	+	–	+	
Beauveria bassiana [35]	–	–	–	+	–	–	III
Fuzarium oxysporum[14]	–	–	–	+	–	–	
Neurospora crassa [21]	–	–	–	+	–	–	
Vetricillium fungicola (This study)	–	–	–	+	–	–	
Verticillium psalliotae 176-8 (This study)	–	–	–	+	–	–	
Phitophthora infestans [36]	(+)	–	–	–	–	–	IV
Botriotinia fuckeliana [37]	–	–	–	–	–	–	V
Ustilago maydis [34]	–	–	–	–	–	–	
Verticillium psalliotae 247A (This study)	–	–	–	–	–	–	

Fig. 1. A comparison of intron position and number in the nitrate reductase genes from various filamentous fungi results in the generation of five distinct groups. + and – indicate presence or absence at each intron position. The intron of the *P. infestans* nitrate reductase gene indicated by (+), is closest to intron position one, but is not perfectly aligned (adapted and updated from Kitamoto et al. [30]).

of intron IV from other fungal species such as *Fusarium oxysporum* (Fig. 1) [14] and *Giberella fujikuroi* [24]. Interestingly, the intron number and position has previously been observed to correlate closely with phylogenetic analysis of nitrate reductase gene sequences [23]. BLAST analysis using nucleotide sequence data corroborates this phenomenon for *V. fungicola*, as high identities to nitrate reductase genes from *F. oxysporum* (81 % identity; 4×10^{-7}) and *G. fujikuroi* (80% identity; 3×10^{-5}) were observed. Analysis of the 5' flanking region identified several potential binding sites for areA/nit2 like proteins and also a probable niiA/nit4 binding site at –420 bp, showing strong similarities to those identified in *Neurospora crassa* and *Aspergillus nidulans*. Two potential creA binding sites are also present within the promoter. Together this suggests that the *V. fungicola* nitrate reductase gene is likely to be subject to similar regulatory processes as those characterized in the model ascomycetes *A. nidulans* and *N. crassa*.

BLAST analysis for putative protein domains was performed and yielded five domains (Fig. 2):

(1) An oxidoreductase molybdopterin-binding domain. This domain of 229 residues is found in a variety of oxidoreductases and binds to a molybdopterin cofactor.

(2) A molybdopterin cofactor (Mo-co) oxidoreductase dimerization domain of 124 residues involved in dimer formation having Ig-fold structure.

(3) An oxidoreductase FAD-binding domain of 99 residues.

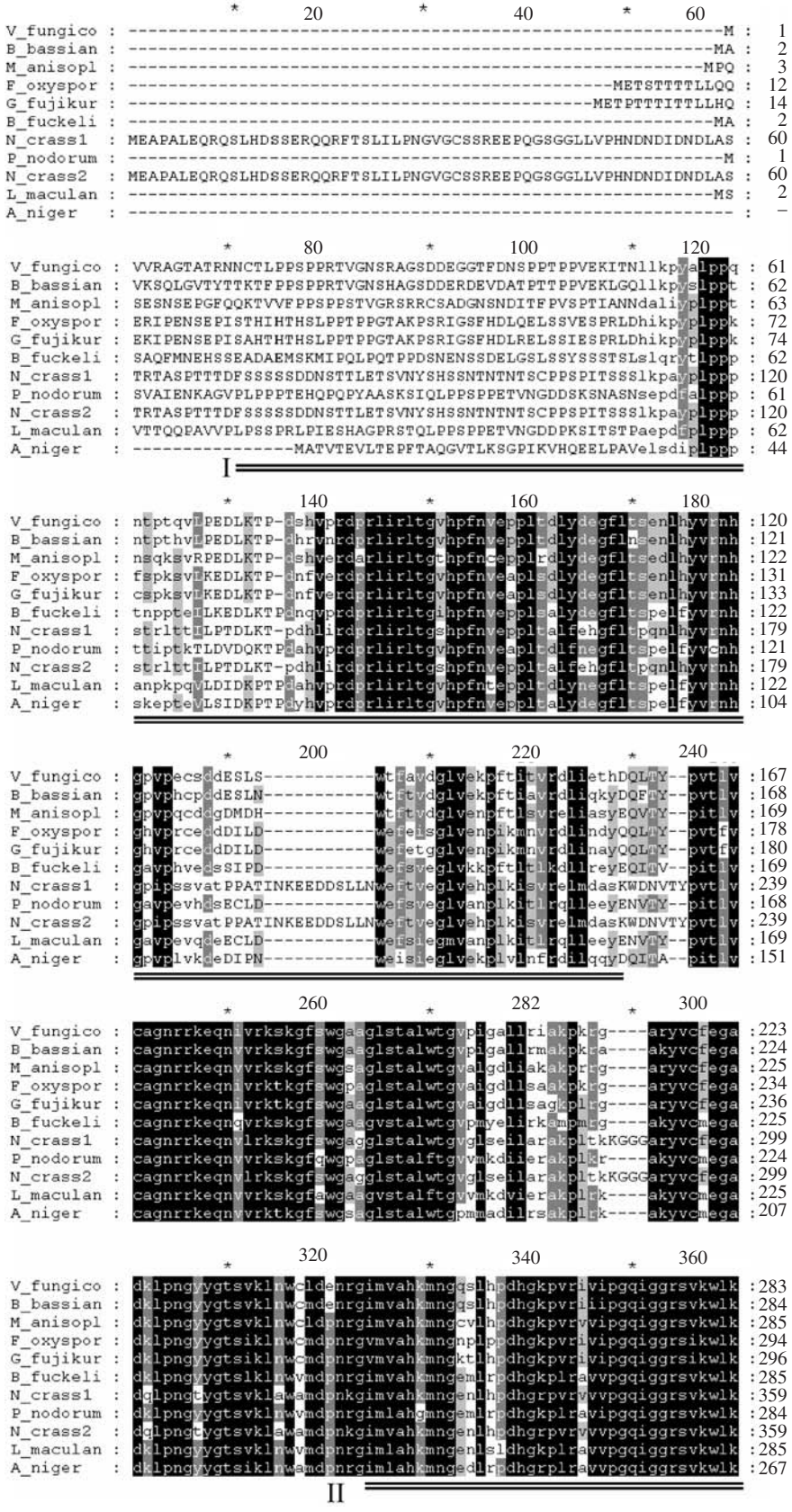
(4) An oxidoreductase NAD-binding domain.

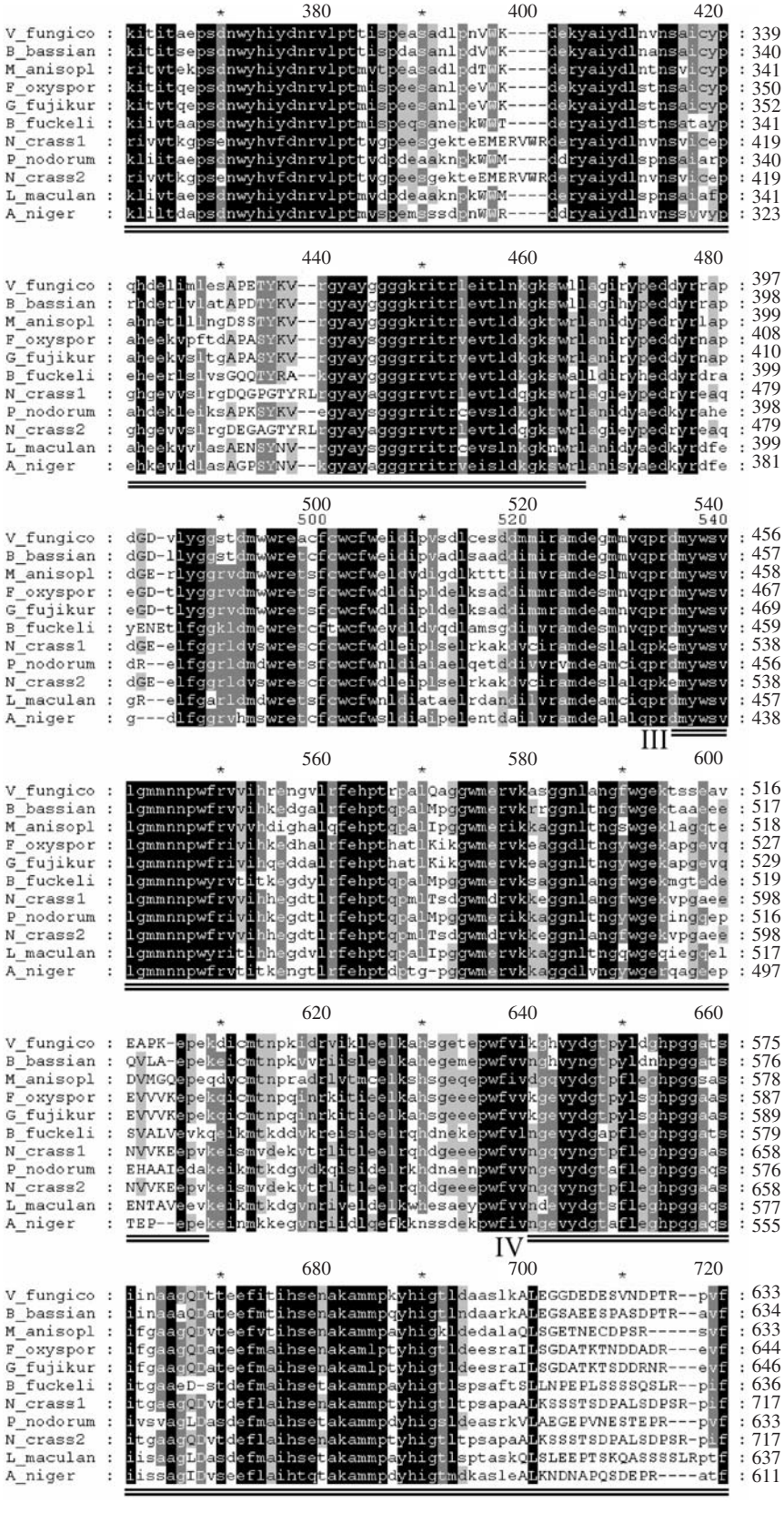
(5) A heme/steroid-binding domain. This domain includes heme-binding domains from a diverse range of proteins and proteins that bind to steroids.

The presence of these domains clearly indicates the role of VfNr1 as a nitrate reductase-encoding gene and correlates with observations by Kleinhofs et al. [25], who identified the conserved NAD, FAD and molybdopterin domains within fungi.

The nitrate reductase gene has been widely used as both a transposon trap and a selectable marker for fungal transformation. With this in mind, attempts were made to isolate nitrate reductase mutants on the basis of resistance to chlorate [19]. However, this proved to be

Fig. 2. Multiple alignment of *VfNr1* to similar nitrate reductase proteins. V_fungico *Verticillium fungicola VfNr1* (Accession No. **AY184495**), B_bassian *Beauveria bassiana* (Accession No. P43100), M_anisopl *Metarhizium anisopliae* (Accession No. CAA04554), F_oxyspor *Fusarium oxysporum* (Accession No. P39863), G_fujikur *Giberella fujikuroi* (Accession No. CAA62232), B_fuckeli *Botryotinia fuckeliana* (Accession No. AAC02633), N_crass1 *Neurospora crassa* (Accession No. S16292), P_nodorum *Phaeosphaeria nodorum* (Accession No. CAA74005), N_crass2 *Neurospora crassa* (Accession No. P08619), L_maculan *Leptosphaeria maculans* (Accession No. P36842), A_niger *Aspergillus niger* (Accession No. P36858). Black shading 100% conserved, dark gray shading 80% conserved, light gray shading 60% conserved. The conserved nitrate reductase domains deduced by BLAST analysis are double underlined and indicated by roman numerals at the beginning of each domain. (I) Oxidoreductase molybdopterin binding domain. (II) Molybdopterin cofactor oxidoreductase dimerisation domain. (III) Heme/Steroid binding domain. (IV) Oxidoreductase FAD-binding domain. (V) Oxidoreductase NAD-binding domain.


I
II


380
400
420
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700
720
III
IV

Fig. 2. (Contd.)

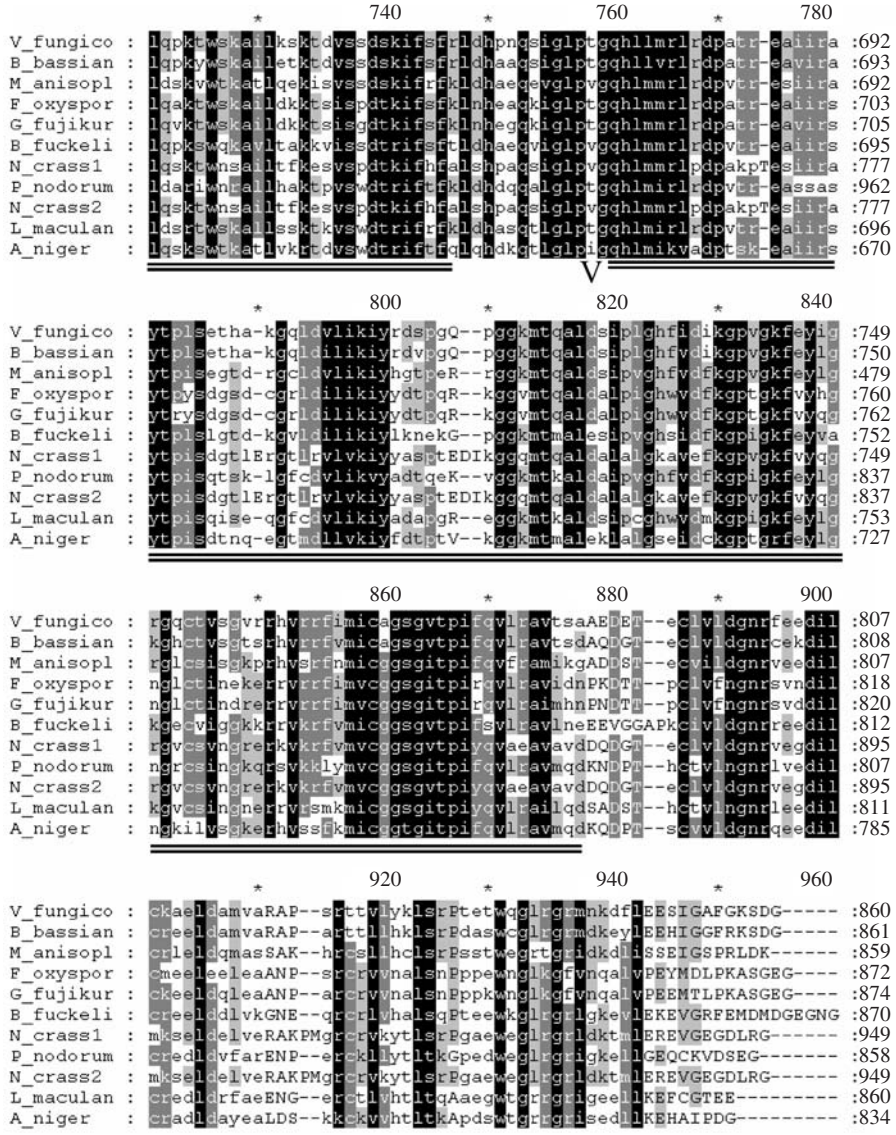

Fig. 2. (Contd.)

unsuccessful due to an unusually high natural resistance to this compound of the *V. fungicola* isolate tested (150-1), thereby preventing the isolation of either spontaneous or UV induced mutants (data not shown). An alternative selectable marker gene for such studies could be ATP sulphurylase, where mutants of some species display selenate resistance [26]; however this was beyond the scope of this study.

The Fungal Phylogeny Based on Nitrate Reductase

PAUP analysis [27] was performed on the encoded amino acid sequences of the NR genes from a number of ascomycete fungi available on the public databases. Parsimony analysis resulted in the dendrogram illustrated in Fig. 3, and indicates that *V. fungicola* clusters with the insect pathogen *B. bassiana*, correlating with the previous study by Mugnier [4] which examined ITS sequences. Furthermore, *V. fungicola* also groups closely with the insect pathogen *Metarhizium anisopliae*, thereby reinforcing the findings of Collopy et al. [10], who observed that *V. fungicola* clusters with the insect pathogens *Cordyceps* and *Paecilomyces*. These data suggest that these fungi shared a common ancestor that utilized chitin as a nutrient source. If *V. fungicola* is capable of infecting insects, it may be possible that the pathogen can infect insects associated with mushroom production. These include sciarids (*Lycoriella* sp.) and phorids (*Megaselia* sp.) [10]. Indeed, phorids such as *Megaselia halterata* are known to be vectors of *V. fungicola* and may act as an intermediary host for the fungus. If *V. fungicola* is capable of infecting such insects, further details of the epidemiol-

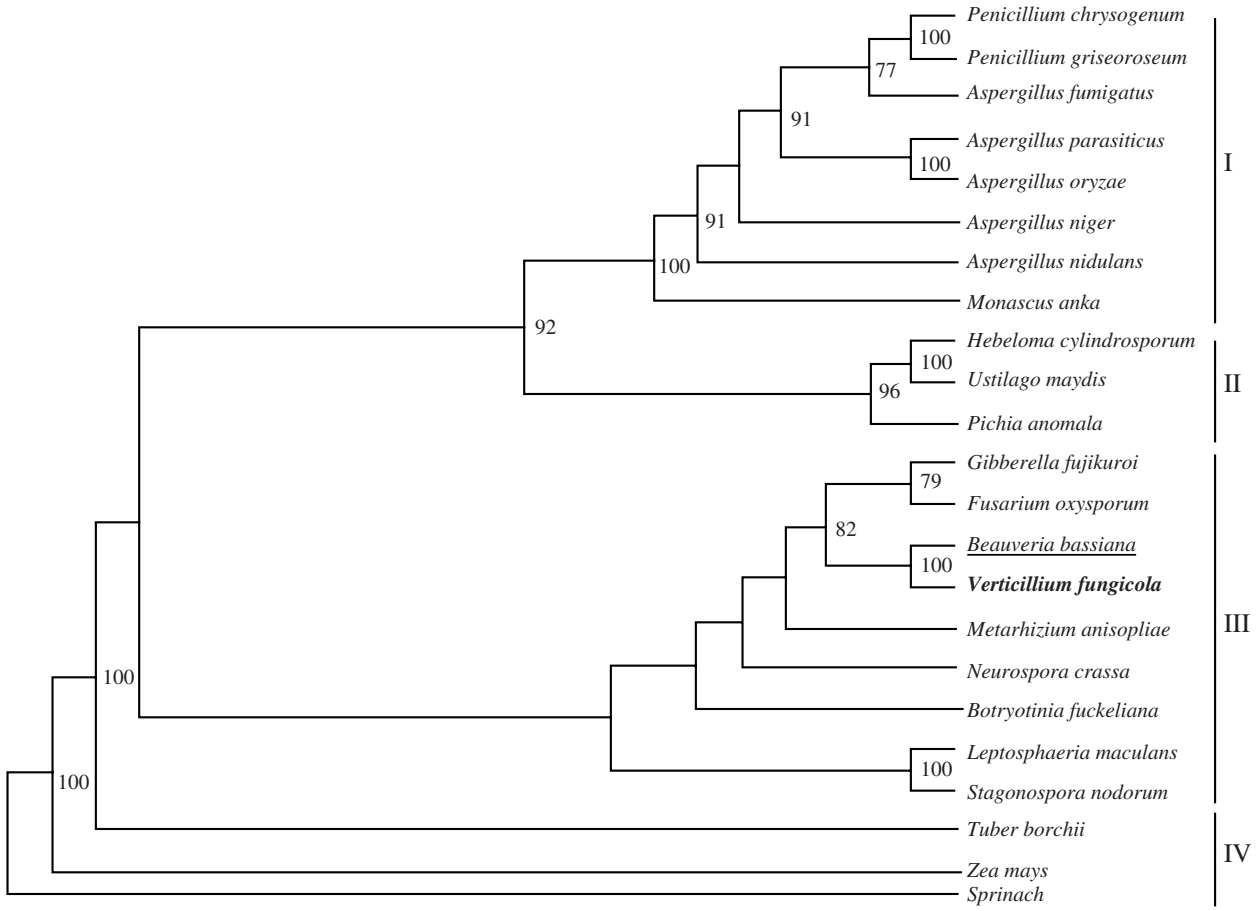

Fig. 3. The relationship of *Verticillium fungicola* isolate 150-1 to other fungi based on amino acid sequences of the entire nitrate reductase proteins deduced from nucleotide sequences. The dendrogram was produced by PAUP Parsimony analysis of the sequences using 1000 bootstraps (% values indicated at branches). Of particular note is that *V. fungicola* (bold) forms a tight group with the insect pathogenic fungus *Beauveria bassiana* (underlined).

ogy and spread of the disease could be obtained by studying this interaction, perhaps using marker gene tagged isolates of *V. fungicola* such as those developed by Amey et al. [28]. The observation that *V. fungicola* clusters with fungal pathogens of insects is useful as the data that have been obtained on these insect-pathogens from previous studies may be applied to the study of *V. fungicola* and could reveal more about the origin of *V. fungicola* and the possible mechanisms it uses to infect *A. bisporus*. As observed by Cutler et al. [23] the species *Fusarium oxysporum* and *Giberella fujikoroi* cluster together, as do *Stagonospora nodorum, Leptosphaeria maculans*, and *Botryotinia fuckeliana*. Neighbor joining analysis was also performed with similar results observed (data not shown). A comparison of the groupings obtained from the intron analysis (Fig. 1) and the entire NR phylogeny (Fig. 3) illustrate that in general, the groupings correlate. *V. fungicola* and *B. bassiana* are clustered in group III of the intron analysis and group III of the NR analysis along with *F. oxysporum* and *N. crassa*. Although *Leptosphaeria maculans* and *Stagonospora nodorum* also cluster in group III of the NR analysis, they have sequences with high similarity and could be separated easily from the other species in group III. This would marry well with the intron data which clusters them in group II. Furthermore, the *Aspergillus* and *Penicillium* species all cluster together in group I of the NR analysis and group I of the intron analysis. The only anomaly is with *G. fujikoroi*, which does not contain an intron and therefore falls into group V of the intron analysis, yet clusters with *V. fungicola, B. bassiana*, and *F. oxysporum* from the NR analysis. These data indicate that for quick taxonomic analysis of organisms, intron position and number are ideally suited, but full length gene analysis should be performed for more accurate results.

Investigations of the Beauveria/Verticillium Clade by ITS and NR Sequence Analysis

To examine the *V. fungicola* species complex in further detail, and to obtain an improved understanding of the relationship between *V. fungicola* and other related

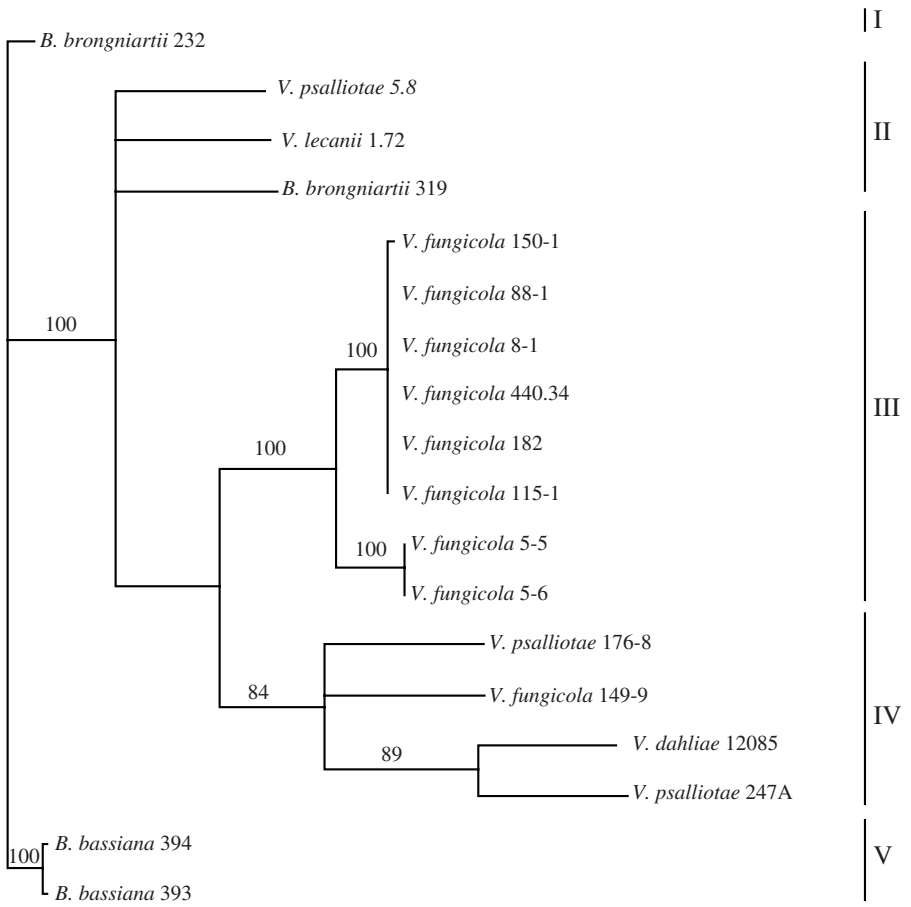

Fig. 4. The relationship of *Verticillium fungicola* isolates to other fungi based on nucleotide sequences of partial nitrate reductase genes. The dendrogram was produced by PAUP Parsimony analysis of the sequences using 1000 bootstraps (% values indicated at branches).

fungi, the central portion of the nitrate reductase gene was PCR amplified from a number of isolates as indicated in table, as was the rDNA ITS region. These were cloned and two representatives from independent reactions sequenced for each isolate. PAUP analysis was used to create phylogenies for the predicted amino acid sequence of the nitrate reductase fragment (Fig. 4) and on the ITS region (Fig. 5). These analyses split the isolates into five groups on each tree as indicated by roman numerals.

The presence of the majority of *V. fungicola* isolates in one section of the tree (group III NR; Group I ITS) indicates that these isolates form a monophyletic group. The group contains isolates from a diverse range of geographical areas, including Europe, the USA, and New Zealand. However, the NR group is split into two and, furthermore, isolate 149-9 is situated on a separate section of the tree with *V. psalliotae* 176-8. Interestingly, isolates 5-5 and 5-6 are both from New Zealand, which may explain why the isolates cluster together. In fact, their partial NR sequences are identical, perhaps indicating that they are clonal. On the ITS tree, *V. fungicola* isolate 895 [5] clusters with *V. lamellicola* and *V. dahliae* isolate 2341. Although Collopy et al. [10] found a total of four groups within the isolates identified as *V. fungicola* from analysis of rDNA sequences, they also found that all *V. fungicola* var. *fungicola* isolates grouped together, similar to the results shown in Figs. 3 and 4. Therefore, as the isolates studied here are *V. fungicola* var. *fungicola* isolates, these data concur with the observations of Collopy et al. [10], who found a lack of diversity in fungi isolated from mushroom farms using a neighbor joining analysis. Collopy et al. [10] propose that this may be due to changes in practices regarding the casing of mushroom crops. As pasteurized peat moss has become more prevalent since the 1970s, the use of soil became less common, and subsequently the import of local isolates onto farms reduced. This increased the uniformity of casing material between mushroom farms and, hence, may have resulted in the same isolates having a selective advantage and, hence, being isolated on the different farms.

The *V. fungicola* isolates are seen to cluster separately from the *Beauveria bassiana* isolates in both phylogenies, as expected due to their original host ranges. This reinforces the distinction that the two are

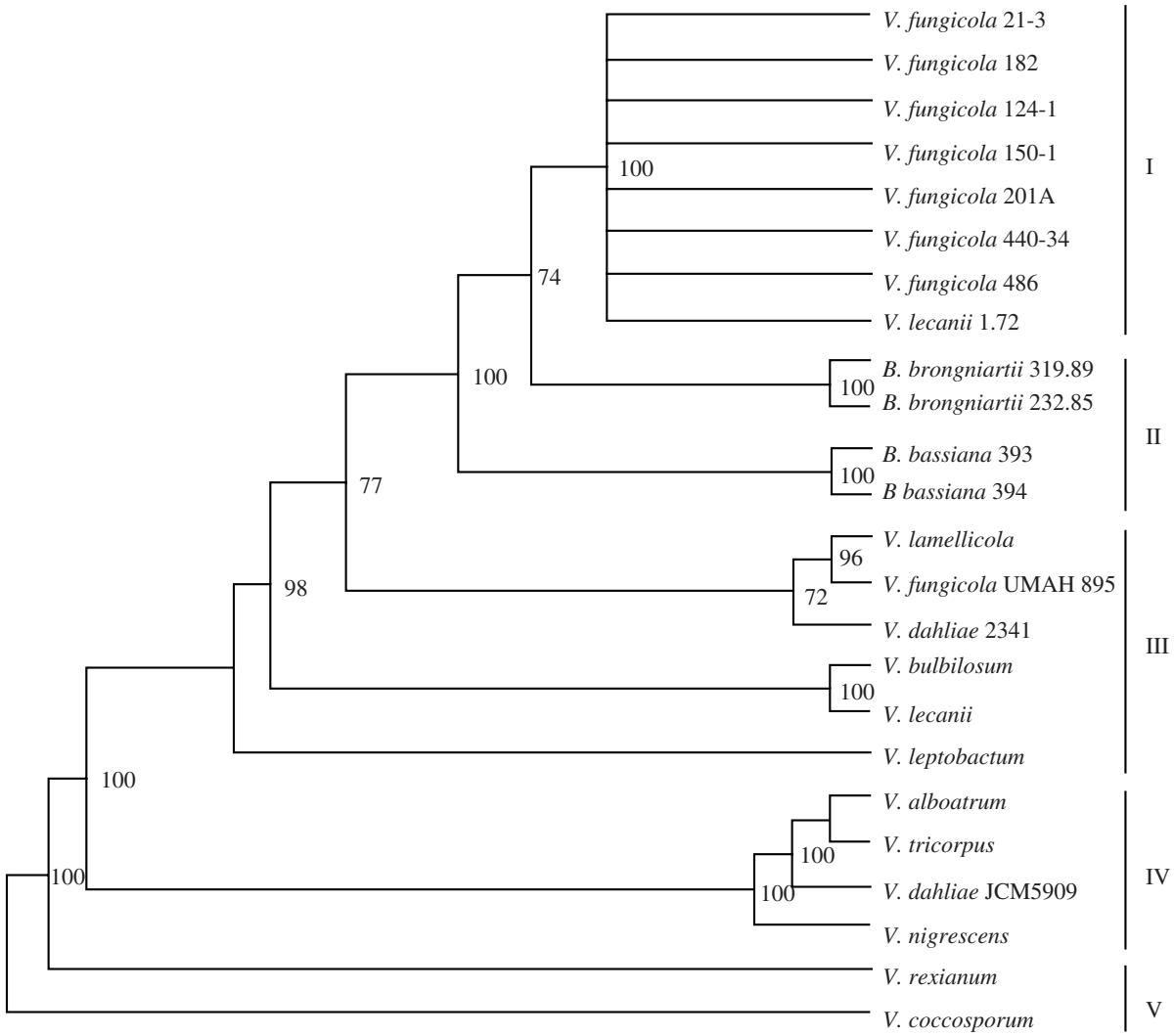

Fig. 5. The relationship of Verticillium fungicola isolates to other fungi based on rDNA ITS sequences. The dendrogram was produced by PAUP Parsimony analysis of the sequences using 1000 bootstraps (% values indicated at branches).

not the same species, although they may have evolved from a common ancestor. Figure 3 indicates that from a wider perspective, the two fungi are closely related and may form part of the same genus, especially as the fungi demonstrate morphological similarities at the microscopic level. However, this focused analysis indicates that clear differences are evident between the individual isolates.

It is interesting that the *V. psalliotae* isolates do not cluster with the majority of the *V. fungicola* isolates (Fig. 4). One isolate (*V. psalliotae* 5-8) clusters with the insect pathogens *V. lecanii* 1.72 and *B. brongniartii* isolates 319 and 232. This may suggest that this *V. psalliotae* isolate is an insect-pathogenic fungus or may have originated from one. Similarly the *V. psalliotae* isolate 247A clusters with *V. dahliae* isolate 12085, a plant pathogenic fungus, which may suggest that *V. psalliotae* isolate 247A may be plant pathogenic also. However, it may also suggest that *V. psalliotae* is very poorly defined or even misidentified, thus warranting further investigation into this poorly studied group. Furthermore, it may be that *V. dahliae* is wrongly classified, as it should be situated well away from this group. It is worth noting that the ITS sequence analysis separates the *V. dahliae* isolate 2341 from JCM5909, which clusters with the plant pathogens *V. alboatrum, V. tricorpus* and *V. nigrescens*. As these sequences were obtained in other studies we cannot comment on their accuracy, so it would be beneficial to examine these isolates at both the morphological level and with alternative sequence data. Indeed, these contradictions demonstrate quite clearly why multi-gene sequence analyses are so vital to taxonomic investigations.

In the nitrate reductase analysis, the observation that *V. psalliotae* isolate 5-8 clusters with the insect pathogens *V. lecanii* 1.72, *B. brongniartii* 319 and *B. brongniartii* 232 suggests that it may have evolved from a common ancestor of these species and therefore war-

rants further investigation. It would be of interest to determine the host range of this *V. psalliotae* isolate along with the host ranges of the other isolates studied to date such as *V. psalliotae* isolates 247A and 176-8, and *V. fungicola* isolate 149-9, which cluster with the plant pathogen *V. dahliae* 12085. The indication that common ancestors exist for these isolates illustrates the need to examine all aspects of an organism's biology to determine the final taxonomic status of fungi. Interestingly, the *V. psalliotae* isolate 247A lacks an intron in the nitrate reductase gene (Fig. 1). In all other isolates examined, an intron of approximately 70 bp was observed which suggests that this lack of an intron may be evolutionally significant. Significantly, in both nitrate reductase and ITS trees, the plant pathogens fall on different branches of the trees to most *V. fungicola* isolates, indicating very little similarity with *V. fungicola*. This finding agrees with the study of Carder and Barbara [3], who also observed this phenomenon.

These data illustrate that *V. fungicola* should be classified separately from other *Verticillium* species. Although high similarities are observed with the insect pathogenic *Beauveria* species it is clear that differences are evident at the genetic and biological levels and that it should therefore be placed in a form genus of its own. Indeed a new genus called *Lecanicillium* has been proposed to include the majority of entomogenous and fungicolous taxa [12].

Recently, a polymerase chain reaction-based test for *V. fungicola* was developed [29]. This specifically tests for *V. fungicola* var. *aleophilum* and not *V. fungicola* var. *fungicola*. As vigilant monitoring and early detection of disease is important to prevent yield-threatening outbreaks of *V. fungicola*, this test may prove of some use in monitoring the efficiency of hygiene practices and screening for sources of infection. As stated by Romaine et al. [29], a similar experimental approach could be adopted to develop a test specific for *V. fungicola* var. *fungicola*. Therefore, the nitrate reductase sequence data obtained in this study could be used to develop diagnostic kits for the detection of *V. fungicola* var. *fungicola*.

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