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Molecular characterisation of *Alternaria linicola* and its detection in linseed

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Abstract

Nucleotide sequences of the ribosomal DNA (rDNA) internal transcribed spacers (ITS) 1 and 2 and a 1068 bp section of the beta-tubulin gene divided seven designated species of *Alternaria* into five taxa. *Stemphylium botryosum* formed a sixth closely related taxon. Isolates of *A. linicola* possessed an identical ITS sequence to one group of *A. solani* isolates, and two clusters of *A. linicola* isolates, revealed from beta-tubulin gene data to show minor variation, were as genetically similar to isolates of *A. solani* as they were to each other. We suggest, therefore, that *A. linicola* falls within the species *A. solani*. Similar results suggest that *A. lini* falls within the species *A. alternata*. RAPD analysis of the total genomic DNA from the *Alternaria* spp. concurred with the nucleotide sequence analyses. An oligonucleotide primer (ALP) was selected from the rDNA ITS1 region of *A. linicola*/*A. solani*. PCR with primers ALP and ITS4 (from a conserved region of the rDNA) amplified a c. 536 bp fragment from isolates of *A. linicola* and *A. solani* but not from other *Alternaria* spp. nor from other fungi which may be associated with linseed. These primers amplified an identical fragment, confirmed by Southern hybridization, from DNA released from infected linseed seed and leaf tissues. These primers have the potential to be used also for the detection of *A. solani* in host tissues.

Introduction

Alternaria linicola Groves and Skolko (Groves and Skolko, 1944) is a commonly occurring pathogen of linseed (*Linum usitatissimum*, L.) in north western Europe (Evans et al., 1997). The pathogen is responsible for a range of symptoms on its host including poor germination, damping-off of seedlings, leaf spotting and a form of head blight in the capsules, which can result in a loss in yield and a reduction in oil quality (Mercer et al., 1989). *A. linicola* exerts its effect early in the season, initially at the seedling stage, by its presence on the lower leaves. However, disease symptoms are rarely discernible on the growing crop until capsule formation is almost complete later in the season (Mercer et al., 1994). Previous investigations (Vloutoglou et al., 1995) have demonstrated the ability of the pathogen to overwinter in naturally infected seed, crop debris and the weed *Veronica persica*, thus providing a potential source of inoculum and an alternative host.

Molecular techniques have been used previously in the systematics of phytopathogenic fungi to assess intra- and interspecific variation and to determine phylogenetic relationships (Gaudet et al., 1989; O'Donnell, 1992; Muthumeenakshi et al., 1994; Sreenivasaprasad et al., 1996). Kusaba and Tsuge (1995) used rDNA internal transcribed spacer (ITS) sequence analysis to redefine several *Alternaria* fungi known to produce host specific toxins. *A. kikuchiana*, *A. mali* and *A. longipes* were reported as *formae speciales* of *A. alternata*, rather than retaining separate species delimitation. Similar techniques were used to compare *Alternaria* species pathogenic to crucifers and to investigate the close relationship between *Pleospora*

herbarum (anamorph *Stemphylium botryosum*), a saprophyte commonly found on linseed seed, and the genus *Alternaria* (Jasalavich et al., 1995). Previous studies have reported close similarities in morphological characteristics (Johansen, 1943; Neergaard, 1945) and metabolite spectra (Evans et al., 1996) between *A. linicola* and *A. solani*. The aim of this study was to establish the relationship between *A. linicola* and other species of the genus associated with both linseed and other arable crops, in order to provide a better understanding of the phylogeny of the genus and to analyse *Alternaria* species delimitation at the molecular level.

Materials and methods

Fungal cultures and DNA extraction

Forty-six isolates of *Alternaria* species were obtained from UK linseed samples assayed for seed health by the Department of Agriculture for Northern Ireland (DANI) as well as from the culture collections, including the International Mycological Institute (IMI, Egham, U.K.), the Centraal Bureau voor Schimmelcultures (CBS, Baarn, The Netherlands), the International Collection of Microorganisms from Plants (ICMP, Auckland, New Zealand) and the Culture Collection Mycotheque De L'Universite Catholique De Louvain (MUCL, Louvain-la-Neuve, Belgium). Several isolates were also provided by Dr. L. Cooke, DANI, the late Dr. R. Wastie, Scottish Crops Research Institute, Dundee, Scotland and Dr. B. Fitt of the Institute of Arable Crops Research Institute, Rothemsted. The origin and original identification of all isolates are given in Table 1.

Fungal cultures were maintained on 2% (w/v) malt agar (MA) at 20 °C. Plugs, taken from the actively growing edge of each culture, were stored long term in sterile distilled water (SDW) at room temperature.

DNA was extracted from aerial fungal mycelium essentially following the protocol of Raeder and Broda (1985) but with an additional phenol/chloroform extraction step. DNA extraction from infected plant material was carried out following the protocol described by Torres et al. (1993).

PCR amplification

For RAPD analysis, primers A3, A11, A13, B6, B7 and B10, supplied by PE Applied Biosystems (Table 2) were used. PCR amplifications (50 µl) were performed using 100 ng of genomic DNA, 1.5 mM $MgCl_2$, 2U AmpliTaq™ polymerase (PE Applied Biosystems), 0.2 mM each of deoxyribonucleotide triphosphates, dATP, dCTP, dGTP, dTTP (dNTPs), 50 mM KCl, 10 mM Tris-HCl (pH 8.3) and 0.4 µM of primer. A PE Applied Biosystems GeneAmp PCR System 2400 was used with the following programme: 1 min at 94 °C, initial denaturation cycle; 15 s at 94 °C, 15 s at 30 °C, 90 s at 72 °C, 45 cycles; 7 min at 72 °C, 1 cycle. After PCR amplification, 15 µl of each product was separated by electrophoresis on 2.0% w/v agarose gel, stained in ethidium bromide and photographed for phylogenetic analysis.

For data analysis, each amplified fragment with all six primers was treated as a separate character. DNA fragments of the same size, obtained with separate isolates with the same primer, were assumed to represent the same genetic locus and scored as either present or absent. The cluster analysis of the data was based on a similarity matrix using the Jaccard coefficient, calculated using the statistical package RAPDistance, version 1.04 (Armstrong et al., 1994).

Initial amplifications of the rDNA gene fragment were performed using the universal primers ITS1 and ITS4 (White et al., 1990) supplied by PE Applied Biosystems, with the following programme; 1 min at 94 °C, initial denaturation cycle; 15 s at 94 °C, 15 s at 55 °C, 30 s at 72 °C, 30 cycles; 7 min at 72 °C, 1 cycle. PCR mixtures were as described for RAPD analysis with the exception that 0.2 µM of each primer was used.

Amplification of a 1068 bp beta-tubulin gene fragment was carried out using the primers, β-tubf1 and β-tubr1 (Table 2), designed from within conserved regions of the beta-tubulin gene from filamentous fungi (McKay et al., 1998). PCR reaction mix and conditions are as described for the amplification of the ITS region of the rDNA.

Nucleotide sequencing

Each PCR product was purified using the Wizard™ DNA Clean Up system (Promega) according to the manufacturer's instructions. Isolates were sequenced using double-stranded DNA template (0.2 µg μl^{-1}) and 1 µM of forward or reverse primers (Table 2) following the protocol supplied with the Prism™ Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (PE Applied Biosystems). Sequencing was conducted

Table 1. * The host and morphological identification of isolates of *Alternaria* species and *Stemphylium botryosum* used in each analysis during this investigation and the subsequent molecular identification

Code	Source	Host	Morpholog Id.	Origin	RFLP	ITS	β-tub	RAPD	Molecular Id.
1A	93-181	Linseed	*A. alternata*	U.K.	*	*	*	*	*A. alternata*
1B	93-182	Linseed	*A. alternata*	U.K.	*	*	*	*	*A. alternata*
1C	93-184	Linseed	*A. alternata*	U.K.	*	*	*	*	*A. alternata*
1E	93-211	Linseed	*A. alternata*	U.K.	*	—	*	*	*A. alternata*
1F	93-262	Linseed	*A. alternata*	U.K.	*	—	—	*	*A. alternata*
1G	93-266	Linseed	*A. alternata*	U.K.	*	—	—	*	*A. alternata*
2A	PM	O.S.R.	*A. brassicae*	N. Ireland	*	*	*	*	*A. brassicae*
2B	PM	O.S.R.	*A. brassicae*	U.K.	*	*	*	*	*A. brassicae*
2C	IMI289963	Brassica spp.	*A. brassicae*	U.S.A.	*	*	*	*	*A. brassicae*
2E	CBS239.73	Brassica spp.	*A. brassicae*	Denmark	*	—	*	*	*A. brassicae*
3A	93-212	Linseed	*A. linicola*	U.K.	*	*	*	*	*A. linicola*
3B	93-215	Linseed	*A. linicola*	France	*	*	*	*	*A. linicola*
3C	93-248	Linseed	*A. linicola*	U.K.	*	*	*	*	*A. linicola*
3D	87-437	Linseed	*A. linicola*	U.K.	*	*	*	*	*A. linicola*
3E	87-577	Linseed	*A. linicola*	U.K.	*	*	*	*	*A. linicola*
3F	87-585	Linseed	*A. linicola*	U.K.	*	*	*	*	*A. linicola*
3G	86-605	Linseed	*A. linicola*	U.K.	*	*	*	*	*A. linicola*
3H	87-631	Linseed	*A. linicola*	U.K.	—	—	*	—	*A. linicola*
4A	BF	Linseed	*A. infectoria*	U.K.	*	*	*	*	*A. infectoria*
4B	BF	Linseed	*A. infectoria*	U.K.	*	*	*	*	*A. infectoria*
5A	93-186	Linseed	*S. botryosum*	U.K.	*	*	*	*	*S. botryosum*
5B	93-209	Linseed	*S. botryosum*	U.K.	*	*	*	*	*S. botryosum*
5C	93-211	Linseed	*S. botryosum*	U.K.	*	*	*	*	*S. botryosum*
5D	93-434	Linseed	*S. botryosum*	U.K.	*	—	*	*	*S. botryosum*
5E	ICMP5620-77	Carrot	*S. botryosum*	Unknown	*	—	—	*	*S. botryosum*
6A	PM	O.S.R.	*A. brassicicola*	U.K.	*	*	*	*	*A. brassicicola*
6B	ICMP1120-77	Cabbage	*A. brassicicola*	Unknown	*	*	*	*	*A. brassicicola*
6C	ICMP1103-78	Choumoellier	*A. brassicicola*	Unknown	*	*	*	*	*A. brassicicola*
7A†	LC	Potato	*A. solani*	Unknown	—	*	—	—	*A. alternata*
7B	ICMP6519-79	Tomato	*A. solani*	Unknown	*	*	*	*	*A. solani*
7C†	ICMP7742-82	Pepino	*A. solani*	Unknown	—	*	—	—	*A. alternata*
7D	RW	Potato	*A. solani*	Unknown	*	*	*	*	*A. solani*
7E	RW	Potato	*A. solani*	Unknown	*	*	*	*	*A. solani*
7F	CBS110.41	Potato	*A. solani*	Unknown	*	*	*	*	*A. solani*
7G	CBS111.44	Ageratum	*A. solani*	Unknown	*	*	*	*	*A. solani*
7H	CBS105.51	Tomato	*A. solani*	Unknown	*	*	*	*	*A. solani*
7I	CBS107.61	Unknown	*A. solani*	Belgium	*	*	*	*	*A. solani*
7J	CBS166.77	Capsicum	*A. solani*	N. Zealand	*	*	*	*	*A. solani*
7K	LC	Potato	*A. solani*	U.S.A.	—	*	—	—	*A. solani*
7L	LC	Potato	*A. solani*	U.S.A.	—	*	—	—	*A. solani*
7M	MUCL28928	Potato	*A. solani*	U.S.A.	—	*	—	—	*A. solani*
7N	MUCL30508	Tomato	*A. solani*	U.K.	—	*	—	—	*A. solani*
7O	MUCL30971	Tomato	*A. solani*	U.K.	—	*	—	—	*A. solani*
7P	MUCL31371	Unknown	*A. solani*	Unknown	—	*	—	—	*A. solani*
7Q	MUCL31389	Tomato	*A. solani*	Unknown	—	*	—	—	*A. alternata*
8A	CBS106.34	Linseed	*A. lini*	Unknown	*	*	*	*	*A. alternata*

†Isolates misidentified.
PM = Dr. P. Mercer; LC = Dr. L. Cooke; RW = Dr. R. Wastie; BF = Dr. B. Fitt.

Table 2. PCR, RAPD and nucleotide sequencing primers used in the present study. 'F' and 'R' signifies forward and reverse respectively

Primer name	Primer sequence
ITS1-F	TCCGTAGGTGAACCTGCGG
ITS2-R	GCTGCGTTCTTCATCGATGC
ITS3-F	GCATCGATGAAGAACGCAGC
ITS4-R	TCCTCCGCTTATTGATATGC
ALP-F	GGCACCTCCCGGGGTGGC
β-tubf1-F	CAGCTCGAGCGTATGAACGTCT
β-tubr1-R	TGTACCAATGCAAGAAAGCCTT
β-tubr2-R	CGGAAGTCGGAAGCAGCCATC
OP-A3	AGTCAGCCAC
OP-A11	CAATCGCCGT
OP-A13	CAGCACCCAC
OP-B6	TGCTCTGCCC
OP-B7	GGTGACGCAG
OP-B10	CTGCTGGGAC

using a PE Applied Biosystems Model 373A DNA Sequencer as recommended by the manufacturer. The sequence data of complementary strands were compared visually and aligned using CLUSTAL V (Higgins et al., 1992). A neighbor joining phylogenetic tree was constructed from genetic distance values calculated using the DNA sequence analysis software package, MEGA (Kumar et al., 1993).

Selection of an A. linicola*-specific primer*

Nucleotide sequence of the rDNA ITS1 and 2 regions of *A. linicola* was aligned with the same region from the other *Alternaria* species used in this study together with sequences obtained from the EMBL database. Additional *Alternaria* spp. were *A. alternata* (IFO 4026), *A. alternata* (*A. citri*-ATCC 38962), *A. alternata* (*A. mali*-IFO 8984), *A. bataticola* (IFO 6187), *A. dianthi* (DA-1), *A. panax* (AP-1), *A. porri* (AP 1) and *A. sesami* (Se-1) (Kusaba and Tsuge, 1995) and *A. raphani* (IMI 354205) (Jasalavich et al., 1995). A sequence within the ITS1 region was identified as unique to *A. linicola* and also to *A. solani*. A PCR primer (ALP), 18 bp in length, was selected from this region (Table 2). PCR reactions were performed with primers ALP (forward) and ITS4 (reverse) and were as previously described except that 15 mM $MgCl_2$, 750 mM KCl and 100 mM Tris-HCl (pH 8.3) were used to encourage specific annealing of the primers (0.2 μM) to the appropriate template in a 50 μl reaction. PCR amplification conditions were: 1 min at 94 °C, initial denaturation cycle; 15 s at 94 °C, 15 s at 62 °C, 30 s at 72 °C, 30 cycles; 7 min at 72 °C. To check the specificity of the primers, DNA from linseed, *Alternaria* spp. and pathogens and saprophytes obtained from linseed (*Botrytis cinerea*, *Fusarium* spp., *Penicillium* spp., *Epicoccum nigrum*, *Phoma exigua* and *Cladosporium herbarum*) were also analysed. Primers ITS1 and ITS2 were used to verify the efficacy of the DNA preparations for PCR.

Detection of A. linicola *in infected linseed seed and leaf tissue*

To test for the presence of *A. linicola* on linseed seeds, seeds were taken from a sample with 38% *A. linicola* infection as determined by the Ulster method (Muskett and Colhoun, 1947). In each assay five of these seeds were placed in 1 ml SDW in a 1.5 ml microcentrifuge tube, heated to 100 °C for 10 min and cooled briefly on ice. Five seeds from uninfected linseed samples were similarly tested. DNA was extracted from infected linseed leaf tissue using a CTAB-based method (Torres et al., 1993). Genomic DNA from *A. linicola* and *A. solani* were included in the assay as positive controls. A 5 μl aliquot from each sample was added to 45 μl of PCR reaction mixture and subjected to the annealing conditions described.

Following gel electrophoresis, the PCR products generated with primers ALP and ITS4 and universal primers ITS1 and ITS2 were transferred to a nylon membrane (Hybond N^+, Amersham, U.K.) by capillary transfer (Sambrook et al., 1989). The ITS1 and ITS2 primers were again included to verify the efficacy of all DNA preparations for PCR. A probe, labelled with [α-^{32}P] deoxyadenosine 5-prime-triphosphate (Amersham) using the Prime-It® RmT labelling kit (Stratagene), was prepared from the PCR product amplified from *A. linicola* genomic DNA with primers ALP and ITS4. Unincorporated nucleotides were removed by push column chromatography (Stratagene) as described by the manufacturer. Hybridization was performed in a hybridization oven (Hybaid) at 65 °C as described by Sambrook et al. (1989). The prehybridization wash was for 3 h (6× SSC, 5× Denhardts solution, 0.5% (w/v) SDS and 100 μg ml^{-1} denatured salmon sperm DNA) and three post-hybridization washes of 30 min in 2× SSC and 0.1% (w/v) SDS and three washes of 30 min in 0.1× SSC and 0.1% (w/v) SDS. Membranes were sealed in thin plastic film and exposed to Hyperfilm β-max (Amersham) at −70 °C using intensifying screens.

Results

Analysis of nucleotide sequence data

Isolates of *Alternaria* species for which ITS and β-tubulin gene nucleotide sequence data was obtained are indicated in Table 1. Nucleotide sequence data for each species can be accessed through EMBL accession numbers (Table 3). All distance values calculated using the Kimura 2-Parameter distance algorithm (Kumar et al., 1993) are shown (Figures 1 and 2).

ITS nucleotide sequence analysis

ITS nucleotide sequence data divided *Alternaria* and *Stemphylium* isolates into six distinct clades. Analysis of seven isolates of *A. linicola* showed that they all possessed an identical DNA sequence for the ITS region of the rDNA. This sequence showed 100% homology with eight isolates of *A. solani* (representative isolate, 7G) as shown in Figure 1. *A. linicola* differed from the remaining six *A. solani* isolates by a single insertion/deletion of thymidine at position 528 in the ITS2 region. This section of the ITS2 region was found to

Table 3. EMBL Accession numbers identifying DNA sequence data for *Alternaria* species and *Stemphylium botryosum*

Isolate id	Description	EMBL Accession No.
A. infectoria 4A	ITS1, 5.8S, ITS2	Y17066
A. infectoria 4B	ITS1, 5.8S, ITS2	Y17067
S. botryosum 5E	ITS1, 5.8S, ITS2	Y17068
A. solani 7B	ITS1, 5.8S, ITS2	Y17069
A. solani 7G	ITS1, 5.8S, ITS2	Y17070
A. lini 8A	ITS1, 5.8S, ITS2	Y17071
A. linicola 3B	ITS1, 5.8S, ITS2	Y17086
A. alternata 1E	beta-tubulin gene	Y17072
A. alternata 1A	beta-tubulin gene	Y17073
A. brassicae 2E	beta-tubulin gene	Y17074
A. infectoria 4B	beta-tubulin gene	Y17075
A. linicola 3B	beta-tubulin gene	Y17076
S. botryosum 5E	beta-tubulin gene	Y17077
A. solani 7B	beta-tubulin gene	Y17078
A. solani 7F	beta-tubulin gene	Y17079
A. solani 7H	beta-tubulin gene	Y17080
A. solani 7J	beta-tubulin gene	Y17081
A. lini 8A	beta-tubulin gene	Y17082
A. infectoria 4A	beta-tubulin gene	Y17083
A. brassicicola 6B	beta-tubulin gene	Y17084
A. linicola 3A	beta-tubulin gene	Y17085

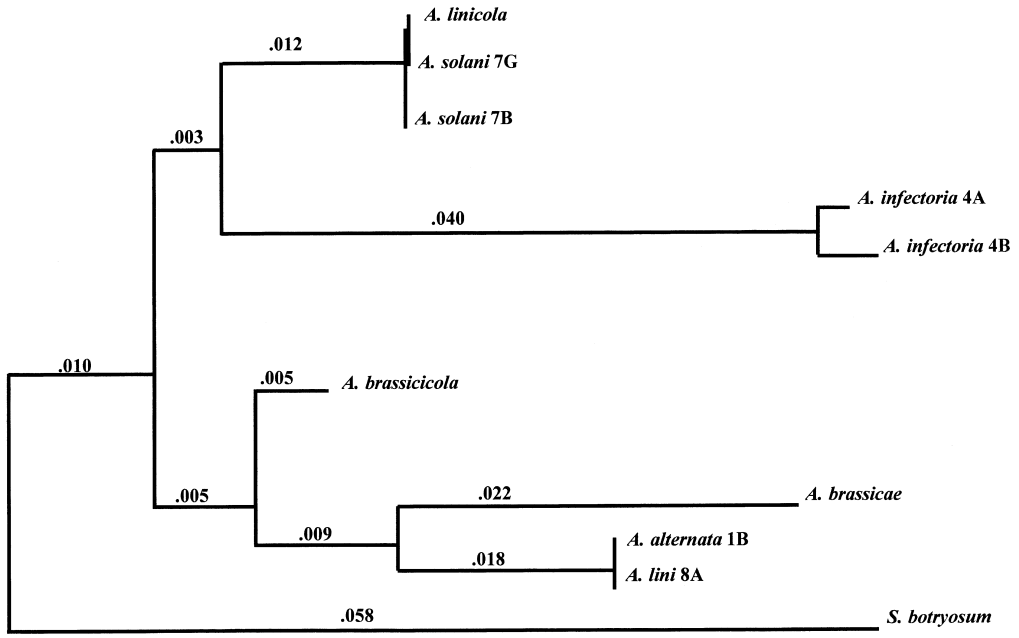

Figure 1. Neighbor joining phylogenetic tree produced from nucleotide sequence analysis of the ITS1, 5.8S gene and ITS2 region of *Alternaria* species using the MEGA statistical package (Kumar et al., 1993). The pairwise genetic distance values were produced using the Kimura 2-Parameter algorithm. Representative isolates are as follows: *A. lini* 8A = *A. alternata* 1A; *A. alternata* 1B = *A. alternata* 1C; *A. solani* 7G = *A. solani* 7H, 7I, 7K, 7L, 7N, 7O, 7P and *A. linicola*; *A. solani* 7B = *A. solani* 7D, 7E, 7F, 7J and 7M.

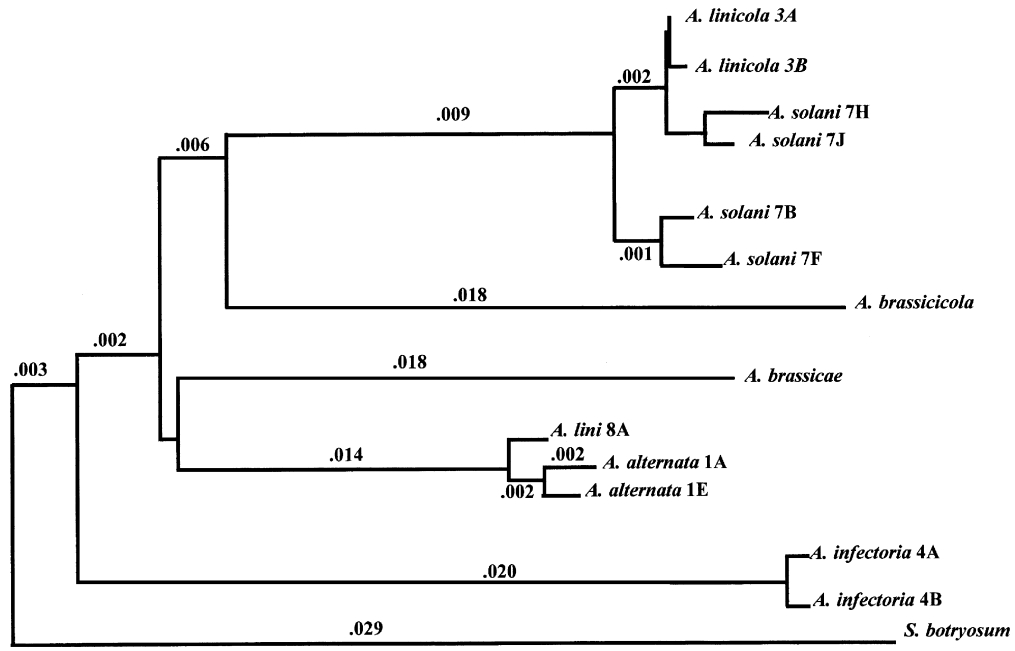

Figure 2. Neighbor joining phylogenetic tree produced from nucleotide sequence analysis of part of the beta-tubulin gene from *Alternaria* species using the MEGA statistical package (Kumar et al., 1993). The pairwise genetic distance values were produced using the Kimura 2-Parameter algorithm. Representative isolates are as follows: *A. linicola* 3A = *A. linicola* 3C and 3E; *A. linicola* 3B = *A. linicola* 3D, 3F, 3G and 3H; *A. solani* 7B = *A. solani* 7D and 7E; *A. solani* 7F = *A. solani* 7G; *A. solani* 7H = *A. solani* 7I; *A. lini* 8A = *A. alternata* 1B and 1C.

exhibit length variation among all the *Alternaria* isolates due to insertion/deletion of thymidine nucleotides. The genetic distance value between *A. linicola* and *A. solani* isolates was negligible, with the exception of the isolates 7A, 7C and 7Q which had been misidentified. The latter three isolates gave an identical sequence to *A. alternata* isolates and were subsequently designated as such.

The ITS sequence data of the one isolate of *A. lini* was compared with those of three *A. alternata* isolates. *A. alternata* isolate 1A showed 100% homology with the isolate *A. lini* 8A. *A. alternata* isolate 1B and 1C were identical but differed from isolates 1A and 8A due to a thymidine insertion/deletion at position 526 in the ITS2 region.

DNA sequence analysis of two isolates of *A. infectoria* showed that they possessed a slightly different sequence to each other, corresponding to a genetic distance value of 0.66%. Three isolates each of *A. brassicae, A. brassicicola* and *S. botryosum* were sequenced, demonstrating that isolates of each species possessed identical sequence data for this region of the rDNA unique to that species.

Beta-tubulin gene nucleotide sequence analysis

Beta-tubulin gene nucleotide sequence data similarly divided the isolates studied into six distinct clades but with greater diversity among the *A. linicola*/*A. solani* and *A. alternata* clades (Figure 2). Results indicated that the 8 isolates of *A. linicola* studied were divided into two sub-groups, representative isolates being 3A and 3B (Figure 2).

A. linicola isolate 3A differed from isolate 3B by a distance value of 0.09% due to a single C/T transition mutation. Both isolates 3A and 3B were found to be genetically closest to *A. solani* 7J. *A. linicola* isolate 3A and *A. solani* 7J were separated by a distance value of 0.09%, an identical value to that which separated the two *A. linicola* groupings, due to a single base A/G transition mutation.

DNA sequence analysis of eight isolates of *A. solani* produced four sub-groups, representative of which were isolates 7B, 7F, 7H and 7J (Figure 2).

The lowest distance value between these isolates of *A. solani* was 0.19% (7H and 7J), due to two C/T transition mutations. Isolates 7B and 7H showed the highest

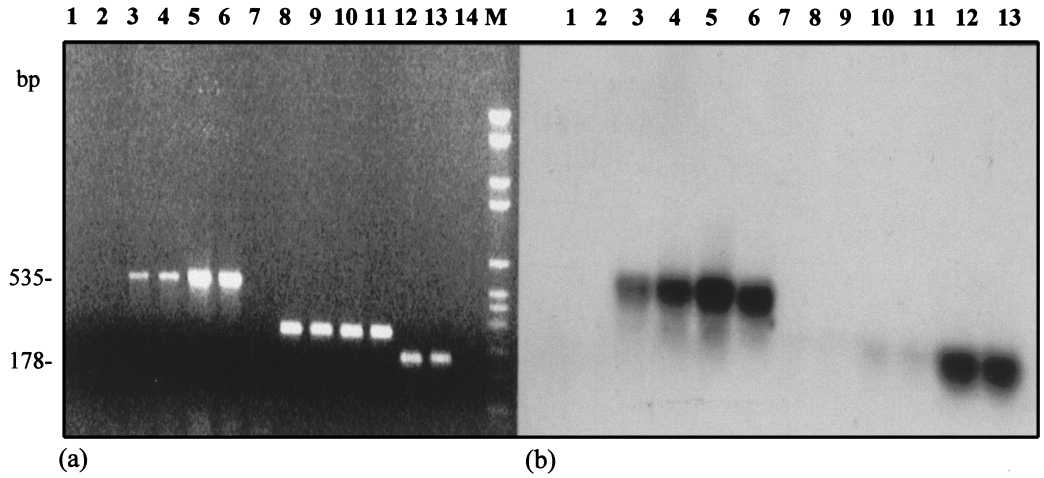

Figure 3. (a) Amplification of a fragment from *Alternaria linicola*-infected linseed seeds and linseed leaf tissue with primers ALP (forward) and ITS4 (reverse) (lanes 1–6) and products amplified with universal primers ITS1 and ITS2 as controls (Lanes 8–13) and (b) Southern hybridization analysis of the fragment. Amplification products in lanes 1 and 8 – non-infected linseed seed, lanes 2 and 9 – non-infected linseed leaf tissue, lanes 3 and 10 – infected linseed seeds, lanes 4 and 11 – infected linseed leaf tissue, lanes 5 and 12 – *A. linicola* genomic DNA, lanes 6 and 13 – *A. solani* genomic DNA, lanes 7 and 14 were negative control (SDW).

distance value 0.85% within the species, due to a single A/G transition and eight T/C transition mutations. Variation among the other *A. solani* isolates were similarly due to A/G and T/C transition mutations and also C/G transversion mutations.

DNA sequence analysis of the β-tubulin gene of four isolates of *A. alternata* and *A. lini* isolate 8A produced three subgroups each with identical sequence data (Figure 2). The distance value between these isolates ranged from 0.47% between 1A and 1E, due to three A/G and two T/C transition mutations and the highest distance value of 0.66% was between the *A. alternata* isolates 1E and *A. lini* 8A, due to one A/G and six T/C transition mutations.

Two isolates of *A. infectoria* analysed differed slightly from each other with a genetic distance value of 0.38% due to four T/C transition mutations. No intraspecific variation was detected within the 1068 bp section of the beta-tubulin gene analysed among the four isolates of *A. brassicae*, three isolates of *A. brassicicola* or three isolates of *S. botryosum*.

Analysis of RAPD data

The RAPD profiles produced with six random primers differentiated the *Alternaria* isolates into the same six distinct clades (phylogenetic tree not shown, data available upon request) as the nucleotide sequence analyses but revealing greater intraspecific variation. Nevertheless, isolates of *A. linicola* and *A. solani* clustered within the same clade but with *A. linicola* forming a sub-group showing minor intraspecific variation, and the single isolate of *A. lini* clustered within the *A. alternata* grouping.

Detection of A. linicola *in planta*

Primers ALP and ITS4 amplified a fragment of c. 535 bp with DNA from *A. linicola* and *A. solani* (Figure 3). These primers did not cross-react with linseed DNA nor DNA from the other fungi tested. A c. 535 bp product was also produced when primers ALP and ITS4 were used to PCR amplify DNA released from infected linseed seeds and leaf tissue (Figure 3). Hybridization analysis of the PCR products from infected linseed seeds and leaves using the PCR product from *A. linicola* DNA as a probe, yielded a strong signal (Figure 3), confirming that the product amplified contained the target sequence of the fungal DNA.

Discussion

Nucleotide sequence analysis of the ITS regions of the rDNA and the beta-tubulin gene classified isolates of seven named species of *Alternaria* and *Stemphylium*

botryosum into six molecular taxa at different taxonomic ranks. RAPD analysis of total genomic DNA categorised isolates into the same six molecular taxa as the nucleotide sequence data. Hence, the molecular data used in the phylogenetic analysis of *Alternaria* isolates categorised them into the same distinct taxa regardless of the marker used. Our data shows the usefulness in phylogeny of the nucleotide sequence data generated from the beta-tubulin gene, an important house-keeping gene essential for cell growth (Davidse and Flach, 1977). This data showed more intraspecific variation among some of the isolates studied than the data generated from the ITS region of the rDNA with even greater variation observed from the RAPD-PCR data; the three types of analyses thus separating isolates at different taxonomic ranks depending on the level of specificity required. The breakdown of species using the different techniques identified *A. solani*, *A. alternata*, *A. brassicae*, *A. brassicicola*, *A. infectoria* and *Stemphylium botryosum* as the six molecular taxa. The molecular phylogeny confirmed that *Stemphylium botryosum* (teleomorph *Pleospora herbarum*) and the genus *Alternaria* are closely related. Most species of *Alternaria* lack a known sexual stage, although *A. infectoria* is known to have a *Pleospora* teleomorph. The nucleic acid characteristics present in both morphs of a fungus are identical (Reynolds and Taylor, 1991), therefore, the polymorphisms observed between isolates are consistent regardless of the sexual or asexual state. Our results concur with those of Simmons (1967) and Jasalavich et al. (1995) who also suggest that the *Alternaria*, being so closely related to *Pleospora*, should be included in the Pleosporaceae.

Kusaba and Tsuge (1994, 1995) used molecular methods to determine the phylogenetic relationship among isolates of *A. alternata*, *A. citri*, *A. mali*, *A. kikuchiana* and *A. longipes*. Based upon the results from their investigation, isolates from these species should not be separated at the species level but should be classified as *A. alternata*, with differentiation at the sub-species level as *formae speciales* (Kusaba and Tsuge, 1995). In a similar manner, the results from the present study would suggest that *A. lini* should not be differentiated from *A. alternata* at the species level. Reservations about the differentiation of *A. lini* and *A. alternata* at the species level have been expressed in previous investigations (Arya and Prasada, 1953; Saharan, 1988). *A. lini* has been reported only in India and, although only one isolate could be obtained for analysis in the present study, it may represent an intraspecific variant of *A. alternata* located only in the sub-continent.

The phylogenetic study of the relationship between *A. linicola* and *A. solani*, although similar to that observed between *A. alternata* and *A. lini*, was more comprehensive. Nucleotide sequence analysis of the ITS region of the rDNA showed that the isolates of *A. linicola* and several of the *A. solani* isolates had an identical sequence for this region. The beta-tubulin gene sequence analysis showed greater variation among the *A. linicola* and *A. solani* isolates than the ITS sequence data but the genetic distance between *A. linicola* and the nearest *A. solani* isolate was the same as the distance between the two groups of *A. linicola* isolates. Both sets of nucleotide sequence data strongly suggest that *A. linicola* falls within the species *A. solani*. RAPD analyses concurred with the sequence analyses and provided findings similar to previous reports (Cooke et al., 1998; Sharma and Tewari, 1998) on their use for species delimitation within the genus *Alternaria*. The isolates of *A. linicola* clustered closely together showing a very low level of genetic variation, within the larger, more divergent *A. solani* clade suggesting that the former may be clonal. At the very least, pathogenic specialisation of *A. linicola* for linseed is a strong possibility, a fact previously reported for isolates of *A. solani* pathogenic to tomato and potato (Weir et al., 1998). The greater variation observed within *A. solani* may relate to the fact that it has a broad host range with a greater ability to cause infection within different host plants (Rotem, 1994). Isolates of *A. solani* used in the present study were obtained from at least five different hosts and, indeed, some were found to be capable of infecting linseed, although less aggressively than *A. linicola* (McKay, 1998). Intraspecific divergence has similarly been seen in *Colletotrichum gleosporioides* and *C. acutatum* (Sreenivasaprasad et al., 1996), both of which have a very broad host range.

Molecular analyses of related taxa suggest that in evolutionary terms, those exhibiting less variation at a molecular level are commonly derived from those with greater variation (Avise et al., 1987; Avise, 1989; Harrison, 1991). *A. linicola*, which shows little intraspecific divergence, has undoubtedly evolved from an isolate of *A. solani* which developed a particular host preference for linseed and one reported alternative host, *Veronica persica* (Vloutoglou et al., 1995). Indeed, Gudrun Johanson (1943) reported the infection of flax plants by *A. solani* and since then various

studies (Neergaard, 1945; Evans et al., 1996) have discussed the ambiguous taxonomic relationship between *A. linicola* and *A. solani* at the species level. Our results suggest that *A. linicola* should not be designated as a separate species but should rather be classified as a *formae speciales* of *A. solani*.

The similarity between *A. linicola* and *A. solani* nucleotide sequence data prevented the selection of an *A. linicola*-specific PCR primer. Nevertheless, the *A. linicola*/*A. solani*-specific primer facilitates rapid detection of *A. linicola* on linseed and its possible use in a multiplex PCR, to include other seed-borne pathogen-specific primers could be a valuable diagnostic tool for linseed seed testing. The primer also has the potential to be used for the rapid detection of *A. solani* in its many host plants.

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