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Molecular studies on intraspecific diversity and phylogenetic position of *Coniothyrium minitans*

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Simple sequence repeat (SSR)–PCR amplification using a microsatellite primer $(GACA)_4$ and ribosomal RNA gene sequencing were used to examine the intraspecific diversity in the mycoparasite *Coniothyrium minitans* based on 48 strains, representing eight colony types, from 17 countries world-wide. *Coniothyrium cerealis*, *C. fuckelii* and *C. sporulosum* were used for interspecific comparison. The SSR–PCR technique revealed a relatively low level of polymorphism within *C. minitans* but did allow some differentiation between strains. While there was no relationship between SSR–PCR profiles and colony type, there was some limited correlation between these profiles and country of origin. Sequences of the ITS 1 and ITS 2 regions and the 5.8S gene of rRNA genes were identical in all twenty-four strains of *C. minitans* examined irrespective of colony type and origin. These results indicate that *C. minitans* is genetically not very variable despite phenotypic differences. ITS and 5.8S rRNA gene sequence analyses showed that *C. minitans* had similarities of 94% with *C. fuckelii* and *C. sporulosum* (which were identical to each other) and only 64% with *C. cerealis*. Database searches failed to show any similarity with the ITS 1 sequence for *C. minitans* although the 5.8S rRNA gene and ITS 2 sequences revealed an 87% similarity with *Aporospora terricola*. The ITS sequence including the 5.8S rRNA gene sequence of *Coniothyrium cerealis* showed 91% similarity to *Phaeosphaeria microscopica*. Phylogenetic analyses using database information suggest that *C. minitans*, *C. sporulosum*, *C. fuckelii* and *A. terricola* cluster in one clade, grouping with *Helminthosporium* species and '*Leptosphaeria*' *bicolor*. *Coniothyrium cerealis* grouped with *Ampelomyces quisqualis* and formed a major cluster with members of the *Phaeosphaeriacae* and *Phaeosphaeria microscopica*.

INTRODUCTION

Coniothyrium minitans is a well-known sclerotial mycoparasite and biocontrol agent of several sclerotium-forming pathogens, mainly species of *Sclerotinia* and *Sclerotium* (Campbell 1947, Whipps & Gerlagh 1992, Grendene & Marciano 1999). It has been shown to control *Sclerotinia sclerotiorum*, a widespread soil-borne plant pathogen, in both field and glasshouse trials (Huang 1980, Whipps & Budge 1990, Budge & Whipps 1991, Budge *et al*. 1995, Evenhuis *et al*. 1995, McQuilken & Whipps 1995, McQuilken, Budge & Whipps 1997a, Gerlagh *et al*. 1999) and currently there are two commercial products, Contans® and KONI (Whipps & Davies 2000). There has been considerable recent interest in inoculum production, formulation and application (McQuilken, Budge & Whipps 1997b, c, Weber, Tramper & Rinzema 1999, 2000), ecology (Williams, Whipps & Cooke 1998a, b, Jones *et al*. 1999) and physiology (Smith *et al*. 1998, 1999, Ooijkaas *et al*. 1999) of *C. minitans*.

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Taxonomically, *C. minitans* is considered a coelomycetous member of the *Leptosphaeriaceae*, in the *Dothideales* (Hawksworth *et al*. 1995). Campbell (1947) first described *C. minitans* and Punithalingam (1982) provided a slight modification to the original description. More recently, Sandys-Winsch *et al*. (1993) described the world-wide distribution of *C. minitans* and characterised *C. minitans* strains into seven groups based on colony morphology and production of pycnidia. However, the variation within these different morphological types of *C. minitans* derived from an international collection was not assessed further.

Molecular methods involving the production of DNA fingerprints have proved useful tools for analysing genetic diversity in fungi. RAPD, RFLP, AFLP (amplified fragment length polymorphisms), UP–PCR (universally-primed–PCR), AP–PCR (arbitrarily-primed–PCR), ERIC–PCR (enterobacterial repetitive intergenic consensus–PCR) and REP–PCR (repetitive extragenic palindromic–PCR) have all been used with success for a range of fungi (Arora, Hirsch & Kerry 1996, Chiu *et al*. 1996, Majer *et al*. 1996, Bryan *et al*. 1999, Lübeck *et al*. 1999). However, in some cases little variation has been detected and reproducibility has been a problem (Majer *et al*. 1996). Another procedure using simple sequence repeat (SSR)

DNA sequences as PCR primers was introduced to differentiate 36 yeast isolates into 10 species and 2 genera (Lieckfeldt *et al*. 1993). Subsequently, a set of six SSR primers and the phage M13 core sequence was used to differentiate over 70 fungal species representing 18 genera of filamentous fungi and five genera of yeasts (Meyer *et al*. 1993) and this technique has been used more recently to examine diversity in mycorrhizal fungi and *Colletotrichum gloeosporiodes* (Freeman, Katan & Shabi 1996, Longato & Bonfante 1997). These initial studies demonstrated that SSR–PCR was a powerful and easy to employ method for distinguishing large samples of fungal isolates at both the inter and intraspecies level.

Recently, PCR amplification of a dispersed repetitive element (DRE) has been used to distinguish a specific New Zealand isolate of *C. minitans* (Goldstein *et al*. 2000). This DRE was initially isolated from a 1.4 kb RAPD fragment that was specifically amplified in the biological control strain A69. When the RAPD fragment was sequenced it was found to contain two 114 bp direct repeats and a number of shorter 8–11 bp repeated elements. Assays based both upon Southern hybridization using the 1.4 kb RAPD fragment as a probe, and PCR using primers flanking the 114 bp, and shorter repeated elements, were able to produce DNA fingerprints that distinguished 33.3% (3/9) and 39.1% (9/23) of the isolates examined respectively. However, use of other molecular techniques to investigate intraspecific variation in this fungus has not been considered.

Consequently, to determine whether the different morphological types of *C. minitans* could be separated easily on a molecular basis, we used a simple sequence repeat (SSR)–PCR technique using the microsatellite primer $(GACA)_4$ to analyse a world-wide collection of over 40 *C. minitans* strains, including all morphological types, and also sequenced the internal transcribed spacers 1 and 2 and 5.8S rRNA gene from 24 representative strains. Further, as it has been proposed to accommodate *C. cerealis*, *C. fuckelii*, *C. minitans*, and *C. sporulosum* in a different genus from other species of *Coniothyrium* on the basis of conidial production (Domsch, Gams & Anderson 1980, Sutton 1980) we also sequenced single strains of *C. cerealis* and *C. fuckelii*, and two strains of *C. sporulosum* including the ex-type strain. The SSR–PCR technique was also carried out with *C. fuckelii* and *C. sporulosum*. This also enabled some interspecific comparisons to be made. On the basis of this molecular information and that available from databases the relationship between these *Coniothyrium* species and their phylogeny is described.

MATERIALS AND METHODS

Fungal strains

The *Coniothyrium* strains used in this study are listed in Table 1. The majority were obtained from the culture collections of Horticulture Research International (Sandys-Winsch *et al*. 1993) and Lincoln University (Goldstein *et al*. 2000) with two strains of *C. minitans*, two of *C. sporulosum* and one each of *C. cerealis* and *C. fuckelii* obtained from CBS (Centraalbureau voor Schimmelcultures, Utrecht). All strains used were characterised and identified according to the criteria of Domsch *et al*. (1980). Forty-three strains of C. *minitans* (encompassing eight colony types originating from 16 countries), two strains of *C. sporulosum* and one of *C. fuckelii* were used in the SSR–PCR based analysis. Twenty-four strains of *C. minitans* (encompassing eight colony types from 14 countries), two strains of *C. sporulosum*, and single strains of *C. cerealis* and *C. fuckelii* were used for ITS sequence analysis. *Coniothyrium minitans*, *C. sporulosum*, *C. fuckelii*, and *C. cerealis* ITS sequences were deposited in GenBank and accession codes are given in Table 2. These sequences were compared in detail to 24 other fungal sequences from databases, the majority of which were chosen for their high similarity value in an Advanced BLAST search using the program supported by NCBI (National Center for Biotechnology Information). These fungal sequences were largely from the *Dothideales*, encompassing anamorphic and teleomorphic representatives of three families (*Leptosphaeriaceae*, *Phaeosphaeriaceae* and *Pleosporaceae*): *Alternaria alternata* (GenBank accession code J276059, U05195), *Ampelomyces quisqualis* (AF126818, AF035783, U82451), *Aporospora terricola* (AF049088), *Helminthosporium* species (AF073910, AF120262), *Leptosphaeria* species (U04203, M96383, M96384, U04207, U04234), *Ophiosphaerella* species (U04861, AJ246157), *Phaeosphaeria* species (U77359, U04237, AF181710), *Pleospora herbarum* (U05202), *Phoma* species (L38711, AF149934) and *Stagonospora arenaria* (U77360). ITS sequences of *Neurospora crassa* (*Sordariales*, M13906) and *Sclerotinia sclerotiorum* (*Leotiales*, M96382) were also included in the analysis as outgroups.

Fungal cultures were maintained on potato dextrose agar (Oxoid) at 20 °C in the dark. Mycelium for DNA extraction was grown in liquid culture in malt extract medium (Oxoid). Briefly, malt extract liquid medium (1 ml) was placed onto a fully colonised agar plate culture of *Coniothyrium* and the surface of the culture was disturbed gently to release conidia and mycelial fragments. This inoculum was collected and was added to Petri dishes containing malt extract liquid medium (15 ml). The Petri dishes were incubated at 20 ° in dark and after 8–10 d the fungus formed a uniform mat covering the entire surface of the medium. The mycelial mat was collected aseptically, washed twice with sterile distilled water and lyophilised.

DNA extraction

Isolation of genomic DNA for microsatellite analysis was carried out as described elsewhere (Goldstein *et al*. 2000). For ITS amplification and sequencing, DNA was extracted from 100 mg of freeze-dried mycelial powder essentially following the method of Raeder & Broda (1985) to enable extraction in a 2 ml microfuge tube.

SSR–PCR analysis

To explore the use of microsatellite primers to detect the intraspecific diversity in *Coniothyrium minitans*, an initial screening with genomic DNA from three *C. minitans* strains (Cm6, Cm7 and Conio) was carried out using six different microsatellite oligonucleotides, [$(CA)_8$, $(CT)_8$, $(CAC)_5$, $(GTG)_5$ $(GACA)_4$ and $(GATA)_4$] as primers. These oligomers were

Table 1. Strains of *Coniothyrium* species used for molecular analysis.

Species	Code	Source[a]	Origin	Colony type[b]	ITS sequencing	SSR–PCR group[c]	DRE group[d]
C. cerealis	CBS 672.68	CBS	Germany	nd	+	nd	nd
C. fuckelii	CBS 132.26	CBS	Germany	nd	+	l	nd
C. minitans	A2/206/3	HRI	Australia	2	+	d	A
C. minitans	A2/212/1	HRI	Australia	2	nd	e	nd
C. minitans	A2/208/1	HRI	Australia	4	+	e	A
C. minitans	A2/256/1	HRI	Bulgaria	4	+	d	A
C. minitans	B/279/1	HRI	Canada	4	+	nd	nd
C. minitans	B/278/1	HRI	Canada	5	+	b	E
C. minitans	A2/330/1	HRI	Denmark	5	nd	d	nd
C. minitans	A1/327/1	HRI	Denmark	7	+	d	A
C. minitans	A2/1042/1	HRI	Germany	1	+	d	A
C. minitans	A2/1030/2	HRI	Germany	2	+	d	A
C. minitans	CBS 641.80	CBS	Germany	4	+	nd	nd
C. minitans	C106 (R425)	HRI	Germany	4	+	f	D
C. minitans	C105	HRI	Germany	5	+	d	A
C. minitans	A2/506/2	HRI	Israel	2	+	d	A
C. minitans	A2/509/1	HRI	Israel	2	nd	d	nd
C. minitans	A2/561/2	HRI	Japan	2	+	d	A
C. minitans	A2/558/3	HRI	Japan	5	+	c	E
C. minitans	A2/592/1	HRI	Korea	2	+	nd	nd
C. minitans	R391(G8)	HRI	Netherlands	1	+	h	B
C. minitans	C10A	HRI	Netherlands	1	nd	h	nd
C. minitans	C10	HRI	Netherlands	1	nd	k	nd
C. minitans	CmMgC9	HRI	Netherlands	4	nd	h	nd
C. minitans	G3	HRI	Netherlands	4	nd	h	nd
C. minitans	G4	HRI	Netherlands	4	nd	h	nd
C. minitans	C18	HRI	Netherlands	5	nd	h	nd
C. minitans	G2	HRI	Netherlands	5	nd	h	B
C. minitans	G9	HRI	Netherlands	5	nd	k	nd
C. minitans	A69	Lincoln	New Zealand	2	+	e	F
C. minitans	A70	Lincoln	New Zealand	2	nd	b	nd
C. minitans	Cm7	Lincoln	New Zealand	4	nd	a	G
C. minitans	Cm8	Lincoln	New Zealand	4	nd	g	H
C. minitans	ITS	Lincoln	New Zealand	4	nd	i	nd
C. minitans	L2E3	Lincoln	New Zealand	4	nd	i	nd
C. minitans	TSR42i	Lincoln	New Zealand	5	nd	f	nd
C. minitans	TSR42d	Lincoln	New Zealand	5	nd	d	nd
C. minitans	ST3 (A71)	Lincoln	New Zealand	6	+	h	B
C. minitans	A2/704/3	HRI	Portugal	2	nd	d	nd
C. minitans	A2/960/1	HRI	Sao Tome & Principe	1	nd	d	nd
C. minitans	A2/1049/1	HRI	South Africa	5	+	j	I
C. minitans	A2/787/2	HRI	Sri Lanka	1	+	d	nd
C. minitans	A2/792/1	HRI	Sudan	1	+	nd	nd
C. minitans	A2/806/3	HRI	Switzerland	4	nd	d	nd
C. minitans	CH1	HRI	UK	2	nd	d	nd
C. minitans	CONIO(IMI134523)	HRI	UK	3	+	e	A
C. minitans	CH3	HRI	UK	3	nd	d	nd
C. minitans	CH4	HRI	UK	3	nd	e	nd
C. minitans	CBS 859.71	CBS	UK	4	+	nd	nd
C. minitans	CONIO BUN	HRI	UK (mutant)	8	+	e	A
C. sporulosum	CBS 218.68	CBS	Germany	nd	+	m	nd
C. sporulosum	CBS 358.75A	CBS	Netherlands	nd	+	l	nd

[a] Culture collections: CBS, Centraalbureau voor Schimmelcultures; HRI, Horticulture Research International; Lincoln, Lincoln University.

[b] The colony morphology type of *C. minitans* is based on Sandys-Winsch *et al*. (1993). Colony morphology types 1, 2 and 3 are dark-coloured with dense pycnidial numbers; 4, 5, 6 and 7 are light coloured with relatively few pycnidia; 8 is a unique natural mutant derived from CONIO with very restricted mycelial growth.

[c] SSR–PCR group determined in this study.

[d] Dispersed repetitive element group based on Goldstein *et al*. (2000). nd, Not done.

supplied by Life Technologies (Paisley). Based on the number of bands produced and reproducibility of the banding patterns obtained following gel electrophoresis, $(GACA)_4$ was chosen to screen the rest of the isolates.

SSR–PCRs were performed in 50 µl reactions containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 3.0 mM $MgCl_2$, 100 µM each dNTP, 25 ng primer, 25 ng genomic DNA template and 2.5 U Taq DNA polymerase (Roche Diagnostics,

Table 2. Characteristics of ITS and 5.8S rRNA gene sequences of *Coniothyrium* species.

Species	Length			GenBank accession code	Species compared	% similarity			
	ITS 1	5.8S	ITS 2			ITS 1	5.8S	ITS 2	Total
C. minitans (Cm) CBS 641-80	195	157	156	AJ293809	Cm → Cs	90	100	97	94
C. sporulosum (Cs) CBS 358-75a	197	157	156	AJ293815	Cm → Cf	90	100	97	94
C. fuckelii (Cf) CBS 132-26	197	157	156	AJ293813	Cm → Cc	69	99	76	66
C. cerealis (Cc) CBS 672-68	191	157	157	AJ293812	Cs → Cf	100	100	100	100
					Cs → Cc	65	99	75	65

Lewes). After an initial denaturation for 2 min at 93 °, the reactions were subjected to 40 cycles each consisting of 20 s at 93 °, 60 s at 50 ° and 20 s at 72 °, followed by a final extension time of 6 min at 72 °. Each reaction was done twice to check the reproducibility. The PCR products were fractionated on a 1.4% agarose TAE buffered gel (Sambrook, Fritsch & Maniatis 1989).

Monomorphic PCR products, or bands, that were in every sample were not scored. All other polymorphic bands were scored binomially irrespective of band intensity as long as the band was consistently present or absent in at least two independent PCR amplifications of the isolate.

PCR amplification of ITS region and sequencing

The primers ITS 1-ext (5′-GTAACAAGGTTTCCGTAGG-TG-3′) and ITS4-ext (5′-TTCTTTTCCTCCGCTTATTGAT-ATGC-3′) were used to amplify rRNA gene spacers and the 5.8S rRNA gene sequence for each strain. 100 µl PCR reactions were performed, each containing appropriately diluted genomic DNA (∼10 ng), 1× reaction buffer (Promega, Southampton), 200 µM each of dNTPs, 0.4 µM each of primers and 2.5 U of Taq DNA polymerase (Promega). PCR amplification consisted of 94 ° for 2 min, followed by 30 cycles of 94 ° for 1 min, 60 ° for 1 min and 72 ° for 1 min and a final extension at 72 ° for 10 min.

PCR products were separated electrophoretically and visualised in 1.5% (w/v) agarose gels containing ethidium bromide (0.4 µg ml^{-1}). PCR products were purified using QIAquick™ PCR purification spin columns following the manufacturer's protocol (QIAGEN, Crawley).

Direct sequencing of PCR amplified products was carried out using primers. ITS 1 and ITS4 (White *et al*. 1990). Sequencing reactions were carried out using the ABI Prism™ Dye terminator cycle sequencing ready reaction kit (ABI/Perkin–Elmer). The products were purified by ethanol precipitation following the manufacturer's protocol and nucleotide sequences were determined by the ABI automated sequencing technology.

Data analysis

The binomial matrix data from the SSR–PCR procedure was analysed with Phylogenetic Analysis using Parsimony (PAUP), version 4.0 software (Swofford, 1999) which uses the cluster analysis of the unweighted pair group method using arithmetic averages (UPGMA). The tree was rooted with two strains of *Coniothyrium sporulosum* and one of *C. fuckelii*. The length of the tree was computed as the (weighted) sums of the lengths of the individual characters:

$$L = \sum_{j=1}^{c} wjlj,$$

where L = length of the full tree, C = total number of characters, wj = weighted sums and lj = lengths of weighted characters.

DNA sequences from ITS 1 and ITS 2 regions and the 5.8S rRNA gene were edited using the Editseq program (DNASTAR, Madison). DNA sequences were aligned with the programme CLUSTAL W (Thompson, Higgins & Gibson 1994) of the UWGCG package. All positions were considered for further analysis. The data set consisted of 543 alignable positions in total (the alignment is available from the authors upon request and is accessible from the HRI web site at http://www.hri.ac.uk/site2/research/path/sclerpat/scleropa.htm). Phylogenetic analyses of the aligned sequences were performed with Distance and Parsimony methods in the PHYLIP package (Felsenstein 1993). DNADIST programme was used to compute distance matrix using Kimura 2-parameter model (Kimura 1980). The distance tree was drawn using NEIGHBOR (data not shown). For parsimony analysis, the same multiple alignment used for distance analysis was used to generate 1000 bootstrap resamplings using SEQB-OOT to determine the branch support for trees (Felsenstein 1985). DNAPARS was used to search for the best tree by ordinary parsimony. The dataset were subjected to 10 randomisations of sequence input order. The resultant trees were analysed using the program CONSENSE to calculate a majority rule consensus tree. The treefile then was read by TREEVIEW (http://taxonomy.gla.ac.uk/rod/treeview.html; Page 1996) to generate the final dendrogram (Fig. 4). Similarity (%) between various *Coniothyrium* spp. and related sequences was calculated using BESTFIT on GCG, Wisconsin.

RESULTS

SSR–PCR analysis

Of the six microsatellite primers examined, only $(GACA)_4$ produced a number of reproducible bands that might be useful for intraspecific differentiation. Of the remaining primers, $(CT)_8$ and $(GATA)_4$ did not amplify *Coniothyrium minitans* DNA; $(GTG)_5$ produced a smear for all three isolates; $(CAC)_5$ did not produce any intraspecific differentiation between the isolates; and $(GA)_8$ amplified poorly producing faint PCR products (data not shown).

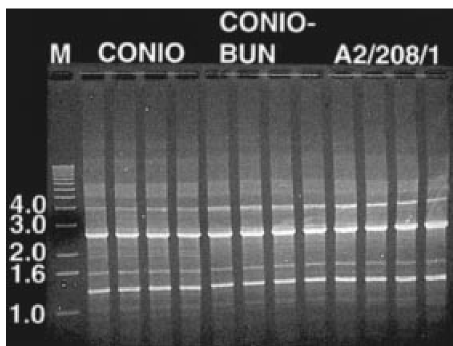

Fig. 1. Microsatellite primer $(GACA)_4$ produces reproducible DNA fingerprints in the SSR–PCR technique. Each *Coniothyrium minitans* strain (Conio, ConioBun and A2/208/1) was amplified in four separate reactions and the reaction products were separated on a 1.4% agarose TAE gel. The ConioBun and A2/208/1 PCR products were fractionated on a separate gel from the Conio products. M, marker.

Primer $(GACA)_4$ was used to amplify genomic DNA from *C. minitans* strains Conio, ConioBun and A2/208/1. Each strain was amplified in four separately mixed PCR reactions to assess the reproducibility of DNA fingerprints with this microsatellite primer. Fig. 1 shows that *C. minitans* genomic DNA amplified with microsatellite primer $(GACA)_4$ produced reproducible fingerprints, even at hybridisation temperatures at 50 °. In addition, independent duplicate reactions of 38 *C. minitans*, two *C. fuckelii* and a single *C. sporulosum* isolate were amplified using the $(GACA)_4$ primer and the banding pattern on 1.4% agarose TAE gels compared, confirming that fingerprints using the microsatellite primer $(GACA)_4$ were consistent and reproducible (data not shown). Fig. 2 shows single reactions of each of forty-one samples fractionated in 1.4% agarose gels in TAE buffer. A total of 11 differently sized bands were observed when all strains of *Coniothyrium* were considered.

The PAUP analysis software program was used to facilitate grouping strains together that had identical simple sequence repeat profile and each group was assigned a lower case letter group name. The SSR–PCR groups are compared to DRE groups (Goldstein *et al*. 2000) and colony type in Table 1. The DRE groups were previously assigned an upper case letter group name based upon identical DRE profiles. No attempt was made to match group names between SSR–PCR and DRE profiles. There was generally good agreement between the SSR–PCR groups and DRE groups. For example, all DRE group B strains coincided with SSR–PCR group h strains. In addition, all of the DRE group A strains coincided with SSR–PCR groups d or e. These groups differ only by the presence of an additional 1.4 kb band in the group d strains. The separation of DRE group A strains into two SSR–PCR groups suggests that SSRs provide finer resolution of *C. minitans* strains than DREs.

While there was no clear correlation between SSR–PCR profiles and colony type, there was some correlation between these profiles and strain origin. For example, all of the group h and k strains, with the exception of a single isolate from New Zealand, were from the Netherlands. Nevertheless, other SSR–PCR groups, such as d and e, contain strains of *C. minitans* from a widely distributed series of countries, so that generalities concerning such SSR–PCR relationships must be treated with caution.

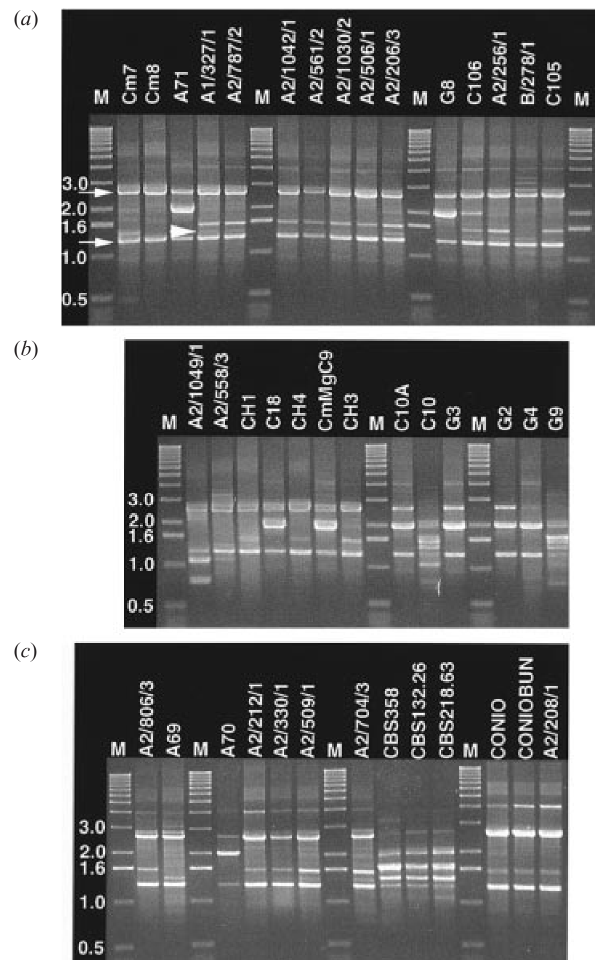

Fig. 2. Products of PCR using microsatellite primer $(GACA)_4$ and genomic DNA from *Coniothyrium* strains. PCR products were fractionated on 1.4% agarose TAE gels. See Table 1 for strain details. The arrows indicate bands found in every *C. minitans* isolate. The arrowhead shows an example of a faintly amplified band that was nevertheless consistently detected in repeated amplifications. M, marker.

Coniothyrium fuckelii and one strain of *C. sporulosum* produced identical SSR–PCR profiles (Table 1) but both *C. sporulosum* strains and *C. fuckelii* were readily distinguishable from *C. minitans*.

Sequence analysis of ITS region

The length of the ITS and 5.8S rRNA gene sequences, the GenBank accession codes and the similarity comparisons of the four *Coniothyrium* species are given in Table 2. The nucleotide sequence of ITS 1-5.8S-ITS 2 region was identical for all twenty-four *C. minitans* strains used in this study. The sequences of the ITS spacer regions and 5.8S gene were identical for the two *C. sporulosum* strains (CBS 218.68 and CBS 358.75A) and these were well distinguished from *C. minitans* sequence, at 94% similarity. The sequence from the *C. fuckelii* strain CBS 132.26 was identical to that of *C.*

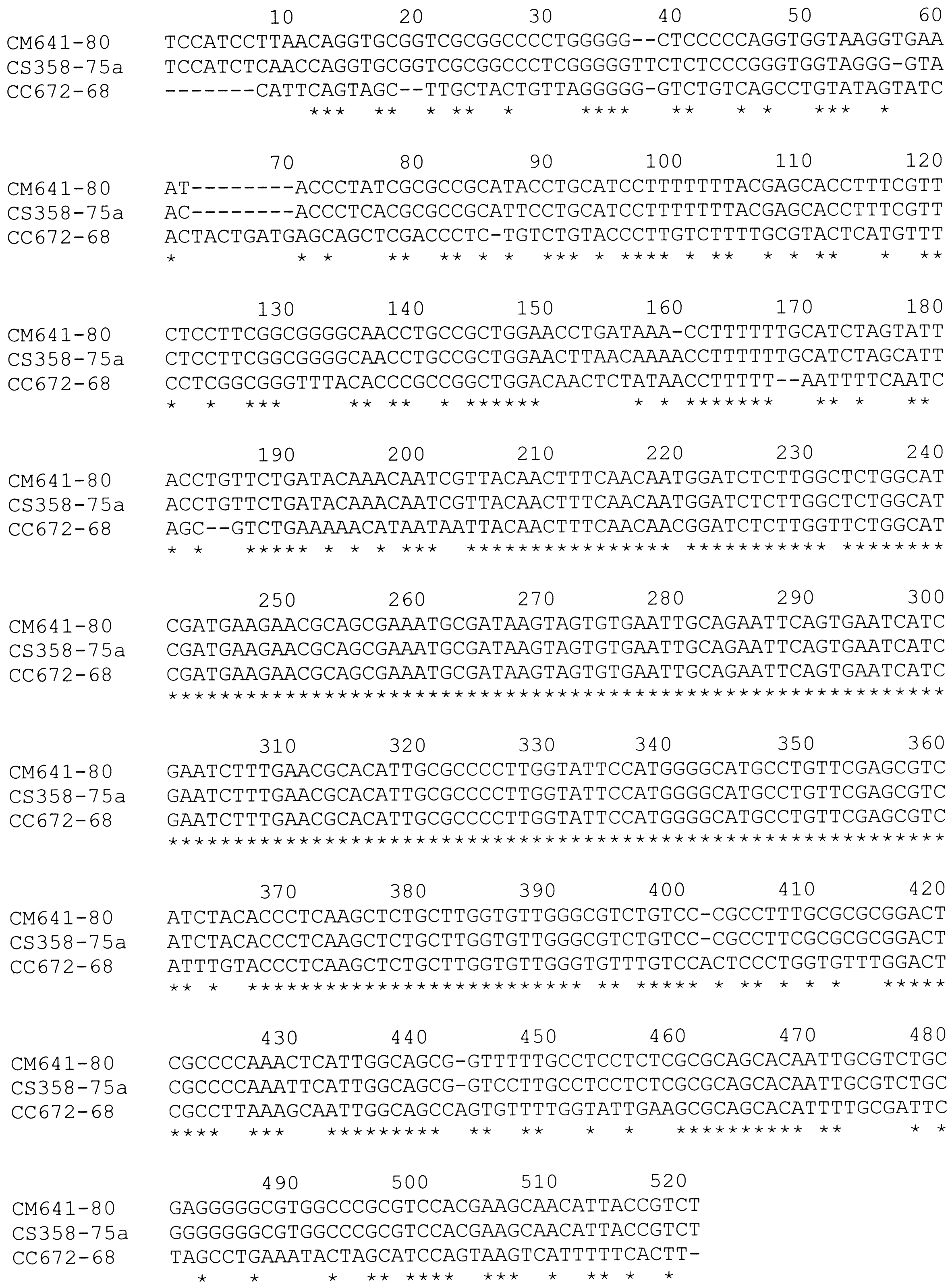

Fig. 3. Alignment of the 5.8S rRNA gene sequences and their flanking internal transcribed spacers (ITS 1 and ITS 2) of *Coniothyrium minitans* (CM) CBS 641-80, *C. sporulosum* (CS) CBS 358-75a and *C. cerealis* (CC) CBS 672-68. ITS 1 encompasses bp 1–206; the 5.8S coding region extends from bp 207–363 and the ITS 2 from bp 364–521. A hyphen represents an introduced gap and positions identical in all three sequences are marked with an asterix. The sequences are written 5′ to 3′.

sporulosum. The single strain of *C. cerealis* CBS 672–68 showed only 65–66% similarity to *C. minitans* and *C. sporulosum*. In general, the base sequence of ITS 1 was more variable than ITS 2 (Table 2).

An alignment of the DNA sequences of ITS 1, ITS 2 and the 5.8S RNA gene of *C. minitans, C. sporulosum* and *C. cerealis* is shown in Fig. 3. The DNA sequence of *Coniothyrium cerealis* was quite different from that of *C. minitans* and *C. sporulosum*

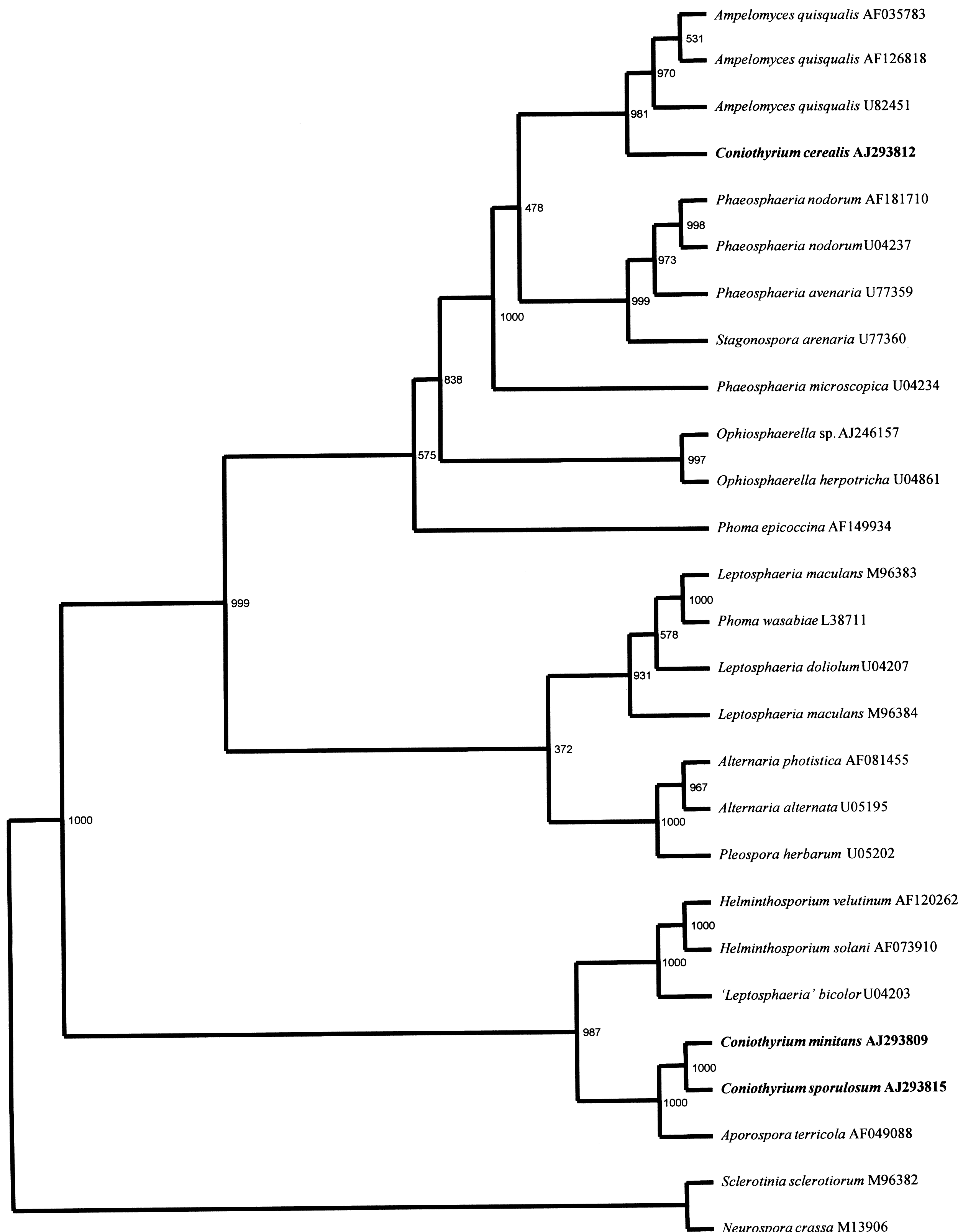

Fig. 4. Parsimony tree based on the 5.8S rRNA gene sequences and their flanking internal transcribed spacers (ITS 1 and ITS 2) of *Coniothyrium minitans* CBS 641–80 (AJ293809), *C. sporulosum* CBS 358-75a (AJ293815) and *C. cerealis* CBS 672-68 (AJ293812), closely related species, and outgroups (*Neurospora crassa* and *Sclerotinia sclerotiorum*). Majority rule consensus tree generated by the CONSENSE program included in the PHYLIP package. The numbers at the nodes are boostrap values based on 1000 resamplings.

and therefore the alignment contained a large proportion of ambiguously aligned sites. The base composition of ITS spacers and 5.8S gene was uniform between *C. sporulosum* and *C. minitans* with G-C content ranging from 53 to 55% but different from that of *C. cerealis* which had only 46% G-C content.

To examine the phylogeny of *Coniothyrium*, a database search was carried out with all three sequences, ITS 1, ITS 2

and the 5.8S rRNA gene, separately and as a single stretch using the Advanced BLAST search program (NCBI). The search using ITS 1 of *C. minitans* and *C. sporulosum* did not show similarity to any other sequences in databases, whereas 5.8S rRNA gene and ITS 2 spacer region revealed high similarity to *Aporospora terricola*. BESTFIT of *C. minitans* sequence including the spacers and 5.8S to *Aporospora terricola* resulted in 87% identity. *C. cerealis* sequences showed greatest similarity to *Phaeosphaeria microscopica* (syn. *Leptosphaeria microscopica*) (91% identity).

Phylogenetic analysis

Coniothyrium minitans, *C. sporulosum* and *Aporospora terricola* grouped in one clade in the phylogenetic analysis using both distance (data not shown) and parsimony methods (Fig. 4) and formed a sister group with *Helminthosporium* spp. and '*Leptosphaeria*' *bicolor*. These two groups were statistically well supported and formed a separate branch from other *Leptosphaeriaceae* members. In both analysis, *Phaeosphaeriaceae* (*Phaeosphaeria nodorum*, *P. avenaria* and *Stagnospora arenaria*), *Pleosporaceae* (*Alternaria* spp., *Pleospora* sp.), *Leptosphaeriaceae* (*L. maculans*, *L. doliolum*, and *Phoma wasabiae*) and *Ophiosphaerellaceae* formed tight clusters. The only strain of *C. cerealis* used in this study did not group with other species of *Coniothyrium* but was strongly supported as a clade with *Ampelomyces quisqualis*, a mitosporic fungus, and formed a major cluster with other *Phaeosphaeriaceae* members.

DISCUSSION

The appropriateness of any DNA-fingerprinting technique relies upon the ability of the technique to reflect genetic distances between individuals (Soll 2000). One way to assess whether a DNA-fingerprinting technique fulfils this requirement is to compare the data produced by that method with data produced by an unrelated method (Tibayrenc *et al*. 1993, van Belkum *et al*. 1994, Pujol *et al*. 1997). To this end we compared the relatedness of *Coniothyrium minitans* isolates by both SSR–PCR and with an unrelated dispersed repetitive element (DRE) (Goldstein *et al*. 2000). In the experiments reported here, 25.6% (11/43) of the isolates had unique DNA fingerprints using the $(GACA)_4$ primer. Importantly, as described in the Results section, there was excellent agreement between genetic distances assigned by SSR–PCR and DRE fingerprinting. For example, the two largest DRE groups, A and B, were completely contained within SSR–PCR groups h and d or e respectively. Since SSR–PCR could resolve the largest DRE fingerprinting group into two groups, it appears that SSRs provides better resolution of *C. minitans* isolates than DREs. Furthermore, the fact that these two independent means of assaying genetic relatedness agree closely, validates the use of SSR–PCR to distinguish *C. minitans* isolates and strongly supports the hypothesis that there is little world-wide genetic diversity in this species. There were no polymorphisms in the ITS region of *C. minitans* as the sequences were identical in all 26 strains examined despite being of wide geographical origin and differing colony type. This further indicates that genetic diversity is limited amongst *C. minitans* strains and that which is present in these genetic regions is not related to phenotype or place of origin.

The degree of intraspecific variation in the ITS region can vary considerably in different fungi. For example, it has been found to be highly variable in some such as *Beauveria brongniartii* (Neuveglise *et al*. 1994), *Colletotrichum lindemuthianum* (Balardin *et al*. 1999), *Fusarium fujikuroi* (O' Donnell & Cigelnik 1997), *Metarhizium anisopliae* (Curran *et al*. 1994), *Puccinia monoica* (Roy *et al*. 1998), and some groups of *Trichoderma harzianum* (Muthumeenakshi *et al*. 1994), or very conserved in others including *Oidiodendron maius* (Hambleton *et al*. 1998), some *Penicillium* species (LoBuglio *et al*. 1994), *Phytophthora quercina* (Cooke *et al*. 1999), and some other groups of *Trichoderma harzianum* (Muthumeenakshi *et al*. 1998). However, the finding that strains of species from geographically distinct areas have identical ITS sequences as with *C. minitans* in this study and with some *Ascosphaera* species (Anderson *et al*. 1998) are rare. This begs the question of the reason for this conservation. One possibility is that *C. minitans* is a relatively new species having recently overcome some constraint or bottleneck that has subsequently allowed rapid spread. In part, this may be related to the specialised life cycle of *Coniothyrium minitans*. It is an ecologically obligate necrotrophic mycoparasite and is restricted to growth in host sclerotia or plant tissues infected by *Sclerotinia* species. In the absence of its host, *C. minitans* survives in soil as conidia although it can be dispersed by rain splash and mesofauna (Whipps & Gerlagh 1992, Williams *et al*. 1998a, b). This could suggest that the world-wide distribution of *C. minitans* is associated with the spread of its host *Sclerotinia* species and susceptible crops. Another possible reason for the low levels of polymorphism is that the species reproduces asexually only.

The SSR–PCR technique and ITS sequencing demonstrated that *C. minitans* was closely related to both *C. fuckelii* and *C. sporulosum* but the latter two species were virtually identical based on data from both molecular tests. Domsch *et al*. (1980) noted that *C. sporulosum* and *C. fuckelii* were indistinguishable on the basis of conidial characteristics and our work corroborates the view that these species may be conspecific. *Coniothyrium cerealis* was less closely related to *C. minitans*, *C. sporulosum* and *C. fuckelii* (65–66% similarity based on ITS sequencing) and also appeared in a different lineage in the phylogenetic analysis although more strains would need to be examined to be certain of this difference in lineage.

Coniothyrium is currently considered an anamorphic member of the *Leptosphaeriaceae* with *Leptosphaeria* as teleomorph (Hawksworth *et al*. 1995). However, no teleomorphic stage has been reported for *C. minitans*. We included *Leptosphaeria* species and other dothidealean fungi from databases in our phylogenetic analysis to determine the relationship of the *Coniothyrium* species used in this study. *Leptosphaeriaceae* (*Leptosphaeria* species with *Phoma* as their anamorph), *Pleosporaceae* (*Pleospora* and *Alternaria*) and *Ophiosphaerella* species were well distinguished by phylogenetic analysis and this result agrees with previous analyses (Morales *et al*. 1995, Jasalavich *et al*. 1995). Phylogenetic analysis indicates the distinct phylogenetic position of *C. minitans* and *C. sporulosum* from other *Leptosphaeriaceae*, *Pleosporaceae* and *Phaeo-*

sphaeriaceae. The closest relationship of these two *Coniothyrium* species was with *Aporospora terricola* but little information other than the sequence and culture deposition data appear available for this species. Morales *et al*. (1995) distinguished '*L.*' *bicolor* as a separate group phylogenetically distant from *Leptosphaeriaceae*, and, interestingly, *C. minitans* and *C. sporulosum* formed a sister group with '*L.*' *bicolor* with a high bootstrap value in our analysis. The only strain of *C. cerealis* used in our study grouped with *Ampelomyces* and was placed along with other *Phaeosphaeriaceae*. Our study also indicates that *C. cerealis* is more closely related to *Phaeosphaeria nodorum* and *P. microscopica* than to the other *Coniothyrium* species. Even though the conidial formation was found to be sparse, we are confident that *C. cerealis* culture used in this study matched the description (Domsch *et al*. 1980). Consequently, it would appear from our studies that *C. cerealis* is phylogenetically distinct from *C. minitans*, *C. fuckelii* and *C. sporulosum*.

In general, our tree extends the phylogeny of *Leptosphaeriaceae* and *Phaeosphaeriaceae* by the placement of four anamorphic *Coniothyrium* species. The genus *Coniothyrium* consists of a large number of species and so, it is not surprising to see a polyphyletic nature of this anamorphic genus. More molecular characterisation studies on a greater number of species and strains of *Coniothyrium*, as well as the closely related *Microsphaeropsis* (Morgan-Jones 1974, Sutton 1980), is required to establish the phylogeny of this genus.

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REFERENCES

Anderson, D. L., Gibbs, A. J. & Gibson, N. L. (1998) Identification and phylogeny of spore-cyst fungi (*Ascosphaera* spp.) using ribosomal DNA sequences. *Mycological Research* **102**: 541–547.

Arora, D. K., Hirsch, P. R. & Kerry, B. R. (1996) PCR-based molecular discrimination of *Verticillium chlamydosporium* isolates. *Mycological Research* **100**: 801–809.

Bryan, G. T., Labourdette, E., Melton, R. E., Nicholson, P., Daniels, M. J. & Osbourn, A. E. (1999) DNA polymorphism and host range in the take-all fungus, *Gaeumannomyces graminis*. *Mycological Research* **103**: 319–327.

Budge, S. P., McQuilken, M. P., Fenlon, J. S. & Whipps, J. M. (1995) Use of *Coniothyrium minitans* and *Gliocladium virens* for biocontrol of *Sclerotinia sclerotiorum* in glasshouse lettuce. *Biological Control* **5**: 513–522.

Budge, S. P. & Whipps, J. M. (1991) Glasshouse trials of *Coniothyrium minitans* and *Trichoderma* species for the biological control of *Sclerotinia sclerotiorum* in celery and lettuce. *Plant Pathology* **40**: 59–66.

Campbell, W. A. (1947) A new species of *Coniothyrium* parasitic on sclerotia. *Mycologia* **39**: 190–195.

Chiu, S.-W., Ma, A.-M., Lin, F.-C. & Moore, D. (1996) Genetic homogeneity of cultivated strains of shiitake (*Lentinula edodes*) used in China as revealed by the polymerase chain reaction. *Mycological Research* **100**: 1393–1399.

Cooke, D. E. L., Jung, T., Williams, N. A., Schubert, R., Bahnweg, G., Obwald, W. & Duncan, J. M. (1999) Molecular evidence supports *Phytophthora quercina* as a distinct species. *Mycological Research* **103**: 799–804.

Curran, J. F., Driver, J.W., Ballard, O. & Milner, R. H. (1994) Phylogeny of *Metarhizium*: analysis of ribosomal DNA sequence data. *Mycological Research* **98**: 547–552.

Domsch, K. H., Gams, W. & Anderson, T.-H. (1980) *Compendium of Soil Fungi*. Academic Press, London.

Evenhuis, A., Verdam, B., Gerlagh, M. & Goosen-van de Geijn, H. M. (1995) Studies on major diseases of caraway (*Carum carvi*) in the Netherlands. *Industrial Crops and Products* **4**: 53–61.

Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Felsenstein, J. (1993) *PHYLIP* (*Phylogeny Inference Package*). Version 3.5c. University of Washington, Seattle.

Freeman, S., Katan, T. & Shabi, E. (1996) Characterization of *Colletotrichum gloeosporioides* isolates from avocado and almond fruits with molecular and pathogenicity tests. *Applied and Environmental Microbiology* **62**: 1014–1020.

Gerlagh, M., Goosen-van de Geijn, H. M., Fokkema, N. J. & Vereijken, P. F. G. (1999) Long-term biosanitation by application of *Coniothyrium minitans* on *Sclerotinia sclerotiorum*-infected crops. *Phytopathology* **89**: 141–147.

Goldstein, A. L., Carpenter, M. A., Crowhurst, R. N. & Stewart, A. (2000) Identification of *Coniothyrium minitans* isolates using PCR amplification of a dispersed repetitive element. *Mycologia* **92**: 46–53.

Grendene, A. & Marciano, P. (1999) Interaction between *Sclerotinia sclerotiorum* and *Coniothyrium minitans* strains with different aggressiveness. *Phytoparasitica* **27**: 201–206.

Hambleton, S., Egger, K. N. & Currah, R. S. (1998) The genus Oidiodendron: species delimitation and phylogenetic relationships based on nuclear ribosomal DNA analysis. *Mycologia* **90**: 854–869.

Hawksworth, D. L., Kirk, P. M., Sutton, B. C. & Pegler, D. N. (1995) *Ainsworth* & *Bisby's Dictionary of the Fungi*. 8th edn. CAB International, Wallingford.

Huang, H. C. (1980) Control of Sclerotinia wilt of sunflower by hyperparasites. *Canadian Journal of Plant Pathology* **2**: 26–32.

Jasalavich, C. A., Morales, V. M., Pelcher, L. E. & Seguin-Swartz, G. (1995) Comparison of nuclear ribosomal DNA sequences from *Alternaria* species pathogenic to crucifers. *Mycological Research* **99**: 604–615.

Jones, E. E., Carpenter, M., Fong, D., Goldstein, A., Thrush, A., Crowhurst, R. & Stewart, A. (1999) Co-transformation of the sclerotial mycoparasite *Coniothyrium minitans* with hygromycin B resistance and B-glucuronidase markers. *Mycological Research* **103**: 929–937.

Kimura, M. (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies on nucleotide sequences. *Journal of Molecular Evolution* **2**: 87–90.

Kuhls, K., Lieckfeldt, E., Samuels, G. J., Meyer, W., Kubicek, C. P. & Borner, T. (1997) Revision of *Trichoderma* sect. *Longibrachiatum* including related teleomorphs based on analysis of ribosomal DNA internal transcribed spacer sequences. *Mycologia* **89**: 442–460.

Lieckfeldt, E., Meyer, W. & Börner, T. (1993) Rapid identification and differentiation of yeasts by DNA and PCR fingerprinting. *Journal of Basic Microbiology* **33**: 413–426.

LoBuglio, K. F., Pitt, J. I. & Taylor, J. W. (1994) Independent origins of the synnematous *Penicillium* species, *P. duclauxii*, *P. clavigerum* and *P. vulpinum* as assessed by two ribosomal DNA regions. *Mycological Research* **98**: 250–256.

Longato, S. & Bonfante, P. (1997) Molecular identification of mycorrhizal fungi by direct amplification of microsatellite regions. *Mycological Research* **101**: 425–432.

Lübeck, M., Alekhina, I. A., Lubeck, P. S., Funck Jensen, D. & Bulat, S. A. (1999) Delineation of *Trichoderma harzianum* into two different genotypic groups by a highly robust fingerprinting method, UP–PCR, and UP–PCR product cross-hybridization. *Mycological Research* **103**: 289–298.

Majer, D., Mithen, R., Lewis, B. G., Vos, P. & Oliver, R. P. (1996) The use of AFLP fingerprinting for the detection of genetic variation in fungi. *Mycological Research* **100**: 1107–1111.

McQuilken, M. P., Budge, S. P. & Whipps, J. M. (1997a) Effects of culture media and environmental factors on conidial germination, pycnidial production and hyphal extension of *Coniothyrium minitans*. *Mycological Research* **101**: 11–17.

McQuilken, M. P., Budge, S. P. & Whipps, J. M. (1997b) Production, survival and evaluation of liquid culture-produced inocula of *Coniothyrium minitans* against *Sclerotinia sclerotiorum*. *Biocontrol Science and Technology* **7**: 23–36.

McQuilken, M. P., Budge, S. P. & Whipps, J. M. (1997c) Biological control of *Sclerotinia sclerotiorum* by film-coating *Coniothyrium minitans* on seed and sclerotia. *Plant Pathology* **46**: 919–929.

McQuilken, M. P. & Whipps, J. M. (1995) Production, survival and evaluation of solid-substrate inocula of *Coniothyrium minitans* against *Sclerotinia sclerotiorum*. *European Journal of Plant Pathology* **101**: 101–110.

Meyer, W., Lieckfeldt, E., Kuhls, K., Freedman, E. Z., Börner, T. & Mitchell, T. G. (1993) DNA- and PCR-fingerprinting in fungi. In *DNA Fingerprinting: state of the science* (S. D. J. Pena, R. Chakraborty, J. T. Epplen & A. J. Jeffreys, eds): 311–320. Birkhäuser Verlag, Basel.

Morales, V. M., Jasalavich, C. A., Pelcher, L. E., Petrie, G. A. & Taylor, J. L. (1995) Phylogenetic relationship among several *Leptosphaeria* species based on their ribosomal DNA sequences. *Mycological Research* **99**: 593–603.

Morgan-Jones, G. (1974) Concerning some species of *Microsphaeropsis*. *Canadian Journal of Botany* **52**: 2575–2579.

Muthumeenakshi, S., Mills, P. R., Brown, A. E. & Seaby, D. A. (1994) Intraspecific molecular variation among *Trichoderma harzianum* isolates colonizing mushroom compost in the British Isles. *Microbiology* **140**: 769–777.

Muthumeenakshi, S., Mills, P. R. & Brown, A. E. (1998) Genetic comparison of the aggressive weed mould strains of *Trichoderma harzianum* from mushroom compost in North America and the British Isles. *Mycological Research* **102**: 385–390.

Neuveglise, C., Brygoo, Y., Vercambre, B. & Riba, G. (1994) Comparative analysis of molecular and biological characteristics of strains of *Beauveria brongniartii* isolated from insects. *Mycological Research* **98**: 322–328.

O'Donnell, K. & Cigelnik, E. (1997) Two divergent intragenomic rDNA ITS 2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* **7**: 103–116.

Ooijkaas, L. P., Wilkinson, E. C., Tramper, J. & Buitelaar, R. M. (1999) Medium optimization for spore production of *Coniothyrium minitans* using statistically-based experimental designs. *Biotechnology and Bioengineering* **64**: 92–100.

Page, R. D. M. (1996) TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* **12**: 357–358.

Pujol, C., Joly, S., Lockhart, S., Noel, S., Tibayrenc, M. & Soll, D. R. (1997) Parity of MLEE, RAPD and Ca3 hybridization as fingerprinting methods for *Candida albicans*. *Journal of Clinical Microbiology* **35**: 2348–2358.

Punithalingam, E. (1982) *Coniothyrium minitans*. *CMI Descriptions of Pathogenic Fungi and Bacteria* **732**: 1–2.

Raeder, U. & Broda, P. (1985) Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* **1**: 17–20.

Roy, B. A., Vogler, D. R., Burns, T. D. & Szaro, T. M. (1998) Cryptic species in the *Puccinia monoica* complex. *Mycologia* **90**: 846–853.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning – A Laboratory Manual*. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Sandys-Winsch, C., Whipps, J. M., Gerlagh, M. & Kruse, M. (1993) World distribution of the sclerotial mycoparasite *Coniothyrium minitans*. *Mycological Research* **97**: 1175–1178.

Smith, S. N., Armstrong, R. A., Barker, M., Bird, R. A., Chohan, R., Hartel, N. A. & Whipps, J. M. (1999) Determination of *Coniothyrium minitans* conidial and germling lectin affinity by flow cytometry and digital microscopy. *Mycological Research* **103**: 1533–1539.

Smith, S. N., Chohan, R. A., Armstrong, R. A. & Whipps, J. M. (1998) Hydrophobicity and surface charge of conidia of the mycoparasite *Coniothyrium minitans*. *Mycological Research* **102**: 243–249.

Soll, D. R. (2000) The ins and outs of DNA fingerprinting the infectious fungi. *Clinical Microbiological Reviews* **13**: 332–370.

Sutton, B. C. (1980) *The Coelomycetes*. Commonwealth Mycological Institute, Kew, Surrey.

Swofford, D. L. (1999) *PAUP: Phylogenetic Analysis using Parsimony*. Version 4.0. Sinauer Associates, Sunderland, MA.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673–4680.

Tibayrenc, M., Neubauer, K., Barnabé, C., Guerrini, F., Skarecky, D. & Ayala, F. J. (1993) Genetic characterization of six parasitic protozoa: parity between random-primer DNA typing and multilocus enzyme electrophoresis. *Proceedings of the National Academy of Sciences, USA* **90**: 1335–1339.

van Belkum, A., Melchers, W., de Pauw, B. E., Scherer, S., Quint, W. & Meis, J. F. (1994) Genotypic characterization of sequential *Candida albicans* isolates from fluconazole-treated neutropenic patients. *Journal of Infectious Disease* **169**: 1062–1070.

Weber, F. J., Tramper, J. & Rinzema, A. (1999) A simplified material and energy balance approach to process development and scale-up of *Coniothyrium minitans* conidia production by solid-state cultivation in packed-bed reactor. *Biotechnology and Bioengineering* **65**: 447–458.

Weber, F. J., Tramper, J. & Rinzema, A. (2000) Effect of the availability of magnesium ions in K-carrageenan gels on the formation of conidia by *Coniothyrium minitans*. *Mycological Research* **104**: 73–76.

Whipps, J. M. & Budge, S. P. (1990) Screening for sclerotial mycoparasites of *Sclerotinia sclerotiorum*. *Mycological Research* **94**: 607–612.

Whipps, J. M. & Davies, K. G. (2000) Success in biological control of plant pathogens and nematodes by microorganisms. In *Measures of Success in Biological Control* (G. Gurr & S. D. Wratten, eds): 231–269. Kluwer Academic Publishers, Dordrecht.

Whipps, J. M. & Gerlagh, M. (1992) Biology of *Coniothyrium minitans* and its potential for use in disease biocontrol. *Mycological Research* **96**: 897–907.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A guide to methods and applications* (M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White, eds): 315–322. Academic Press, San Diego.

Williams, R. H., Whipps, J. M. & Cooke, R. C. (1998a) The role of soil mesofauna in dispersal of *Coniothyrium minitans*: mechanisms of transmission. *Soil Biology and Biochemistry* **30**: 1937–1945.

Williams, R. H., Whipps, J. M. & Cooke, R. C. (1998b) Splash dispersal of *Coniothyrium minitans* in the glasshouse. *Annals of Applied Biology* **132**: 77–90.

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