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Molecular taxonomy of the *Alternaria* and *Ulocladium* species from humans and their identification in the routine laboratory

Molekulare Taxonomie humaner Isolate von *Alternaria*- und *Ulocladium*-Arten und ihre Identifizierung im Routinelabor

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Keywords. *Alternaria*, *Ulocladium*, melanized fungi, opportunistic fungi, rDNA, ITS-sequencing, RFLP, identification.

Schlüsselwörter. *Alternaria*, *Ulocladium*, Schwärzepilze, Opportunisten, rDNA, ITS-Sequenzierung, RFLP, Identifizierung.

Summary. The *Alternaria* and *Ulocladium* species reported from humans are studied taxonomically using rDNA internal transcribed spacer (ITS) sequence data. The ITS variability within the genus is relatively limited. The two most important, longicatenate species, *Alternaria alternata* and *A. infectoria*, clearly differ in their ITS domains, due to a 26-bp insert in ITS1 of the latter species. A number of taxa inhabiting particular plant species, such as *A. longipes* on tobacco and *A. mali* on apple, but also the common saprobic species *A. tenuissima* cannot reliably be distinguished from *A. alternata* using this method. The large number of described noncatenate, obligatory plant pathogens are extremely rare as agents of human disease; clinical routine identification does not need to include these taxa. The predictivity of a simplified polymerase chain reaction–restriction fragment length polymorphism procedure of rDNA for the recognition of the relevant species or species aggregates is established in a randomized test. The method was found to be rapid and cost-effective. Its efficacy extended to the identification of sterile and meristematic *Alternaria* strains, some of them previously classified in genera such as *Chmelia* and *Botryomyces*. Microscopic morphology and some additional tests remain necessary to allow identification of the aggregates of potential etiological agents of human disease. About 14% of the sequences deposited in GenBank were found to be misidentified. *Alternaria infectoria* is one of the most common clinical *Alternaria* species, despite its low degree of melanization. The lack of pigmentation has frequently led to misidentification of such isolates.

Zusammenfassung. Die *Alternaria*- und *Ulocladium*-Spezies, die als klinische Isolate beschrieben worden sind, wurden taxonomisch mittels rDNA ITS (Internal Transcribed Spacer-) Sequenzanalysen untersucht. Die ITS-Variabilität innerhalb dieser Gattungen ist relativ begrenzt. Die beiden humanmedizinisch bedeutsamsten Arten, *Alternaria alternata* und *A. infectoria*, unterscheiden sich eindeutig voneinander in ihrer ITS-Domäne, vor allem durch eine Insertion in ITS1 von 26 Bp bei der letztgenannten Spezies. Manche pflanzenpathogene Arten, wie beispielsweise *A. longipes* auf Tabakpflanzen und *A. mali* auf Äpfeln, sowie die häufig isolierte saprobische Art *A. tenuissima*, können mit dieser Methode nicht eindeutig von *A. alternata* unterschieden werden. Die meisten der pflanzenpathogenen Arten ohne Konidienketten werden jedoch extrem selten als Verursacher humaner Mykosen beobachtet, weshalb sie klinisch kaum relevant sind und ihre Identifizierung in Routinelaboratorien als nicht erforderlich erachtet werden kann. Die Identifizierungsfähigkeit einer vereinfachten PCR-RFLP-Analyse der rDNA zur Bestimmung der humanmedizinisch bedeutsamen Spezies oder

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Speziesgruppen wurde in einer randomisierten Studie überprüft. Diese Methode erwies sich als schnell und kostengünstig und ermöglichte sogar die Identifizierung steriler und meristematischer *Alternaria*-Isolate, die zuvor in die Gattungen *Chmelia* und *Botryomyces* eingeordnet worden waren. Die mikroskopische Untersuchung sowie einige zusätzliche Tests bleiben weiterhin notwendig, um einige humanpathogene *Alternaria*-Speziesaggregate zu identifizieren. Etwa 14% der Sequenzdaten, die in der Genbank erhältlich sind, erwiesen sich als Fehlidentifizierungen. *A. infectoria* ist eine der bedeutsamsten *Alternaria*-Arten aus klinischem Material, die jedoch oft nur geringfügig pigmentiert ist. Die fehlende Pigmentierung hat dazu beigetragen, dass entsprechende Isolate mehrfach falsch identifiziert wurden.

Introduction

Members of the genus *Alternaria* are among the most important airborne agents of allergic disorders [1]. In addition, traumatic, localized infections are frequently observed [2], which may take a more severe course in immunocompromised patients [3, 4]. In humans, the species should be regarded as typical opportunists, their natural ecological niche being on living or dead plant material.

Most infections by *Alternaria* species are attributed to the ubiquitous *A. alternata* (Fr.) Keissl. However, six *Alternaria* species have been reported as potential agents of disease [5]. Morphological distinction of species may be difficult due to the wide range of variability of conidia, but some species show clear-cut molecular differences [6, 7]. It was the aim of the present study to provide a rapid and cost-effective methodology for the distinction of clinically relevant entities.

We used the following research strategy. As a reliable starting point was needed prior to development of practical diagnostics, the taxonomy of clinically relevant *Alternaria* species is overviewed. Some *Ulocladium* species were included which are morphologically similar to *Alternaria* and which have occasionally been reported from humans or clinical samples [5]. In addition to morphology (not shown), the rDNA internal transcribed spacer (ITS) domain, which is known to display the observed diversity around the level of species or species aggregates [7], was sequenced. Comparative restriction maps were constructed from which minimum sets of digestions required to recognize the clinically relevant entities were abstracted. The predictive value of this set was verified in a double-blind study.

Materials and methods

Strains, culture conditions and public domain data

Clinical strains representing each of the names used in the literature were collected and supplemented with ex-type and authentic specimens and other strains of the same species from nonhuman sources. Reference strains were taken from the collection of the Centraalbureau voor Schimmelcultures (Utrecht, the Netherlands), or were kindly donated by E.G. Simmons (Amherst, MA, USA). When no authentic live material was available, strains regarded as representative for the species by taxonomic specialists were taken as such. Strains studied (Table 1) were grown on malt extract agar (MEA) and potato carrot agar (PCA). Data generated were supplemented by all identical and closely similar sequences from EMBL/GenBank. Strains finally clustering as *Alternaria alternata* and *A. infectoria* Simmons were grown on potato dextrose agar (PDA), dichloran Rose Bengal yeast extract sucrose agar (DRYES) and Müller–Hinton Agar II (MHA) and their cultural characteristics and gross conidiation were recorded. Recipes of media have been given by De Hoog *et al.* [5]; MHA was obtained from Becton Dickinson (Heidelberg, Germany).

DNA extraction

Approximately 1 g mycelium was transferred to a 2 : 1 mixture of silica gel and Celite 545 with 300 µl cetyltrimethylammonium bromide (CTAB)-buffer added (Tris–HCl, 200 mmol l^{-1}, pH 7.5; Na-EDTA, 200 mmol l^{-1}; NaCl 8.2%; CTAB 2%). The material was ground with a micropestle (Eppendorf, Hamburg, Germany). After addition of 200 µl CTAB-buffer and vigorous shaking, the sample was incubated for 10 min in a 65 °C water bath. Then, 500 µl chloroform was added and the mixture was vortexed briefly and centrifuged for 5 min at 20 800 *g*. After the aqueous supernatant was transferred to a new Eppendorf tube, two volumes (~800 µl) ethanol 96%, −20 °C were added and mixed gently. The DNA was precipitated at −20 °C for at least 30 min. The pellet, obtained by centrifugation for 5 min at 20 800 *g*, was washed twice with 500 µl ethanol 70% at −20 °C. DNA was dried overnight at room temperature and suspended in 97.5 µl TE-buffer (10 mmol l^{-1} Tris, 10 mM Na-EDTA, pH 8.0) with 2.5 µl RNAse-solution (10 mg pancreatic RNase 20 U mg^{-1} was added to 1 ml 0.01 mol l^{-1} sodium acetate, heated at 100 °C for 15 min and cooled slowly to room temperature; the pH was adjusted to 7.4 by adding 100 µl Tris–HCl). Samples were incubated for 5–30 min at 37 °C and then stored in a refrigerator.

Table 1. List of strains and sequences studied with collection and GenBank, EMBL and DDBJ numbers

Name	Numbers	Source	Geography	Isolated by	Authority
A. alternata	AA6 = U05195	*Brassica rapa* spp. *oleifera*	Canada	G.A. Petrie	[27]
A. alternata	CBS 105.49	contaminant blood culture	Italy	M. Poli	–
A. alternata	CBS 101.13, T for *A. geophila*	soil	Switzerland	W. Daszewska	[58]
A. alternata	CBS 103.33	soil	Egypt	F.H. van Beyma	–
A. alternata	CBS 108.41 = ATCC 11892	wood	–	S. Truter	–
A. alternata	IFO 4026 = D38756 + D38764	–	Japan	T. Hasegawa	–
A. alternata	CBS 603.78 = QM 9553, REPR for *A. alternata*	air	USA, Texas	G.H. Meyer	E.G. Simmons
A. alternata	dH 10735 (received as *A. chlamydospora*) case 2	human skin lesion	India	S.M. Singh	[12]
A. alternata	CBS 795.72 = ATCC 24127	*Plantago aristida*	USA, Arkansas	G.E. Templeton	–
A. alternata	BMP 214110 = AF229461	leaf, *Daucus* sp.	USA, California	B.M. Pryor	[30]
A. alternata	BMP 214107 = AF229460	leaf, *Daucus* sp.	USA, California	B.M. Pryor	[30]
A. alternata	CBS 916.96 = EGS34–016 = AF071346, REPR for *A. alternata* (ET)	*Arachis hypogaea*	India	E.G. Simmons	[25]
A. alternata	AJ276055	plasticized PVC	UK	J.S. Webb	[59]
A. alternata	AF218791	soil in penguin colony	Antarctica	J.R. Bradner	–
A. alternata	CBS 109803	human skin lesion	Germany	P. Mayser	[13]
A. alternata	dH 12353	human skin lesion	Germany	P. Nenoff	[60]
A. alternata	GHP 2027	keratitis	Germany	G. Haase	[55]
A. arborescens	ATCC 28329 = AF229459, T for *A. alternata* f. sp. *lycopersici*	*Lycopersicon esculentum*	USA, California	R.G. Grogan	[61]
A. bataticola	CBS 531.63 = IFO 6187 = D38760 + D38769	*Ipomoea batatas*	Japan	–	[7]
A. brassicae	BMP 216102 = AF229463	leaf, *Armoracia rusticana*	USA, California	B.M. Pryor	[30]
A. brassicae	AB11 = U05253	*Brassica rapa* ssp. *oleifera*	Canada	G.A. Petrie	[27]
A. brassicicola	EEB 2232	*Brassica oleracea*	USA, California	–	[30]
A. brassicicola	Thai = AF201964	*Brassica* sp.	Thailand	–	[62]
A. brassicicola	Abc2 = U05198	*Brassica oleracea* ssp. *capitata*	Canada	G.A. Petrie	[27]
A. carotiincultae	EGS 26–010 = AF229465 (T)	dead leaf, *Daucus carota*	USA, Ohio	E.G. Simmons	E.G. Simmons
A. crassa	DGG Acr1 = AF229464	leaf, *Datura* sp.	–	–	[30]
A. cheiranthi	EGS 41–188 = AF229457	seed, *Cheiranthus cheira*	Italy	E.G. Simmons	[22]
A. chlamydospora	CBS 198.74	salt-marsh soil	Kuwait	A.F. Moustafa	E.G. Simmons
A. chlamydospora	CBS 491.72 = ATCC 20845 (T)	desert soil	Egypt	J. Mouchacca	[63]
A. dauci	Ad1 = D38768	*Daucus carota*	–	–	[7]
A. dauci	BMP 213116 = AF229468	leaf, *Daucus* sp.	USA, California	B.M. Pryor	[30]
A. dauci	BMP 213109 = AF229467	leaf, *Daucus* sp.	USA, California	B.M. Pryor	[30]
A. dauci	ATCC 36613 = AF229466	leaf, *Daucus carota*	USA, Florida	J.O. Strandberg	[64]
A. dianthi	Da1 = D38758 + D38766	*Dianthus* sp.	–	–	[7]
A. dianthicola	CBS 112.38 = IMI 264945 (T)	*Dianthus caryophyllus*	Denmark	P. Neergaard	[65]
A. japonica	ATCC 13618 = QM 1286 = AF229474	*Raphanus sativus*	Canada	E.G. Simmons	E.G. Simmons
A. japonica	AR6 = IMI 354205 = = U05200	*Brassica rapa* spp. *oleifera*	Canada	G.A. Petrie	[27]

Table 1. Continued

Name	Numbers	Source	Geography	Isolated by	Authority
A. lini	CBS 106.34 = DSM 62019 = Y17071 (T)	*Linum usitatissimum*	–	–	[66]
A. linicola	Y17086	*Linum usitatissimum*	France	G.J. McKay	[67]
A. longipes	CBS 540.94 = QM 9589	*Nicotiniana tabacum*	USA, North Carolina	E.G. Simmons	E.G. Simmons
A. longipes	CBS 113.35	*Nicotiniana tabacum*	–	W.B. Tisdale	
A. longipes	CBS 539.94 = QM 8438	*Nicotiniana tabacum*	USA, North Carolina	E.G. Simmons	E.G. Simmons
A. longipes	CBS 916.96 = IMI 254138	*Arachis hypogaea*	India	E.G. Simmons	E.G. Simmons
A. macrospora	DGG Ams1 = AF229469	leaf, *Gossypium* sp.	–	–	[30]
A. mali	IFO 8984 = ATCC 44899	leaf, *Malus* sp.	Japan	–	–
A. mouchaccae	CBS 453.74	seedling death of *Carduus pycnocephalis*	Australia	G. Parbery	W. Gams
A. mouchaccae	CBS 208.74	salt-marsh soil	Egypt	A.F. Moustafa	E.G. Simmons
A. mouchaccae	CBS 490.72, T for *U. chlamydosporum*	desert soil	Egypt	J. Mouchacca	[68]
A. (L.) photistica	CBS 212.86 = AF081455 (T)	*Digitalis purpurea*	UK	U. Allitt	[15]
A. panax	Apx1 = D38759 + D38767	*Panax ginseng*	–	–	[7]
A. petroselini	EGS 09–159 = AF229454	seed, *Petroselinum crispum*	USA, California	J.W. Groves	E.G. Simmons
A. porri	Ap1 = D38760 + D38770	*Allium cepa*	–	–	[7]
A. porri	AB026159	*Allium cepa*	Japan	M. Saito	–
A. porri	ATCC 58175 = AF229470	*Allium fistulosum*	USA, Arizona	P.J. Cotty	[30]
A. radicina	CBS 245.67 = ATCC 6503 = AF307014 (T)	*Daucus carota*	USA, Washington	J.A. Stevenson	[69]
A. radicina	ATCC 58405 = DAR 32156 = AF307015	*Apium graveolens* var. *dulcae*	Australia	A.H. Wearing	[70]
A. radicina	BMP 212114 = AF229473	seed, *Daucus* sp.	Japan	B.M. Pryor	[30]
A. radicina	BMP 212107 = AF229472	seed, *Daucus* sp.	Japan	B.M. Pryor	[30]
A. radicina	ATCC 96831 = AF229471	seed, *Daucus* sp.	France	E.G. Simmons	E.G. Simmons
A. selini	EGS 25–198 = IMI 137332 = AF229455 (T)	*Petroselinum crispum*	Saudi Arabia	H. Martin	E.G. Simmons
A. sesami	Se1 = D38761 + D38771	*Sesamen indicum*	–	–	[7]
A. smyrnii	EGS 37–093 = AF229456	dead stem, *Smyrnium olusatrum*	UK	M.B. Ellis	E.G. Simmons
A. solani	ATCC 58177 = AF229475	*Lycopersicon esculentum*	Mexico	P.J. Cotty	[71]
A. solani	CBS 111.44 = Y17070	seed, *Ageratum houstonianum*	–	P.J. Neergaard	[65]
A. solani	ICMP 6519–79 = Y17069	–	–	–	[67]
A. solani	IFO 7517 = D38762 + D38772	Solanaceae	Japan	–	[7]
A. solani	U80204	(NCBI data incorrect)	–	–	–
A. tenuissima	CBS 966.95 = IMI 79630	*Lycopersicon esculentum*	India	C. Chatuwedi	–
A. tenuissima	CBS 965.95	endophyte *Festuca ovina*	Switzerland	E. Bucheli	–
A. tenuissima	CBS 880.95 = IMI 292915	*Fragaria vesca*	Belgium	–	–
A. tenuissima	CBS 918.96 = IMI 255532, REPR for *A. tenuissima*	*Dianthus* sp.	UK	E.G. Simmons	E.G. Simmons
A. tenuissima	CBS 750.68	pod, *Phaseolus vulgaris*	France	J. Nicot	P. Joly
A. tenuissima	CBS 877.95	human sinusitis	India	S.M. Singh	[12]
A. tenuissima	CBS 879.95 = IMI 300779	*Sorghum* sp.	UK	M. Kalicz	–
A. tenuissima	ATCC 16423 = QM 7137 = AF229476	–	–	S.M. Martin	[30]
B. caespitosus	CBS 177.80 (T)	human skin lesion	Spain	C. Rubio	[11]
Cylindrocarpon lichenicola	ATCC 204306 = AF133843	–	–	P.C. Iwen	–
D. penicillatum	Cf96 = AF102899	*Papaver somniferum*	USA, Maryland	D.F. Farr	[28]
L. infectoria	CBS 137.90	human skin lesion	Germany	U. Schmidt	[72]
L. infectoria	CBS 308.53 = ATCC 12054 = AF229480	straw *Avena sativa*	–	L.E. Wehmeyer	–
L. infectoria	CBS 567.66 = ATCC 24279, T for *Chmelia slovaca*	human otitis	Slovakia	Y. Svobodá	[14, 73]
L. infectoria	CBS 827.68	seed *Lolium* sp.	Germany	U.G. Schlösser	–

Table 1. Continued

Name	Numbers	Source	Geography	Isolated by	Authority
L. infectoria	CBS 106.52	*Paeonia* sp.	Netherlands	I. de Boer	–
L. infectoria	CBS 160.79 = IMI 182035	straw	UK	–	–
L. infectoria	CBS 102692	human skin lesion	Germany	R. Horré	[4]
L. infectoria	4B = Y17067	*Linum usitatissimum*	UK	B. Fitt	[67]
L. infectoria	BMP 211115 = AF229458	seed, *Daucus* sp.	Netherlands	B.M. Pryor	[30]
L. infectoria	CBS 210.86 = EGS 27–193 = AF081456 (T)	*Triticum aestivum*	UK	J. Webster	[15]
L. infectoria	MZ10 = AJ276058	plasticized PVC	UK	–	[59]
L. infectoria	4A = Y17066	*Linum ustitatissimum*	UK	B. Fitt	[67]
L. infectoria	DH 12481	skin of renal transplant recipient	Portugal	R.M.P. Vieira	G.S. de Hoog
L. infectoria	DH 12429 = CBS 109785	skin of transplant recipient	Italy	L. Polonelli	G.S. de Hoog
P. herbarum	DAOM 150679 = U05202	*Trifolium pratense*	Canada	–	[27]
P. papaveracea	Pf96 = AF102888	*Papaver somniferum*	USA, Maryland	D.F. Farr	[28]
P. cf. *papaveracea*	DH 12348 = CBS 109787	human sinusitis	Germany	D. Alber	G.S. de Hoog
U. alternariae	BMP 314105 = AF229485	seed, *Daucus* sp.	–	–	[30]
U. atrum	CBS 102059	*Citrus* sp.	Greece	E.G. Simmons	[32]
U. atrum	CBS 195.67 = QM 8408 = ATCC 18040 = AF 229486, REPR for *U. atrum* (ET)	soil	USA, California	P.M.D. Martin	E.G. Simmons
U. botrytis	CBS 103.47 = IMI 56424, AUT for *A. stemphylioides*	fruit of *Phoenix dactylifera*	USA, California	D.E. Bliss	[74]
U. botrytis	CBS 104.21, T for *A. abietis*	needle, *Abies concolor*	the Netherlands	T.A. Tengwall	[54]
U. botrytis	CBS 197.67 = ATCC 18042 = IMI 124942, REPR for *U. botrytis* (ET)	air	USA, Massachusetts	R.T. Moore	[20]
U. botrytis	CBS 198.67 = ATCC 10843 = AF229487	soil under garbage	USA, California	E.G. Simmons	E.G. Simmons
U. botrytis	CBS 452.72	salt-marsh soil	Kuwait	A.F. Moustafa	–
U. chartarum	CBS 105.32	leaf, *Iris* sp.	USA, Montana	J.A. Stevenson	–
U. chartarum	CBS 199.67 = ATCC 18045 = IMI 116369	manila fibre	Switzerland	O. Wälchi	[20]
U. chartarum	CBS 200.67 = ATCC 18044 = QM 8328 = 229488, REPR for *U. chartarum* (ET)	*Populus* plywood	Canada	S.J. Hughes	E.G. Simmons
U. cucurbitae	CBS 102061	leaf, *Cucumis sativus*	Australia	W.R. Tweedie	[32]
U. dauci	CBS 102062 (T)	seed, *Daucus carota*	USA, Washington	T. Schultz	[32]
U. multiforme	CBS 102060 (T)	beach soil	Canada	J. Reid	[32]

Abbreviations used: A = *Alternaria*, B = *Botryomyces*, D = *Dendryphion*, U = *Ulocladium* (anamorph genera); L = *Lewia*, P = *Pleospora* (teleomorph genera).
T = ex-type strain; AUT = authentic strain; ET = ex-epitype strain; REPR = representative strain.
Recognized culture collections: ATCC = American Type Culture Collection, Manassas, VA, USA; CBS = Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; DAOM = Department of Agriculture (Mycology), Ottawa, Canada, GHP = Gerhard Haase Pilze, Institute for Medical Microbiology RWTH, Aachen, Germany; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand; IFO = Institute for Fermentation, Osaka, Japan; IMI = International Mycological Institute, Kew, UK; QM = Quarter Master Culture Collection, Amherst, MA, USA.

Restriction fragment length polymorphism (RFLP)

The rDNA ITS domains were amplified with 30 cycles in a Biomed 60 thermocycler using primers ITS1 and ITS4 [8], as follows: predwell (94°C, 5 min), denaturation (94°C, 1 min), annealing (48°C, 1 min), elongation (72°C, 2 min), postdwell (72°C, 3 min). One unit Super-*Taq* polymerase (SphaeroQ, Leiden, the Netherlands) was used per reaction. Amplicons were digested with two units of endonucleases (Table 4) at 36°C overnight and electophoresed for 3 h at 6–10 V cm^{-1} on 1.5% agarose gels.

Sequencing

Amplicon V9G–LS 266 [9] was generated as above and purified using the Gel Band Purification Kit (Amersham Pharmacia, Roosendaal, the Netherlands). DNA was bound to GFX-columns,

Table 2. Misidentified strains

Number	Current name	Correct identification
NCBI AF218791	*Alternaria alternata*	*Ulocladium chartarum*
CBS 198.74	*Alternaria chlamydospora*	unidentified species
NCBI D38768	*Alternaria dauci*	*Alternaria porri*
NCBI D38760	*Alternaria porri*	unidentified species
NCBI AF307015	*Alternaria radicina*	*Alternaria petroselini*
NCBI U80204	*Alternaria solani*	unidentified species
NCBI AF133843	*Cylindrocarpon lichenicola*	*Alternaria alternata*
CBS 103.47	*Ulocladium botrytis*	*Ulocladium atrum*
CBS 104.21	*Ulocladium botrytis*	*Ulocladium atrum*
NCBI AF229487	*Ulocladium botrytis*	*Ulocladium atrum*
CBS 452.72	*Ulocladium botrytis*	*Ulocladium atrum*
CBS 453.74	*Ulocladium mouchaccae*	unidentified species

Total number of misidentified strains: GenBank: seven out of 49; CBS: five out of 50.

eluted according to protocols given by the supplier, and collected with TE-buffer. Concentrations of amplicons were estimated by comparison with SmartLadder markers (Eurogentec, Seraing, Belgium) on 1% agarose gels. Sequencing primers were ITS1, ITS4 and ITS5; reactions (96°C for 10 s; 50°C for 5 s; 60°C for 4 min; 25 cycles) were carried out with 15–50 ng of DNA for a 10-µl reaction mixture including 4 pmol primer and 4 µl BigDye RR Mix (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). Subsequently, DNA was precipitated with ethanol and sequenced using an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Alignment and phylogenetic analysis

Sequences were adjusted using the SEQMAN program within the LASERGENE software package (DNASTAR, Madison, WI, USA) and aligned using BIONUMERICS (Applied Maths, Kortrijk, Belgium). A distance tree was constructed using neighbor-joining with a Kimura correction using the TREECON (version 1.3b) software package [10], and a phylogenetic tree was constructed using PAUP version 4.0b8 with heuristic search option and stepwise addition. Bootstrap values were calculated from 100 resampled datasets.

Results

Identity of strains

For each strain included in the tree, an authority for its identification is given (Table 1). Ex-type materials are also indicated in the Table. Authorities are experts in the field who confirmed the identity of the strain. A reference to a taxonomic publication citing the strain is given where appropriate. Correct sequence identity can be judged from the alignment of rDNA ITS sequences using the Ward's averaging option in BIONUMERICS. Conclusions based on these data are supplemented with morphology and literature data. The reference strains provide a reliable framework for the phylogenetic tree. Misidentified strains are listed in Table 2. In GenBank, 14% of the sequences held prove to be incorrectly identified. A marked example is *Cylindrocarpon lichenicola* (Massal.) Hawksworth, AF1 33843. The species is remote from *Alternaria*, belonging to the order Hypocreales rather than Pleosporales, but its sequence appears to be identical to that of *A. alternata*. The authors probably sequenced a contaminant. In the CBS culture collection, made up of strains primarily

Table 3. Species having ITS1–2 rDNA sequences nearly identical to the species printed in bold type

Alternaria alternata
Alternaria arborescens
Alternaria lini
Alternaria longipes
Alternaria mali
Alternaria tenuissima
Alternaria radicina
Alternaria carotiincultae
Alternaria petroselini
Alternaria selini
Alternaria raphani
Alternaria japonica
Alternaria solani
Alternaria linicola
Ulocladium atrum
Ulocladium dauci
Ulocladium multiforme
Ulocladium cucurbitae
Ulocladium botrytis
Ulocladium alternariae

Table 4. Restriction enzymes used

Enzyme	Recognition site
*Acc*II	CG/CG
*Dde*I	C/TNAG
*Hae*III	GG/CC
*Hha*I	GCG/C
*Hinf*I	G/ANTC
*Msp*I	C/CGG
*Rsa*I	GT/AC
*Sau*3A1	/GATC
*Taq*I	T/CGA

identified by morphology, 10% of the *Alternaria* strains have been misidentified (Table 2). Some species have nearly identical ITS sequences (Table 3). They are mostly also morphologically similar and are frequently found on the same host plant. Information on whether these are separate species was not gained in the course of this study.

Cultural characteristics

CBS strains assigned after sequencing to the *Alternaria alternata* and *Lewia infectoria* clusters grew poorly on media with high sugar concentration (Table 7), being highly degenerative on DRYES medium. On this medium cultures were invariably sterile, with white, dense, cushion-shaped aerial mycelium, sometimes deeply furrowed or wrinkled and then of hard texture and olivaceous brown. No consistent difference between the two species was noted in growth on the media.

ITS sequencing

Strains of *Alternaria infectoria/Botryomyces caespitosus* de Hoog & Rubio [11] (Fig. 1) (group 1) were found to compose a clade differing clearly from the remaining species. The ITS of strains contain an indel of about 30 bp starting at position 23 of ITS1. An insertion of similar length but with different base composition is found at the same position in a group of four strains containing *Alternaria mouchaccae* Simmons including the ex-type strain, CBS 490.72 (group 2). The latter indel is also present in strain *Ulocladium botrytis* Preuss, CBS 197.67, albeit a minor 4 bp difference. All remaining *Alternaria* and *Ulocladium* strains analyzed lack an insertion at this position. The cluster *A. infectoria/B. caespitosus* (1) is split at low resolution by differences at two phylogenetically informative sites (i.e. recurrent in different strains). *Botryomyces caespitosus* also differs from *A. infectoria* by an additional 10 unique bases (i.e. found in a single strain only).

A core group (group 3) of 31 strains, including CBS 603.78 and CBS 916.96 are regarded by E.G. Simmons as strains representative for *Alternaria alternata*. Seven of these strains are strictly identical. Among them is CBS 101.13, ex-type strain of *Alternaria geophila* Dawszewska, an established synonym of *A. alternata*, as well as four strains deposited under the name *Alternaria tenuissima* (Kunze:Pers.) Wiltsh., for which no type material is known to be preserved. Group 3, with CBS

Table 5. Overview of restriction sites

Strains with ITS domains of about 600 bp:

	U. botrytis	*B. caespitosus*	*A. infectoria*
ITS1:			
*Hinf*I	57	–	–
*Hha*I	92	85	90
*Acc*II	–	85	91
*Hae*III	–	93	99
*Msp*I	–	94	100
*Hae*III	101	97	103
***Rsa*I**	**138**	**–**	**136**
***Dde*I**	**–**	**162**	**168**
5.8S:			
*Sau*3A1	246	239	234
*Taq*I	265	257	263
*Hinf*I	318	310	316
*Taq*I	324	316	322
*Hinf*I	326	318	324
*Hha*I	347	339	345
*Taq*I	377	369	375
ITS2:			
*Rsa*I	391	383	389
***Hinf*I**	**441**	**–**	**441**
*Msp*I	465	459	465
*Hae*III	468	462	468
*Hha*I	486	–	486
*Acc*II	498	492	498
*Hha*I	500	494	500
Backward primer	603	598	605

Strains with ITS domains of about 570 bp:

	A. alternata	*A. clamydospora*	*U. atrum*	*U. chartarum*
ITS1:				
*Hinf*I	–	–	–	57
*Rsa*I	109	111	108	108
5.8S:				
*Sau*3A1	215	219	215	216
*Taq*I	233	239	234	235
***Hinf*I**	**–**	**292**	**287**	**288**
*Taq*I	293	298	293	293
*Hha*I	317	321	316	317
*Hinf*I	296	300	295	296
*Taq*I	347	351	346	347
ITS2:				
*Rsa*I	362	365	360	361
*Hinf*I	413	415	410	411
*Msp*I	437	439	434	435
*Hae*III	440	442	437	438
*Hha*I	458	460	455	456
***Acc*II**	**–**	**–**	**467**	**468**
*Hha*I	–	–	469	469
Backward primer	578	578	572	573

Sites usable for diagnostics (criteria see text) written in bold type.

Table 6. Fragments generated by diagnostic RFLP markers

Spacer domain 600 bp:				
*Rsa*I	*U. botrytis*	*B. caespitosus*	*A. infectoria*	
	138	383	136	
	212	215	216	
	253		253	
*Dde*I	*U. botrytis*	*B. caespitosus*	*A. infectoria*	
	603	162	168	
		436	437	
*Hinf*I	*U. botrytis*	*B. caespitosus*	*A. infectoria*	
	(8)	(8)	(8)	
	(57)	280	117	
	115	310	164	
	162		316	
	261			
Spacer domain 570 bp:				
*Acc*II	*A. alternata*	*A. clamydospora*	*U. atrum*	*U. chartarum*
	578	578	105	105
			467	468

Bands < 100 bp in brackets; double bands are indicated with slash.

879.95 as the outermost strain, shows somewhat greater variation. Four informative positions are noted within the group. Since, however, there is no discernible pattern at these sites, no clear distinction can be made within group 3. The group contains three strains of *Alternaria longipes* (Ell. & Everh.) Mason, collected from tobacco more than 60 years apart. The strains share a single base substitution near the end of ITS2. In addition, group 3 comprises two strains deposited under the name *A. tenuissima*.

Group 4 contains *Ulocladium* and *Alternaria* species with conidia that are short and mostly show septation in various orientations. The ex-type strains of *Ulocladium dauci* Simmons and *U. multiforme* Simmons are strictly identical in their ITS sequences to those of *A. stemphylioides* Bliss and *A. abietis* Tengwall, which are synonyms of *U. botrytis*. *Ulocladium cucurbitae* (Let. & Roum.) Simmons and *U. atrum* Preuss belong to the same group. Other *Alternaria* strains with chlamydospore-like conidia, namely *Alternaria mouchaccae* and CBS 198.74, identified as *A. chlamydospora*, form a well-delimited cluster (Fig. 1). The *Ulocladium* strains analyzed proved to be heterogeneous, comprising two separate subclusters which were located amidst *Alternaria* species. One of the subclusters (4) contained the ex-type strain of *A. chlamydospora*, CBS 491.72, at a short internal distance. None of the agents of clinical cases attributed to *A. chlamydospora* were available for taxonomic confirmation, except for a strain isolated from skin by Singh *et al.* [12]. This strain was found to be *A. alternata* (Table 1). Cluster 4 contains a subgroup

Table 7. Growth and conidiation of *Alternaria alternata* and *A. infectoria* on PCA, PDA, DRYES and MHA

		PCA	PDA				DRYES				MHA			
		Con	Col	Surf	Con	D	Col	Surf	Con	D	Col	Surf	Con	D
A. alternata	CBS 603.78	+	B	S	–	4.5	X	S	–	4.0	W	S	–	2.0
A. alternata	CBS 795.72	+	B	S	–	8.0	W	S	–	4.5	W	S	+	3.0
A. alternata	CBS 916.96	+	B	S	–	5.5	X	S	–	3.5	X	S	+	1.5
A. alternata	CBS 109803	+	B	S	–	6.0	B	R	–	2.5	W	S	+	1.5
A. longipes	CBS 540.94	–	B	X	–	4.5	W	X	–	3.0	B	S	+	2.0
A. tenuissima	CBS 750.68	+	B	S	–	6.0	W	X	–	3.0	W	S	+	2.0
A. tenuissima	CBS 880.95	+	B	S	–	7.5	B	R	–	3.0	X	S	+	1.5
A. tenuissima	CBS 918.96	+	B	S	–	8.0	W	S	–	4.5	W	S	–	2.5
A. tenuissima	CBS 879.95	+	X	S	–	5.5	B	R	–	2.5	B	S	+	1.5
A. infectoria	CBS 137.90	+	B	S	–	7.5	B	X	–	2.0	W	S	–	2.0
A. infectoria	CBS 308.53	–	B	S	–	6.0	W	S	–	3.0	W	S	–	3.5
A. infectoria	CBS 567.66	–	B	S	–	2.5	B	X	–	1.5	W	S	–	3.0
A. infectoria	CBS 827.68	–	W	S	–	5.0	W	S	–	1.5	W	S	–	1.5
A. infectoria	CBS 106.52	–	B	S	–	5.5	W	S	–	1.5	W	S	–	2.0
A. infectoria	CBS 102692	–	B	S	–	6.5	W	R	–	1.5	W	S	–	4.0
A. infectoria	CBS 210.86	+	B	S	–	5.0	B	S	–	2.5	W	S	–	2.0
A. infectoria	DH 12481	–	B	S	–	4.0	W	S	–	2.0	W	S	–	1.0
A. infectoria	DH 12429	–	B	S	–	5.5	W	S	–	1.5	W	S	–	1.8

DRYES = Dichloran Rose Bengal Yeast Extract Sucrose Agar; PDA = Potato Dextrose Agar; MHA = Müller Hinton Agar II.
Col = colony color and texture: B = grey to olivaceous-black and lanose; W = off-white or whitish and cushion-shaped, X = intermediate between any of the categories.
Surf = colony surface: R = rough, folded; S = without furrows.
Con = conidiation: + = conidia abundantly present; – = conidia degenerate or absent.
D = colony diameter after 10 days in cm.

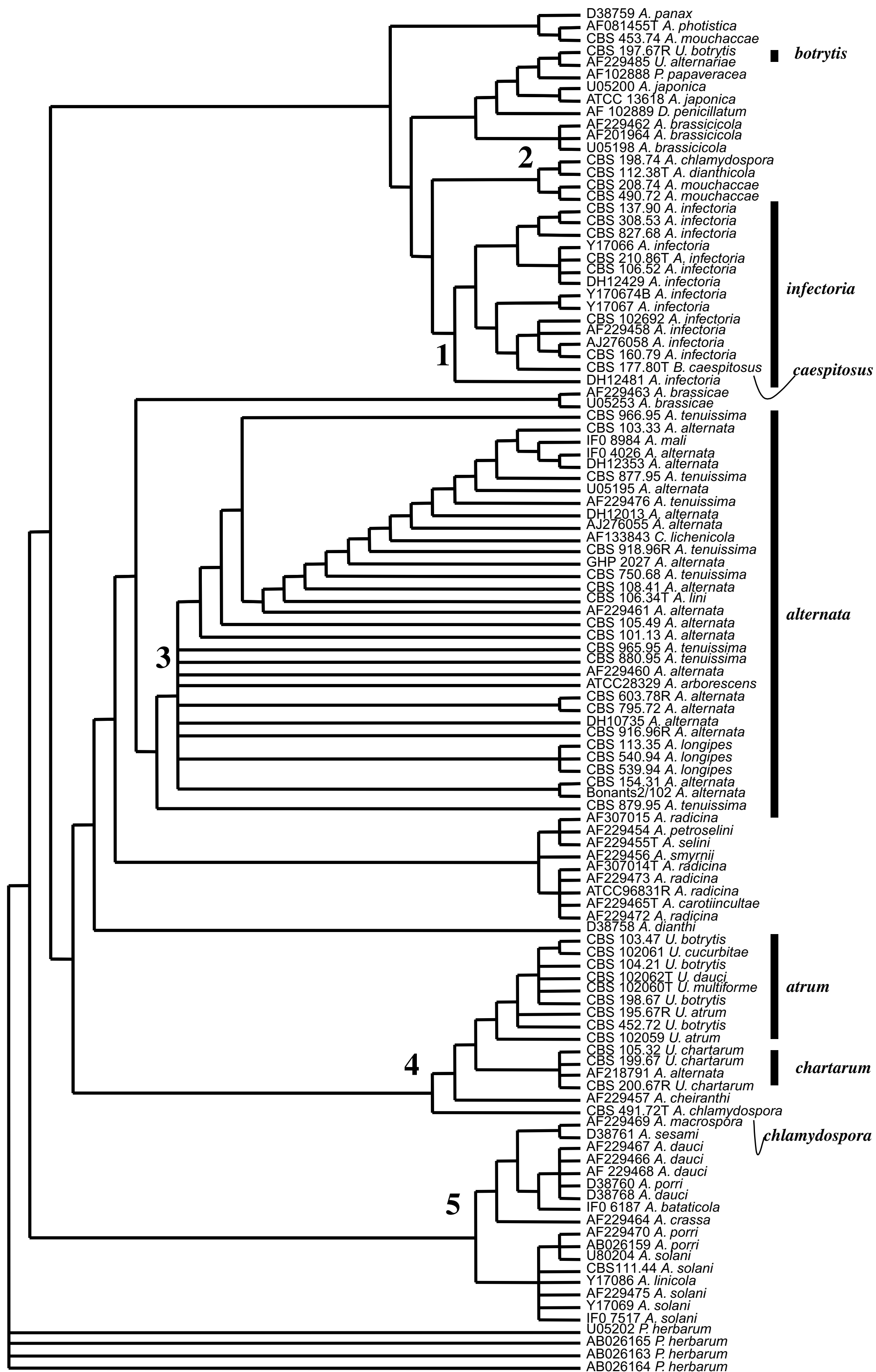

Figure 1. Strict consensus tree of equally most parsimonious trees (312 steps) of *Alternaria/Ulocladium* obtained by heuristic search in the program PAUP, based on confidently aligned ITS1–2 rDNA sequences. *Pleospora herbarum* is selected as outgroup. Strains are listed with their original name of deposition, while groups (1–5) reported in the medical literature are indicated with vertical bars, with final identification at the right hand side.

of strains that were identified morphologically by E.G. Simmons alternatively as *U. atrum* (CBS 195.67, CBS 102059) or as *U. botrytis* (CBS 198.67), as well as the ex-type strains of *U. dauci* Simmons, *U. multiforme* Simmons and *U. cucurbitae* Simmons. In Fig. 2, recognized entities are listed with their prevalent ecology.

Rflp

Restriction sites are summarized in Fig. 3. With primers ITS1 and ITS4, most strains yielded an approximately 570-bp amplicon, including an ITS domain of about 480 bp, while the remainder, a group of which *A. chlamydospora* and *A. infectoria* are the core species, had an approximately 600-bp amplicon with an ITS domain of about 510 bp (Table 5, Fig. 4). *Botryomyces caespitosus*, with a 600-bp amplicon, was similar to the latter group.

The majority of the strains of the group with the 570-bp amplicon yielded identical RFLP patterns with all enzymes used. This group included strains identified as *A. alternata*, the type strain of *A. dianthicola* Neergaard and several tentatively identified *Ulocladium* species. Only two representative strains of *U. chartarum* (Preuss) Simmons (E.G. Simmons, personal communication), CBS 199.67 and 200.67, had a deviating *Hinf*I pattern. The *U. chartarum* strains were identical to one of the strains of *A. tenuissima*, all remaining isolates of the latter species being identical to *A. alternata*.

RFLP patterns of the group with 600-bp amplicons were identical for most of the enzymes used. Some variation was found with *Hinf*I, *Msp*I and *Dde*I. Comparison with available literature data and a morphological re-examination revealed that these differences correlated with the species concepts of *A. chlamydospora*, *A. infectoria* (both with characteristic pattern with *Hinf*I) and *U. botrytis* (characteristic patterns with *Hinf*I and *Msp*I) (Table 6, Fig. 5). *Botryomyces caespitosus* was indistinguishable from *Alternaria infectoria*.

Discussion

Clinically, phaeohyphomycosis caused by *Alternaria* may be difficult to recognize. Lesions in debilitated patients are variable [13]: they may appear as nonhealing ulcers, crusted lesions, erythematous macules, or subcutaneous nodules. Histopathologically, thick-walled (sub)spherical elements 10–15 μm in diameter or short chains of oblong cells may be observed. The rounded elements are mostly unpigmented, and therefore *Alternaria* infection is sometimes misdiagnosed as yeast infection or blastomycosis. Even *in vitro* identification may be highly problematic, since clinical isolates may remain degenerate and sterile. An interesting example of misdiagnosis concerns *Chmelia slovaca* (Svobodová *et al.*) Svobodová, first described as a sterile fungus. The verrucose lesions with thick-walled cells in tissue were interpreted originally as chromoblastomycosis [14]; the chlamydosporic cultures were white, later turning olivaceous [14]. In the course of our study *C. slovaca* was identified as *Alternaria* cf. *infectoria* on the basis of ITS sequence data. We frequently obtain white, sterile or nearly sterile clinical strains which turn out to be *Alternaria* species (Table 1), and thus we are confident that *Chmelia* is nothing but a sterile member of this genus. The name *C. slovaca* predates *A. infectoria* Simmons [15]. The type culture of *C. slovaca* is degenerate and never produced conidia, and one might argue that sequence data alone are insufficient proof of its identity, particularly if *A. infectoria* is found to be a species aggregate. It is remarkable that clinical strains often show low production of a purported virulence factor, melanin. The fact that subcutaneous *Alternaria* infections are present in tissue with hyaline cells may indicate that melanin is less important in invasion by members of this genus.

Diagnostic problems may be encountered in strains with meristematic morphology. *Alternaria infectoria* and *A. chlamydospora* Mouchacca may be clinically relatively important because they easily form muriform, chlamydospore-like cells that are probably more immune resistant and refractory to therapy. The close affinity of the meristematic species *Botryomyces caespitosus* to *Alternaria infectoria* was first reported by de Hoog *et al.* [6]. The ex-type strain of *B. caespitosus*, CBS 177.80, cannot be a meristematic ecotype of *A. infectoria* because the ITS domains of the species differ at 10 positions, but it is likely to be a synanamorph of an as yet undescribed *Alternaria* species. CBS 177.80 is easily distinguished from *A. infectoria* by its extremely slow growth, but as a species *B. caespitosus* is very difficult to recognize. Three additional strains maintained under the name *B. caespitosus* in the CBS culture collection were re-identified by molecular characters as three different entities, a meristematic mutant of *Exophiala dermatitidis* (Kano) de Hoog and two still undescribed, mutually unrelated meristematic black yeasts. The strain remaining identified as *B. caespitosus*, CBS 177.80, originated from a verrucose skin lesion [11]. Meristematic morphology is known to occur in phylogenetically unrelated fungi. Strains of *Aureobasidium pullulans* (De Bary) Arn. may grow meristematically under conditions of low nitrogen availability (P. Zalar, personal communication). The black yeast *Sarcinomyces phaeomuriformis* Matsumoto *et al.* forms meristematic and yeast-like synanamorphs that are genetically

identical [16]. Close molecular resemblance but nonidentity of hyphal and meristematic ecotypes is also known in the basidiomycetous yeast *Trichosporon/Fissuricella* [17]. The ecological function of the meristematic morphology is probably connected to extreme growth conditions, but the evolutionary mechanism maintaining this appearance in the progeny is as yet unknown.

In order to obtain insight into the ecology and correct identification of *Alternaria* species, public domain sequences were included in our study. In routine identification, BLAST searches in GenBank are frequently applied. However, GenBank sequences of less-known fungi should be used with caution. Isolation data are often not provided, and may not even be obtained by cross-reference to public culture collections since many authors sequence their own isolates without depositing vouchers. Occasionally no publication has appeared on the sequences deposited, or authors use a numbering system that cannot be traced back to original data. Occasionally the data provided are simply incorrect, such as with *Alternaria solani* NCBI U80204 referring to a bacterial publication. Several authors compare insufficient numbers of reference strains to calibrate the identity of the material they sequenced. For example, an *Ulocladium* sequence in GenBank (NCBI AF218791) was attributed to the wrong genus, *Alternaria*. A striking case was a sequence identical to *A. alternata* but attributed to *Cylindrocarpon lichenicola*, where the authors probably sequenced a contaminant in their culture. The problem is particularly significant in less common species or in highly deviating sequences, where no or little reference data for comparison are as yet available. The position of a number of taxa in Fig. 1 remains therefore questionable. Some clear-cut misidentifications are listed in Table 2. About 14% of the GenBank sequences proved to be misidentified. This confirms the suggestion by Harmsen *et al.* [18] that automatic identification exclusively based on public data banks cannot be more than an approximation. For strains held in the CBS culture collection, which were identified by classical methods, 10% were incorrectly identified. These figures do not include taxonomically ambiguous identifications discussed below. For the main traits, the data do allow tree-reconstruction with the species (aggregates) for which several representatives were included, but the tree lacks precision in the highly diversified plant-pathogenic taxa.

The genus *Alternaria* may possibly comprise hundreds of species of rapidly growing, melanized hyphomycetes, characterized by sympodially elongating conidiophores bearing large, multiseptate conidia [19–22]. *Alternaria infectoria* and *A. photistica* Simmons are known to belong to the order Pleosporales through *Lewia* teleomorph connections [15]. It is likely that the entire *Alternaria/Ulocladium* complex is of Pleosporalean affinity, as stated by Sivanesan [23]. Small subunit (SSU) rDNA phylogeny [24] has shown that the genus is monophyletic. Correspondence of *Alternaria*-like anamorphs with Pleosporales was confirmed by Berbee *et al.* [25] and Olivier *et al.* [26] based on rDNA ITS and glyceraldehyde-3-phosphate genes. *Pleospora herbarum* (Pers: Fr.) Rabenh., having a *Stemphylium* anamorph, is also related [27], as can be concluded from its position in the ITS tree (Fig. 1). The presence of *Pleospora papaveracea* (De Not.) Sacc. in the *U. botrytis* cluster (6; Fig. 1) further underlines the relationship of *Pleospora*-like teleomorphs and *Alternaria* complex, despite its *Dendryphion* anamorph [28]. The close relationship of *Alternaria* with *Ulocladium* species producing muriform conidia is also demonstrated in the cluster containing *Alternaria*, *Ulocladium* and *Dendryphion* species. A primary culture of *P. cf. papaveracea* with *Dendryphion* anamorph, CBS 109787, initially showed meristematic development. Sclerotial cells were described previously in *P. papaveracea* [29]. With a BLASTn search we found some *Embellisia* sequences which belonged in the same cluster.

Within *Alternaria*, classically, two groups are roughly recognized. Most obligatory plant pathogenic taxa produce large, slender, noncatenate conidia and show attenuated conidiation in culture. A second group consists of species with short conidia arising in long chains. These species generally show abundant conidiation in culture; most of them are saprotrophic, but the group also contains facultative or obligate plant pathogenic members. Some of the saprotrophic species are distinguished with difficulty from the genus *Ulocladium*, which has small, relatively dark conidia arising singly or in short chains. Simmons [20] delimited the two genera by the morphology of immature conidia, which have pointed bases in *Ulocladium* and rounded bases in *Alternaria*. This character is observed with difficulty in catenulate *Ulocladium* species, so that in routine practice the catenate saprotrophs of *Alternaria* and *Ulocladium* are frequently regarded as a single species complex.

This classical segregation of *Alternaria* groups based on morphology, pathogenicity and conidiation is poorly supported by ITS rDNA sequence data (Fig. 1). A separation of *Alternaria* and *Ulocladium* at the generic level is not apparent either. Our data are congruent with those of Pryor & Gilbertson [30]. Their *Alternaria* ITS tree distinguished non-catenate plant pathogens and catenate saprotrophs. The latter clustered a short distance from *Ulocladium* and from a group of plant pathogens with muriform conidia. *Alternaria infectoria*, on the other hand, was

found a substantial distance from all these groups. In Fig. 1, two main clusters of catenate *Alternaria* species are found, centered around *A. alternata* and *A. infectoria*, respectively. The noncatenate plant pathogens constitute two further clusters, while a group containing nearly all *Ulocladium* strains is paraphyletic to one of the plant pathogenic clusters and to *A. chlamydospora*. Strains of *Ulocladium* as well as *A. chlamydospora*-like strains are morphologically characterized by more or less ellipsoidal rather than the obclavate conidia observed in the remainder of *Alternaria*. It should be noted that the *Alternaria* species with ellipsoidal conidia are heterogeneous: a strain from *Carduus* identified as *A. mouchaccae*, CBS 453.74, is probably an undescribed species, while the two strains of *A. chlamydospora* studied are clearly different from each other.

Alternaria alternata is the oldest and most common species of the genus; it is one of the short-conidial saprotrophs. The species was redefined by Simmons [20, 22] on the basis of careful morphological observations. In the absence of usable type material, he indicated strains CBS 916.96 and CBS 603.78 as representative for the species (CBS List of Cultures; E.G. Simmons, personal communication). Simmons' concept of (morphologically) representative isolates is convenient where no living or interpretable material is available (dried material is present at L: Leiden, the Netherlands). We favor the application of a more formal approach, similar to that used by Gräser *et al.* [31] in the dermatophytes. Therefore the status of **epitype** is proposed for CBS 916.96.

In a series of publications [15, 20, 22, 32–34], E. G. Simmons maintained a narrow species concept for the short-chained taxa around *A. alternata*, based on the three-dimensional branching patterns of conidial chains. Yu [35] noted that it is difficult for nonexperts to detect morphological differences between the units; this is certainly true for strains from humans which are often degenerate or even sterile. Most of the entities recognized by Simmons had different Random amplified polymorphic DNA (RAPD) profiles [36] and had specific host plants [36]. RAPD-typing found a large set of strains which originated from closely similar substrates but nevertheless showed pronounced infraspecific variability. Similar variability was found by Morris *et al.* [37] with strains from tomato in California: only 34 out of 137 RAPD markers within this ecologically unified set of strains proved to be monomorphic for all isolates. It is therefore likely that RAPD is poorly suited for diagnosis of nonhost-specific taxa world-wide. On the other hand, some highly specialized species do exist that show remarkably consistent RAPD patterns even in widely geographically distributed populations [38].

The ITS tree is compared with ecological data in Fig. 2. This tree is strongly biased towards strains from economically important host plants. For example, *Daucus carota* is over-represented, being the host plant of six species treated (Table 1). Nevertheless a distinction can be made between species having (nearly) the same ITS sequence and comprising isolates originating from the same host plant, and groups/taxa with an equally low degree of ITS diversity but showing no host specificity. The latter frequently contain strains from dead substrates, such as soil, wood, straw and plastic. This is the case in *Alternaria alternata (s.l.)* and *A. infectoria*, as well as in the saprotrophic genus *Ulocladium*. The mixed origin of strains clustering in these *Alternaria* species suggests that as far as saprotrophs are concerned, their colonization of living host plants is relatively coincidental.

Occurrence on a particular host plant does not necessarily identify the species, since several taxa may occur on the same host. This was particularly demonstrated by Simmons [39], who found many distinct taxa parasitizing *Citrus*. Most of the host-specific taxa may differ in toxin production [33], the toxins playing a role as pathogenicity factors [40]. Among the host-specific *Alternaria* species with ITS sequences almost identical to that of *A. alternata* (Table 3) are *A. longipes* (on tobacco), *A. lini* Dey (on flax), *A. mali* Roberts (on apple) and *A. arborescens* Simmons (on tomato). The last name was introduced by Simmons [39] for the former *A. alternata* f.sp. *lycopersici* Gronan *et al.* Serdani *et al.* [41] listed *A. arborescens* as a main cause of core rot of apples in South Africa, along with *A. tenuissima*; the two species appeared to be very close in all characters analyzed. Evolutionary adaptation and speciation on particular host plants within the basically saprotrophic *A. alternata s.l.* is possible in principle. Indications for host specialization were found in differential host resistance to a single pathovar [42] and in low intraspecific variability of anonymous markers of strains among a single host [43]. Andersen *et al.* [44] found *A. longipes* to deviate consistently from *A. alternata* in its metabolite spectrum. Thus the entities recognized by E. G. Simmons and R. G. Roberts are probably correct, but to conceive these entities as separate species is debatable. The groups of Nishimura [45, 46] and Adachi [47] referred to similar units as pathotypes within a single umbrella-species, *A. alternata*. This view was supported by Kuninaga and Yokosawa [48] who found high DNA reassociation values between *A. alternata* and related species. Our ITS sequencing data indicate that several host-specific plant pathogens belong to the *A. alternata* species aggregate. Population genetic studies are needed to demonstrate whether full

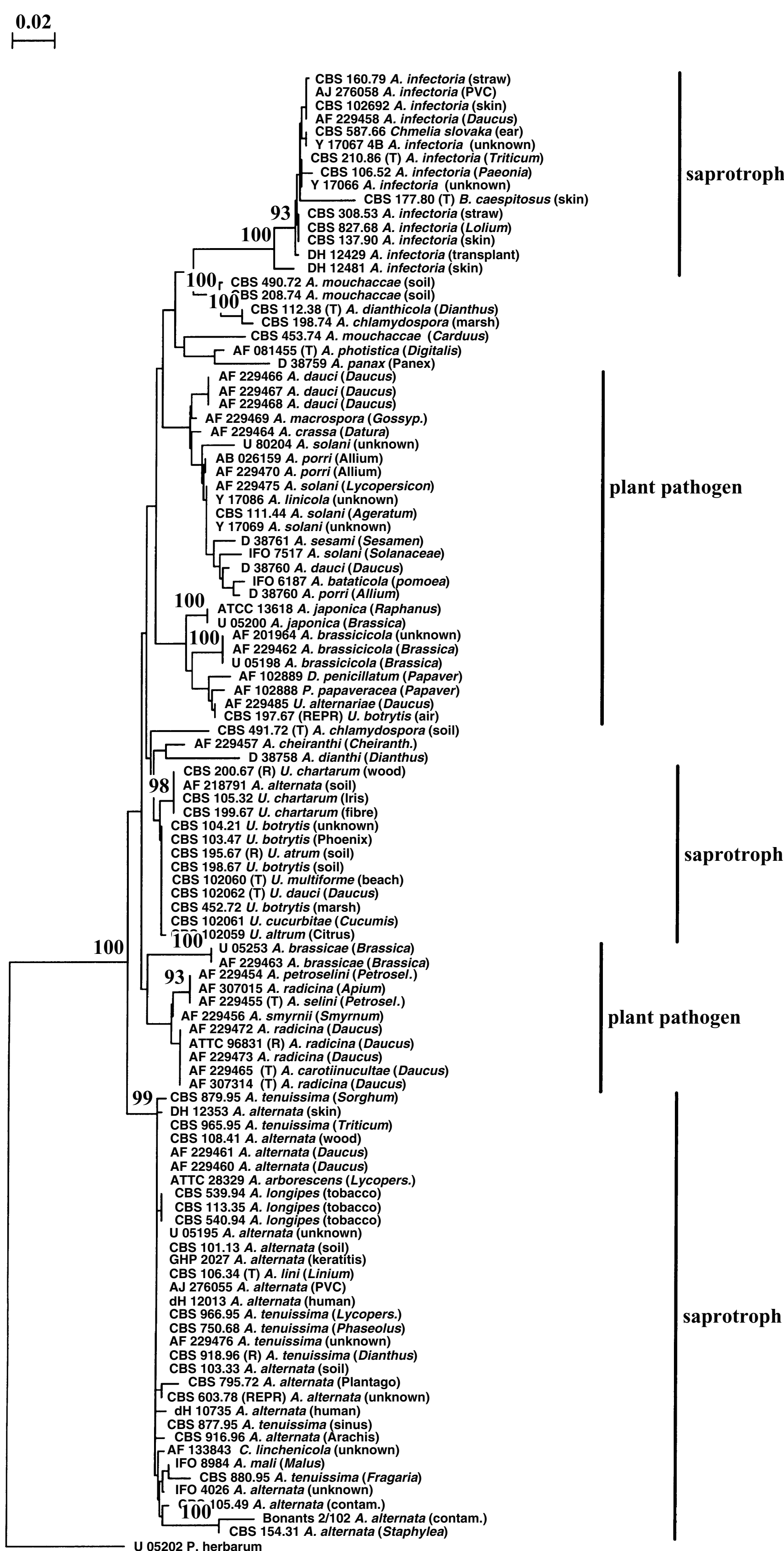

Figure 2. Distance tree of *Alternaria/Ulocladium* based on confidently aligned ITS1–2 rDNA sequences, using neighbor-joining algorithm with Kimura correction in the program TREECON. Bootstrap > 90 from 100 resampled datasets are shown. *Pleospora herbarum* is selected as outgroup. Strains are listed with their original name of deposition and source of isolation. Main ecological categories are indicated with vertical bars, with saprotrophic *vs.* plant pathogenic nature at the right hand side.

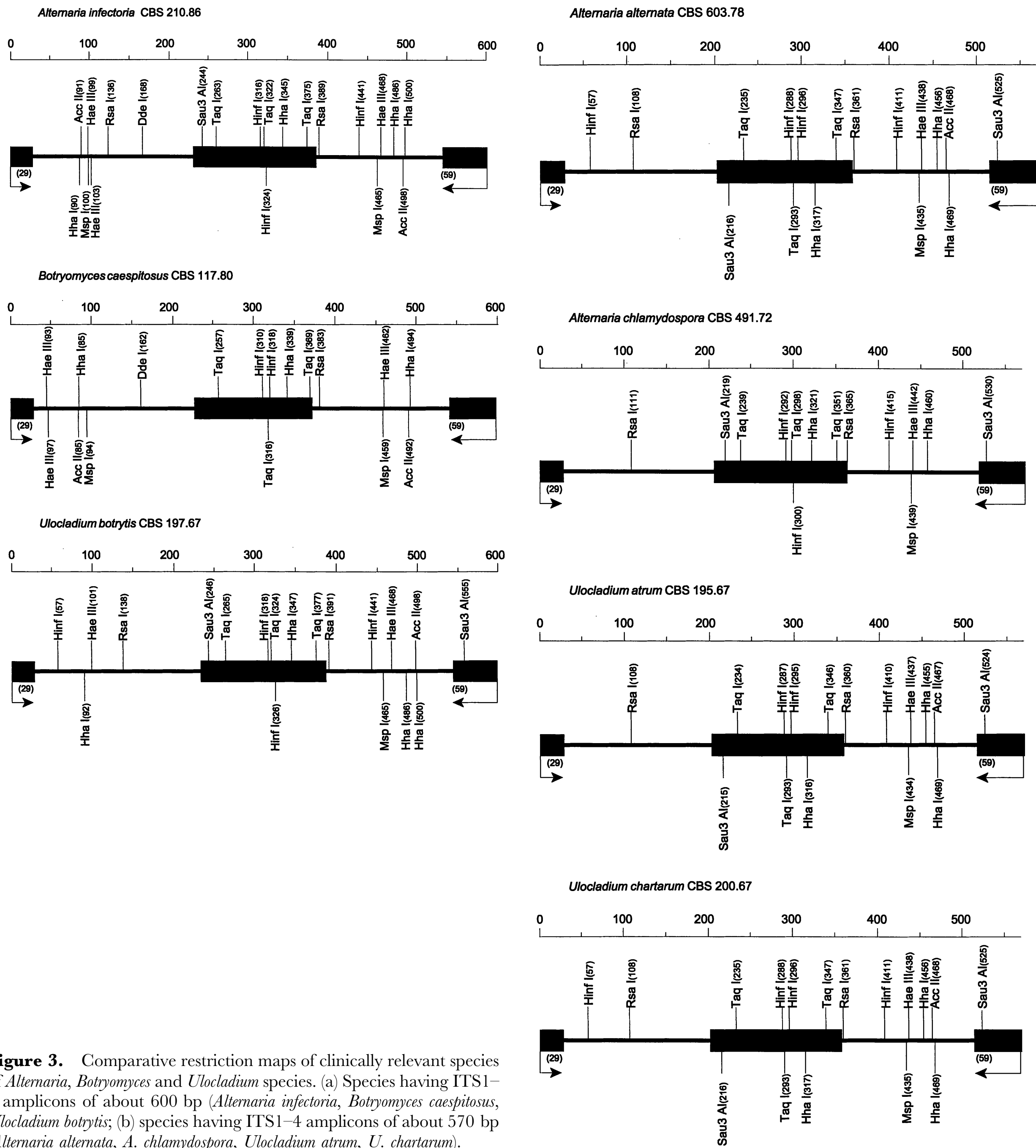

Figure 3. Comparative restriction maps of clinically relevant species of *Alternaria*, *Botryomyces* and *Ulocladium* species. (a) Species having ITS1–4 amplicons of about 600 bp (*Alternaria infectoria*, *Botryomyces caespitosus*, *Ulocladium botrytis*; (b) species having ITS1–4 amplicons of about 570 bp (*Alternaria alternata*, *A. chlamydospora*, *Ulocladium atrum*, *U. chartarum*).

evolutionary separation has been achieved of microspecies distinguished by E. G. Simmons and co-workers.

Most of the clinical cases in the older literature have been attributed to *A. alternata* [2, 49–53] or *A. tenuissima* (see de Hoog *et al.* [5] for review). Very few strains described as etiological agents in clinical case studies have been preserved, and consequently their identities can no longer be verified. *Alternaria alternata* and *A. tenuissima* have short and long conidia, respectively, and differ in conidial branching patterns [22], but in the ITS distance tree (Fig. 2) the strains of the two species are intermingled. The strains studied include IMI 255532, which was treated as representative of *A. tenuissima* by Simmons [22] as well as other isolates morphologically identified as *A. tenuissima* by E. G. Simmons and P. Joly. Given the wide occurrence of the umbrella-species, *A. alternata*, on dying and dead plant material, it is unlikely that any pathotype-specific toxins play a significant role in human disease. Regardless of whether taxa like *A. mali*, *A. longipes* and *A. tenuissima* should be maintained at all (see above), the significance of their recognition in the routine clinical laboratory seems to be very low.

In our study we found that nearly all clinical isolates sequenced so far belong to the predominantly

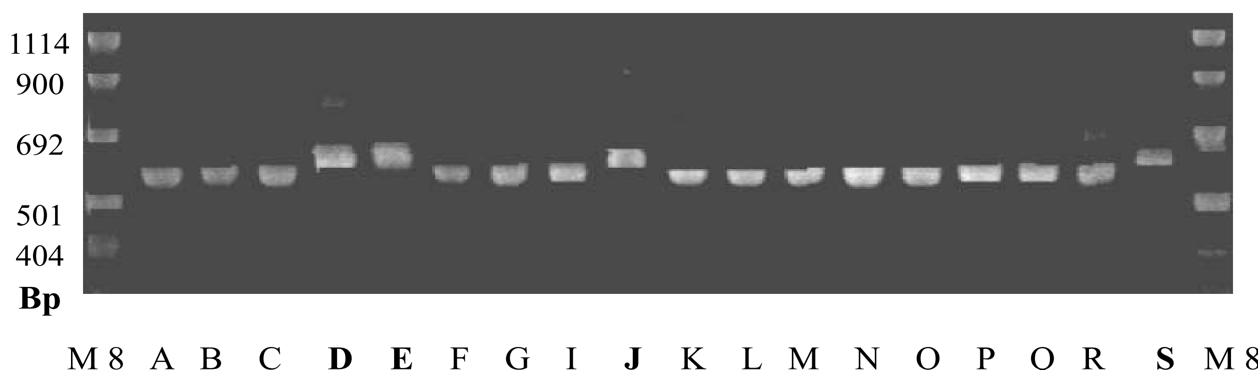

Figure 4. ITS amplicons using primers ITS1 and ITS4. D, E, J and S are about 600 bp, the remaining strains about 570 bp in length. A = CBS 603.78; B = CBS 918.96; C = CBS 795.72; D = CBS 198.74; E. CBS 137.90; *F* = CBS 879.95; *G* = CBS 880.95; I = CBS 103.47; J = CBS 197.67; K = CBS 105.32; L = CBS 104.21; M = CBS 750.68; N = CBS 199.67; O = CBS 916.96; *P* = CBS 102059; Q = CBS 540.94; R = CBS 177.80.

saprotrophic complexes, *A. alternata* and *A. infectoria*. The ITS-verified strains typically showed opportunistic behavior in the immunosuppressed host [4, 13, 54, 55]. In agreement with expectations, human infection by *Alternaria* species other than the saprotrophic aggregates has not been proven. We think mycoses are very unlikely to be caused by the great majority of members of the genus *Alternaria* species, since most of them are plant-pathogenic.

Ulocladium species are saprotrophic, and no specialization on particular host plants has been observed. *U. atrum*, *U. botrytis* and *U. chartarum* are morphologically highly variable, and therefore we believe that the genus is over-classified. In the absence of living type material (dried specimens at B: Berlin, Germany), we follow Simmons' [20] taxonomic treatment of *Ulocladium* and regard CBS 195.67 as **epitype** for *U. atrum*, CBS 200.67 as **epitype** for *U. chartarum*, and CBS 197.67 as **epitype** for *U. botrytis*.

Clinical cases by *Ulocladium* species are exceptional. *Ulocladium botrytis* is regularly found as a contaminant in superficial mycoses, but thus far no case report has been published [5]. De Hoog *et al.* [5] reviewed four cases ascribed to *U. chartarum*, while Magina *et al.* [56] reported a fifth case. Currently no clinical strains are available for molecular comparison.

In conclusion, recognition of *Alternaria alternata*, *A. chlamydospora*, *A. infectoria*, *Botryomyces caespitosus* and *Ulocladium chartarum* is clinically meaningful, since these are potential – though not always proven – agents of disease. This is important as long as the possibility of interference with therapy regimens of different degrees of melanization and the occurrence of meristematic growth has not been excluded. In contrast, molecular distinction of plant-pathogenic species, or of morphological entities or plant-associated pathotypes within *A. alternata*, appears to have little medical significance.

A white colony on DRYES medium is a morphology diagnostic test proposed for *Alternaria alternata vs. A. infectoria* [57]. However, our evaluation of this character showed that growth responses on DRYES of the two species are variable and insufficiently diagnostic (Table 7) to be used for identification. Optimal distinction of relevant species is enabled by rDNA data. ITS RFLP is particularly promising in the Pleosporales, where variability in the spacer domains is moderate, compared to the bewildering diversity found in other groups of fungi such as the order Chaetothyriales [6]. Different degrees of spacer variability have also been observed within the Pleosporales in the family Pleosporaceae, to which the *Alternaria* anamorphs are attributed. Jasalavich *et al.* [27] attributed the variable degrees of species diversity to the existence of differences in reproductive strategies. Within the Pleosporaceae the ITS diversity is large enough to provide distinction of clinically significant species and species aggregates. The low degree of variation within each group maximizes the predictivity of the test.

The molecular diagnostic techniques which are most practical for routine hospital laboratories include PCR using general primers combined with restriction enzyme digests of the amplicons. The method has sufficient resolution [6]. In the species listed as potential agents of mycoses by De Hoog *et al.* [5], a subdivision can be made on the basis of the length of the ITS1–4 amplicon (Fig. 5). Figure 3 gives an overview of restriction maps similar to those published by De Hoog *et al.* [5]. The ITS spacer domain is about 570 bp in the *A. alternata* clade, *A. chlamydospora*, *U. atrum* and *U. chartarum*, and about 600 bp in *B. caespitosus*, *A. infectoria* and *U. botrytis*. The difference can be seen on gel by comparison with molecular weight markers [6]. Restriction sites detected by the panel of enzymes used are listed in Table 6. All species of the 600 bp group can be distinguished easily, but in the 570 bp group *Ulocladium atrum* and *U. chartarum*

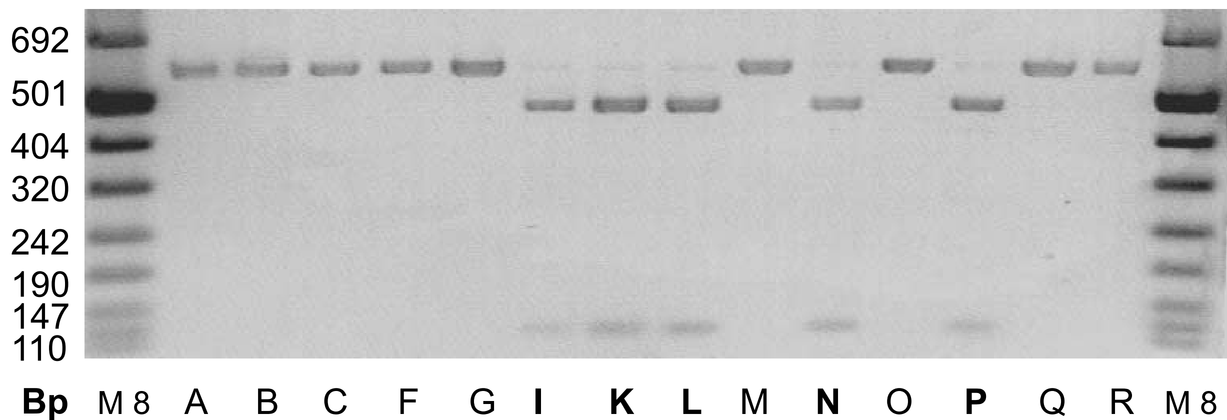

Figure 5. RFLP (ITS×*Acc*II) of randomly selected strains with ITS domain of about 570 bp (expected bands at 578 and 468–9 + 105, resp.). A = *A. alternata* CBS 603.78; B = *A. tenuissima* CBS 918.96; C = *A. alternata* CBS 795.92; *F* = *A. tenuissima* CBS 879.95; *G* = *A. tenuissima* CBS 880.95; I = *U. atrum* CBS 103.47; K = *U. chartarum* CBS 105.32; L = *U. atrum* CBS 104.21; M = *A. alternata* CBS 750.68; N = *U. chartarum* CBS 199.67; O = *A. alternata* CBS 916.96; P = *U. atrum* CBS 102059; Q = *A. longipes* CBS 540.94; R = *A. alternata* CBS 879.95; M = molecular weight marker (Roche).

Table 8. Diagnostics of clinically relevant *Alternaria*, *Botryomyces* and *Ulocladium* species in summary

1a	ITS amplicon about 570 bp.	2
1b	ITS amplicon about 600 bp.	5
2a	*Acc*II digestion largest band 578 bp.	3
2b	*Acc*II digestion largest band 467–468 bp.	4
3a	*Taq*I 114 bp present; conidia obclavate, in long, branched chains.	*A. alternata*
3b	*Taq*I 114 bp absent; conidia meristematic, not in chains.	*A chlamydospora*
4a	Conidia spherical, cruciately septate; conidia never in chains.	*U. atrum*
4b	Conidia ovoidal, most with three transverse septa; some of the conidia in short chains.	*U. chartarum*
5a	*Hinf*I digestion largest band 261 bp.	*U. botrytis*
5b	*Hinf*I digestion largest band 310–316 bp.	6
6a	*Hinf*I digestion smallest bands 117 and 164 bp.	*A. infectoria*
6b	*Hinf*I digestion smallest band 280 bp.	*B. caespitosus*

are identical. In the ITS distance tree (Fig. 1) *U. chartarum* is distinguishable as a paraphyletic branch at a distance of three informative positions, but no restriction enzyme is available for the diagnostic sites. A dichotomous key to the medically important species considered in this study can be prepared using these molecular characters for rapid identification (Table 8).

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