Generic distinction in the *Helminthosporium*-complex based on restriction analysis of the nuclear ribosomal RNA gene

Teik-Khiang Goh[1*], Kevin D. Hyde[1] and Daniel K.L. Lee[2]

[1]Fungal Diversity Research Project, Department of Ecology and Biodiversity, The University of Hong Kong, Pokfulam Road, Hong Kong; * email: tkgoh@hkucc.hku.hk
[2]Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong

Goh, T.K., Hyde, K.D. and Lee, D.K.L. (1998). Generic distinction in the *Helminthosporium*-complex based on restriction analysis of the nuclear ribosomal RNA gene. Fungal Diversity 1: 85-107.

The DNA sequences coding for the nuclear 28S rRNA, 5.8S rRNA, and the internal transcribed spacers (ITS1 and ITS2) for 36 representative fungal species from *Bipolaris*, *Cercospora*, *Corynespora*, *Curvularia*, *Drechslera*, *Exserohilum* and *Helminthosporium* were amplified using the polymerase chain reaction. The amplified products were digested using restriction endonuclease *Rsa* I, and the electrophoretic patterns compared. With the exception of *Bipolaris* and *Curvularia*, cluster analysis of the restriction phenotypes clearly separated the genera. *Bipolaris* and *Curvularia* appeared to be more heterogeneous than the other allied genera within the *Helminthosporium*-complex.

Introduction

The genus *Helminthosporium* Link is heterogeneous and includes species that are economically and ecologically important. There are numerous of binomial names in the genus, and most of them have been disposed in other hyphomycete genera. Their classification is of more than academic interest because some of them cause devastating diseases of important crops.

The basic morphology of *Helminthosporium sensu stricto*, based on the type species *H. velutinum* Link, includes solitary, cylindrical, unbranched, brown conidiophores that produce obclavate, pseudoseptate conidia through small pores in the walls of the distal and intercalary cells of the conidiophores (Luttrell, 1963). Growth of conidiophores cease with the formation of terminal conidia. Graminicolous "*Helminthosporium*" species (Drechsler, 1923; Misra, 1973), however, differ fundamentally from *Helminthosporium sensu stricto* in producing solitary conidia at the apex of the brown, geniculate conidiophores. Resumption of growth occurs by sympodial extension from the subapical region of the conidiophore with distinct conidial scars. These differences in morphology

are the basis of segregating the graminicolous species into four principal genera, namely *Bipolaris* Shoemaker, *Curvularia* Boedijn, *Drechslera* S. Ito and *Exserohilum* Leonard and Suggs (Shoemaker, 1959; Luttrell, 1963, 1964; Ellis, 1971; Alcorn, 1983b, 1988; Sivanesan, 1987). Traditional characters currently used to differentiate these four genera include conidial shape, hilum morphology, type of germination, septum ontogeny and texture of conidiophores. Other criteria useful in differentiation include anamorph-teleomorph connections and culture colony morphology (Alcorn, 1983b, 1988; Sivanesan, 1987).

Although most taxa previously included in *Helminthosporium* have been transferred to other more appropriate genera, some ambiguous species still remain in *Helminthosporium* (*sensu lato*). Some of the criteria used in the present classification of the *Helminthosporium*-complex are artificial, and this has caused taxonomic confusion. The nomenclatural complexities resulting from the evolution of taxonomic concepts in graminicolous *Helminthosporium* species are also confusing to non-specialists. Many taxa in this helminthosporioid group have species epithets included in various genera. For example, the binomial "*Exserohilum turcicum*" may be reported under any one of four anamorphic genera (i.e. *Bipolaris*, *Drechslera*, *Helminthosporium*, and *Luttrellia* Khokhr. and Gornostai) or three teleomorphic genera (i.e. *Keissleriella* Höhn., *Setosphaeria* Leonard and Suggs, and *Trichometasphaeria* Munk) (Sivanesan, 1987).

Bipolaris and *Curvularia* species are similar in many ways, and many species in both genera have been reported to have a *Cochliobolus* Drechsler sexual state. Most *Curvularia* species, however, have curved conidia that have a distinctly swollen central cell. A few workers have considered that these two genera are synonymous (Luttrell, 1979), but others prefer to separate them (Ellis, 1971, 1976; Alcorn, 1983a; Sivanesan, 1987). Ellis (1971, 1976) however, considers *Bipolaris* and *Exserohilum* to be synonymous with *Drechslera*. The plurality of binomial names of taxa in the "*Helminthosporium*-complex" lessens the precision and reliability of information storage and recall associated with the use of a single name for each taxon. The need for a stable classification based on non-artificial characters is therefore paramount in this important group of plant pathogens.

Employment of molecular techniques in fungal systematics is relatively new and has been reviewed by Bruns *et al.* (1991). Restriction enzyme analyses of mitochondrial DNA and nuclear ribosomal DNA (rDNA) have been used widely in fungal systematics (White *et al.*, 1990; Persson, Erland and Jansson, 1996; Nicholson, Bunyard and Royse, 1997). A challenge facing biologists interested in *Helminthosporium* (*sensu lato*) is how to devise a classification in which

natural relationships are recognised, and reliable criteria for differentiating genera and species are available. A plaucible solution might be available at the molecular level. We are therefore examing taxonomic concepts in the four principal genera (i.e. *Bipolaris*, *Curvularia*, *Drechslera*, and *Exserohilum*) segregated from *Helminthosporium sensu stricto* using various molecular techniques. We would like to know whether these four genera are phylogenetically distinct groups, and to test whether the current classification is reasonable and natural. In this paper we report on a restriction analysis of the PCR-amplified portions of the nuclear ribosomal RNA gene for a number of representative taxa from various genera of interest. For comparison, we also included representative species of *Helminthosporium sensu stricto* as well as two other similar hyphomycete genera which are also important plant pathogens: *Cercospora* Fresen. (Hsieh and Goh, 1990) and *Corynespora* Güssow (Ellis, 1957). For simplicity and convenience, abbreviated names of fungal taxa (see Table 1) were used in tables, figures and legends.

Materials and methods

Fungal isolates and cultural conditions

A total of 36 representative species of *Bipolaris*, *Cercospora*, *Corynespora*, *Curvularia*, *Drechslera*, *Exserohilum* and *Helminthosporium* were chosen for this study. Sources and descriptions of the fungal isolates are given in Table 1. Fungal isolates were grown on either potato dextrose agar (PDA) or malt

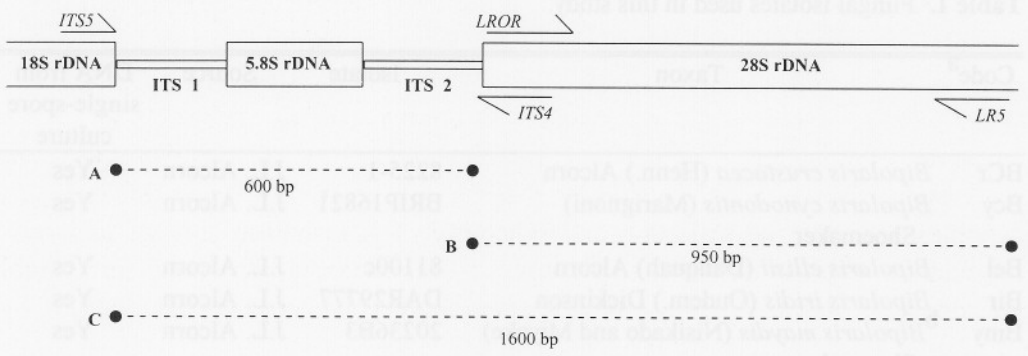

Fig. 1. Diagrammatical representation of the rDNA (partial map) illustrating location of PCR-primers (*ITS5*, *ITS4*, *LROR*, *LR5*) and segments amplified (dotted lines). **A.** Product amplified with primers *ITS5* and *ITS 4*; **B.** Product amplified with primers *LROR* and *LR5*; **C.** Product amplified with primers *ITS5* and *LR5*. Sizes of products indicated are merely approximation.

extract agar (MEA) and incubated at 24 C under cool white fluorescent light. Twenty of the cultures used for DNA extraction were from single-spore isolates, whereas the other sixteen were from mycelial cultures.

DNA isolation

Genomic DNA was isolated according to the methods given by Lee and Taylor (1990). Mycelia from each of the fungal isolates were scraped from the surface of PDA or MEA and ground by hand in a mortar and pestle, with lysis buffer containing 50 mM Tris-HCl (pH 7.2), 50 mM EDTA, 3 % (w/v) sodium dodecyl sulphate, and 1 % (w/v) 2-mercaptoethanol. DNA samples were extracted with chloroform : TE-saturated phenol (1:1, pH 8) and precipitated with cold isopropanol. DNA pellets were resuspended in 100-300 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). The concentration of DNA was examined on 1 % agarose gels (GibcoBRL) and was stored at -20 C until further use.

PCR amplification

Four different primers were used in PCR-amplifications of the nuclear ribosomal RNA gene (rDNA; Fig. 1), viz. *LROR* (5'-ACCCGCTGAACTTA-AGC-3', GibcoBRL), *LR5* (5'-TCCT GAGGGAAACTTCG-3', GibcoBRL), *ITS5* (5'-GGAAGTAAAAGTCGTAACAAGG-3', GibcoBRL), and *ITS4* (5'-TCCTCCGCTTATTGATATGC-3', GibcoBRL). Segments of the rDNA from

Table 1. Fungal isolates used in this study.

Code[a]	Taxon	Isolate	Source	DNA from single-spore culture
BCr	*Bipolaris crustacea* (Henn.) Alcorn	8225-1	J.L. Alcorn	Yes
Bcy	*Bipolaris cynodontis* (Marignoni) Shoemaker	BRIP16821	J.L. Alcorn	Yes
Bel	*Bipolaris ellisii* (Danquah) Alcorn	81100c	J.L. Alcorn	Yes
Bir	*Bipolaris iridis* (Oudem.) Dickinson	DAR29777	J.L. Alcorn	Yes
Bmy	[b]*Bipolaris maydis* (Nisikado and Miyake) Shoemaker	20236B3	J.L. Alcorn	Yes
Bov	*Bipolaris ovariicola* Alcorn	8842-1	J.L. Alcorn	Yes
BPp	*Bipolaris papendorfii* (Aa) Alcorn	9084c	J.L. Alcorn	Yes
BRv	*Bipolaris ravenelii* (M.A. Curtis) Shoemaker	8638-1	J.L. Alcorn	Yes
Ccy	*Curvularia cymbopogonis* (Dodge) Groves and Skolko	88109-2	J.L. Alcorn	No

Table 1. (continued).

Code[a]	Taxon	Isolate	Source	DNA from single-spore culture
CEr	*Curvularia eragrostidis* (Henn.) J.A. Meyer	95/1857a	J.L. Alcorn	Yes
CHe	*Curvularia heteropogonicola* (Sivan.) Alcorn	IMI268958	J.L. Alcorn	Yes
CIq	*Curvularia inaequalis* (Shear) Boedijn	BRIP14448	J.L. Alcorn	No
CLu	[b]*Curvularia lunata* (Wakker) Boedijn	95/1937b	J.L. Alcorn	Yes
COr	*Curvularia oryzae* Bugnic.	MRL1089	J.L. Alcorn	Yes
DAv	*Drechslera avenae* (Eidam) Scharif	DAR33699	J.L. Alcorn	No
DBi	*Drechslera biseptata* (Sacc. and Roum.) Rich. and Fraser	WU353	J.L. Alcorn	Yes
DBr	*Drechslera brizae* (Nisikado) Subram.	PDDCC6183	J.L. Alcorn	No
DCa	*Drechslera campanulata* (Lèv.) B.C. Sutton	BRIP15927	J.L. Alcorn	Yes
DTe	*Drechslera teres* (Sacc.) Shoemaker	8712	J.L. Alcorn	No
DTr	*Drechslera triseptata* (Drechsler) Subram. and Jain	NZ6120	J.L. Alcorn	No
DTR	[b]*Drechslera tritici-repentis* (Died.) Shoemaker	7819-1	J.L. Alcorn	Yes
EFu	*Exserohilum fusiforme* Alcorn	8822b	J.L. Alcorn	Yes
EGe	*Exserohilum gedarefense* (El Shafie) Alcorn	8307	J.L. Alcorn	Yes
EHo	*Exserohilum holmii* (Luttr.) Leonard and Suggs	9084b	J.L. Alcorn	No
ELo	*Exserohilum longirostratum* (Subram.) Sivan.	7728	J.L. Alcorn	No
EMi	*Exserohilum minor* Alcorn	81100b	J.L. Alcorn	No
ERo	*Exserohilum rostratum* (Drechsler) Leonard and Suggs	BRIP23191	J.L. Alcorn	Yes
ETu	[b]*Exserohilum turcicum* (Pass.) Leonard and Suggs	94/1823	J.L. Alcorn	No
H1	*Helminthosporium solani* Durieu and Mont.	CBS359-49	CBS	No
H2	[b]*Helminthosporium velutinum* Link	CBS201-29	CBS	No
H3	*Helminthosporium* sp.	BRIP14521	J.L. Alcorn	Yes
H4	*Helminthosporium* sp.	PWA1-6	K.D. Hyde	Yes
CEap	[b]*Cercospora apii* Fresen.	CBS119.25	CBS	No
CEcn	*Cercospora canescens* Ellis and Martin	CBS153.55	CBS	No
COcs	[b]*Corynespora cassiicola* (Berk. and M.A. Curtis) Wei	CBS296.80	CBS	No
COol	*Corynespora olivacea* (Wallr.) M.B. Ellis	CBS291.74	CBS	No

[a] = Abbreviations of fungal taxa which are used in other tables, figures and captions.
[b] = Type species of the genus.

each of the fungal isolates were amplified by PCR with three different pairings of primers, viz. *LROR/LR5* (amplification of the front portion of the 28S large subunit), *ITS5/ITS4* (amplification of the 5.8S rDNA and the two flanking internal transcribed spacer (ITS) regions), and *ITS5/LR5* (amplification of the ITS, 5.8S rDNA and partial segment of the 28S large subunit). The DNA samples were usually diluted 1:100 in distilled water for use as templates in PCR amplifications. PCR amplifications were performed in 100 µL volumes of reaction buffer (500 mM KCl, 1 % (v/v) Triton X-100, 100 mM Tris-HCl pH 9), 0.2 mM of each deoxyribonucleotide triphosphate, 1.5 mM of $MgCl_2$, 0.3 µM of each primer, *ca* 1-10 µg of fungal DNA, and 5 units of *Taq* DNA polymerase (Promega). The amplifications were performed for 35 cycles, using the following parameters: 95 C denaturation step (1 min), 54 C annealing step (1 min), 72 C primer extension (1-2 min, depending on primers used). A final incubation step at 72 C (10 min) was added after the final cycle. Negative controls (no template DNA) were included to test for the presence of DNA contamination of reagents and reaction mixtures. Usually 5 µL of each PCR reaction was electrophoresed on 1 % agarose gels together with either 100-basepair (bp) ladder (Pharmacia) or 1-kilobasepair ladder (GibcoBRL).

Restriction analysis of PCR products

Generic and interspecific variations in representative species of *Bipolaris*, *Curvularia*, *Drechslera* and *Exserohilum* were examined by means of restriction analysis of the PCR products amplified with different pairs of primers. The restriction endonuclease *Rsa* I was used according to the manufacturer's instructions (GibcoBRL). Usually 20 µL of each PCR product was digested in 40 µL volumes at 37 C overnight (*ca* 10-15 hrs). The restriction fragments were subjected to electrophoresis on 2.5 % NuSieve 3:1 agarose gels (FMC BioProducts). The size of bands was estimated by comparison with 100-bp ladder (Pharmacia). Results were recorded by photographing gels over a UV-transilluminator.

Phenetic analysis of restriction data

The presence or absence of restriction fragments was scored for all taxa digested with the restriction endonuclease *Rsa* I, which resulted in a binary 1/0 matrix. For all pairs of taxa, the S_{xy} values were calculated from the restriction phenotypes using the formula of Nei and Li (1979),

$$S_{xy} = \frac{2\,(N_{xy})}{N_x + N_y}$$

where N_{xy} equals the number of fragments shared between taxa X and Y, and N_x and N_y equal the number of fragments of taxa X and Y respectively. The percentage dissimilarity D between taxa was calculated as $D = (1- S_{xy}) \times 100\ \%$, and a distance matrix was established. Using the TREECON Neighbor-joining program (Van de Peer and De Wachter, 1997), dendrograms were calculated from the distance matrix with UPGMA (unweighted pair group method using arithmetic averages) clustering method.

Results

PCR amplification

Gel electrophoresis of the PCR-amplified rRNA gene segments of 36 fungal taxa exhibited a single band for each taxon which varied in size corresponding to the different pairs of primers used. PCR using primer pairs *LROR/LR5*, *ITS5/ITS4*, and *ITS5/LR5* yielded specific products of approximately 950 bp (28S-PCR products), 600-680 bp (ITS-PCR products), and 1560-1630 bp (ITS/28S-PCR products) respectively. For each pair of primers used in the amplifications, no secondary bands were obtained other than the specific products. In addition, no PCR products were obtained in the controls (result not shown). No clear length polymorphism of the 28S-PCR products was evident amongst taxa when primers *LROR/LR5* were used (see Table 2). When primer pairs *ITS5/ITS4* and *ITS5/LR5* were used, however, both the ITS-PCR and ITS/28S-PCR products exhibited length polymorphism (see Tables 3 and 4), probably due to the variable length of the two internal transcribed spacers.

Restriction analysis of PCR products

When the 28S-PCR products (*ca* 950 bp) of seven *Drechslera* species, eight *Bipolaris* species, six *Curvularia* species and seven *Exserohilum* species. were digested with *Rsa* I, three distinct restriction patterns (phenotypes) were obtained (Fig. 2; Table 2). The first pattern comprised two fragments, one of *ca* 740 bp long and the other of *ca* 210 bp in length, and was exhibited by most of the *Bipolaris* and *Curvularia* species. The second pattern comprised three fragments, viz. approximately 415, 325 and 210 bp in length respectively, and were exhibited by all *Drechslera* as well as *Exserohilum* species. This second phenotype was also exhibited by two *Bipolaris* and two *Curvularia* species, namely *B. cynodontis*, *B. maydis*, *C. eragrostidis*, and *C. oryzae*. The third pattern was exhibited only by a single taxon, *Bipolaris iridis*, and comprised three fragments, approximately 450, 270 and 230 bp in length respectively.

Table 2. Distribution of restriction fragments after digestion of the 28S-PCR product by *Rsa* I.

No.	Size[a]	DAv	DBi	DBr	DCa	DTe	DTr	DTR	BCr	BCy	BEl	BIr	BMy	BOv	BPp	BRv	CCy	CEr	CHe	CIq	CLu	COr	EFu	EGe	EHo	ELo	EMi	ERo	ETu
1	740	0[b]	0	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0
2	450	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	415	1	1	1	1	1	1	1	0	1	0	0	1	0	0	0	0	1	0	0	0	1	1	1	1	1	1	1	1
4	325	1	1	1	1	1	1	1	0	1	0	0	1	0	0	0	0	1	0	0	0	1	1	1	1	1	1	1	1
5	270	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	230	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	210	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Fragment size sum		950	950	950	950	950	950	950	950	950	950	950	950	950	950	950	950	950	950	950	950	950	950	950	950	950	950	950	950
Phenotype		I	I	I	I	I	I	I	II	I	II	III	I	II	II	II	II	I	II	II	II	I	I	I	I	I	I	I	I

Table 3. Distribution of restriction fragments after digestion of the ITS-PCR product by *Rsa* I.

No.	Size[a]	DAv	DBi	DBr	DCa	DTe	DTr	DTR	BCr	BCy	BEl	BIr	BMy	BOv	BPp	BRv	CCy	CEr	CHe	CIq	CLu	COr	EFu	EGe	EHo	ELo	EMi	ERo	ETu
1	390/400[a]	0[b]	0	0	0	0	0	0	1	1	0	0	1	0	1	1	1	1	0	0	0	1	0	1	0	1	1	1	0
2	340	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
3	250/260	1	1	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0
4	230/240	0	0	0	0	0	0	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0	0	1
5	210-220	1	1	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
6	200	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
7	170/180	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	1	0	1
8	150/160	1	0	1	0	1	0	1	0	1	0	1	0	1*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	120/130	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
10	90/100	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	70/80	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	1	0	1
Fragment size sum		630	680	680	650	630	680	630	630	630	620	630	630	640	630	630	630	630	620	620	640	640	630	650	660	650	650	640	610
Phenotype		I	II	I	III	I	II	I	IV	V	VI	I	IV	VII	IV	IV	IV	IV	VI	VIII	IX	IV	X	XI	XII	XI	XIII	XI	VIII

[a] Fragment sizes (in base pairs) estimated by comparison with molecular weight standard.

[b] 0 = restriction fragment absent; 1 = restriction fragment present.

* Double bands of similar size.

Table 4. Distribution of restriction fragments after digestion of the ITS/28S-PCR product by *Rsa* I.

No.	Size[a]	DAv	DBi	DBr	DCa	DTr	DTR	BEl	Blr	BMy	BOv	BPp	BRv	CCy	CEr	CHe	CIq	CLu	COr	EFu	EGe	EHo	EMi	ETu
1	790/800[a]	0[b]	0	0	0	0	0	1	0	0	1	1	1	1	0	1	1	1	0	0	0	0	0	0
2	450/460	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
3	430/440	1	1	1	1	1	1	0	1	1	0	0	0	0	1	0	0	0	1	0	1	0	0	0
4	410/420	0	0	0	0	0	0	0	0	1*	0	1	1	1	1*	0	0	0	1*	0	0	0	0	0
5	380/400	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	0	1	1	1	1	1
6	340	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
7	320	1	1	1	1	1	1	0	0	1	0	0	0	0	1	0	0	0	1	1	1	1	1	1
8	260/270	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	240/250	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
10	210/220	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0
11	190/200	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	1
12	170/180	1	0	1	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0
13	150/160	0	0	0	0	0	1	0	1	0	1*	0	0	0	0	0	0	0	0	0	0	0	0	0
14	120/130	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
15	70/80	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	1
Fragment size sum		1580	1630	1610	1600	1630	1580	1570	1580	1580	1590	1580	1580	1580	1580	1570	1570	1590	1590	1580	1600	1610	1600	1560
Phenotype		I	II	I	I	II	III	IV	V	VI	VII	VIII	VIII	VIII	VI	IV	IX	X	VI	XI	XII	XI	XIII	XIV

[a] Fragment sizes (in base pairs) estimated by comparison with molecular weight standard.

[b] 0 = restriction fragment absent; 1 = restriction fragment present.

* Double bands of similar size.

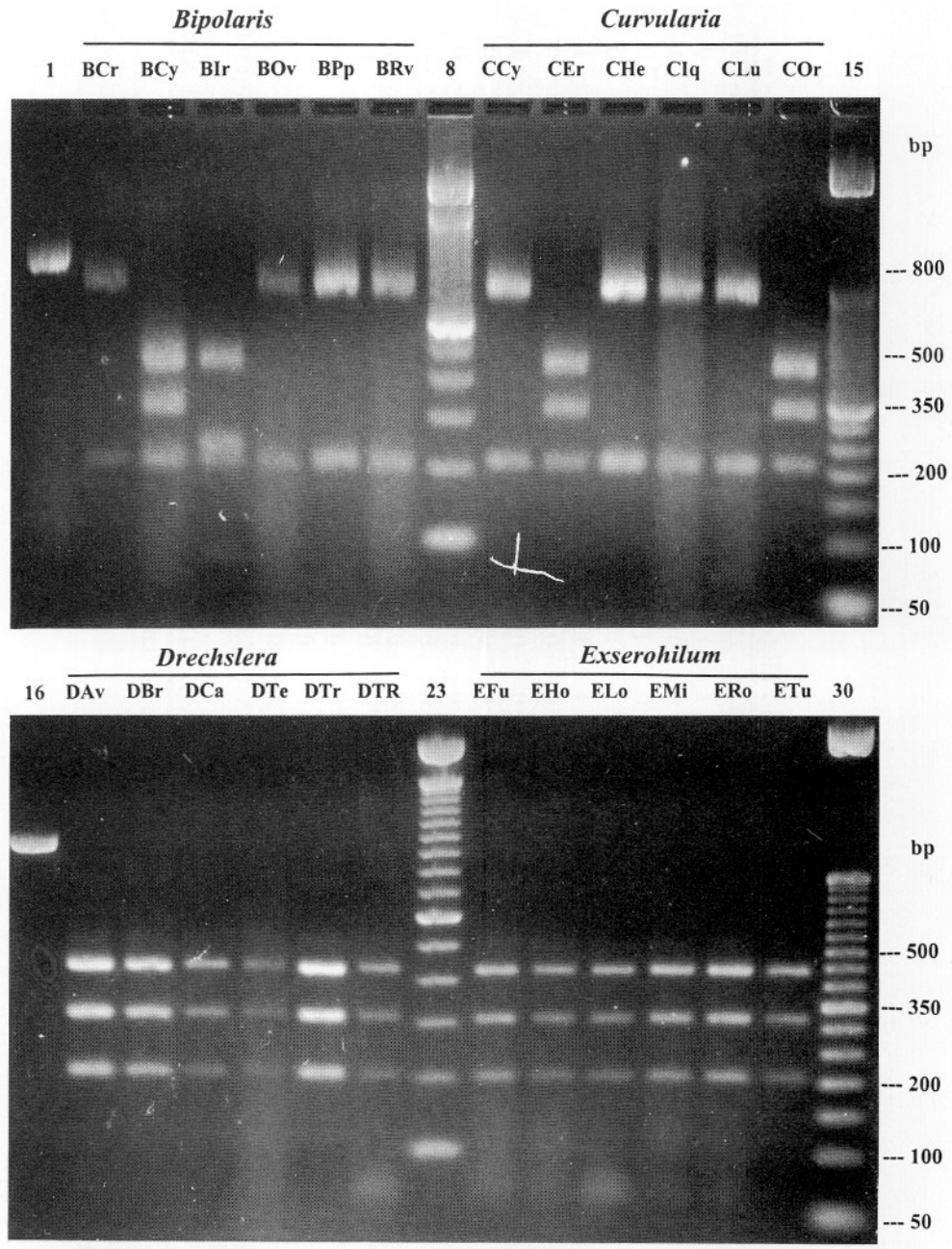

Fig. 2. Restriction fragment patterns obtained from digestion with *Rsa* I of 28S-PCR products amplified from selected isolates of *Bipolaris*, *Curvularia*, *Drechslera* and *Exserohilum*. Lanes 1 and 16 are uncut products of BCr and DAv respectively. Lanes 8 and 23 correspond to 100 bp ladder (GibcoBRL), whereas 15 and 30 correspond to 50 bp ladder (GibcoBRL).

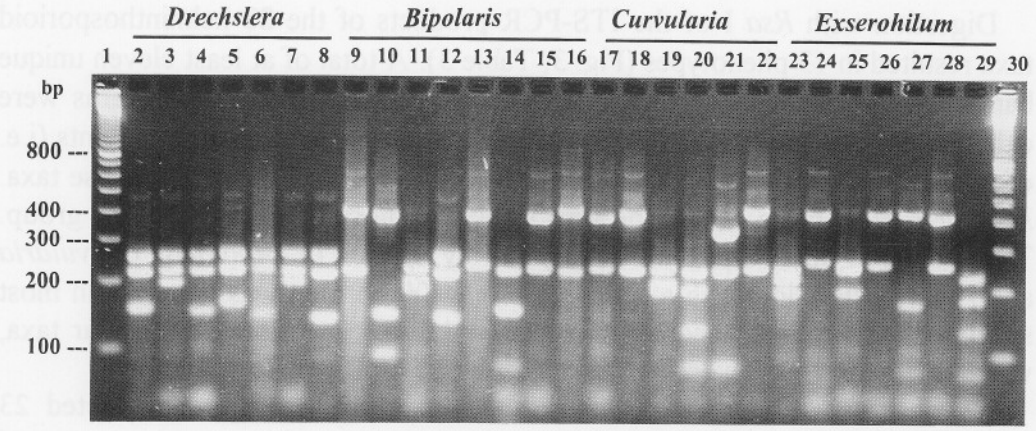

Fig. 3. Restriction patterns of the ITS-PCR products following digestion with *Rsa* I. Lanes 1 and 30 correspond to 100 bp ladder (Pharmacia). Lanes 2-8 correspond to *Drechslera* species, DAv, DBi, DBr, DCa, DTe, DTr and DTR, respectively; Lanes 9-16 correspond to *Bipolaris* species, BCr, BCy, BEl, BIr, BMy, BOv , BPp and BRv, respectively; Lanes 17-22 correspond to *Curvularia* species, CCy, CEr, CHe, CIq, CLu and COr, respectively; lanes 23-29 correspond to *Exserohilum* species, EFu, EGe, EHo, ELo, EMi, ERo and ETu, respectively.

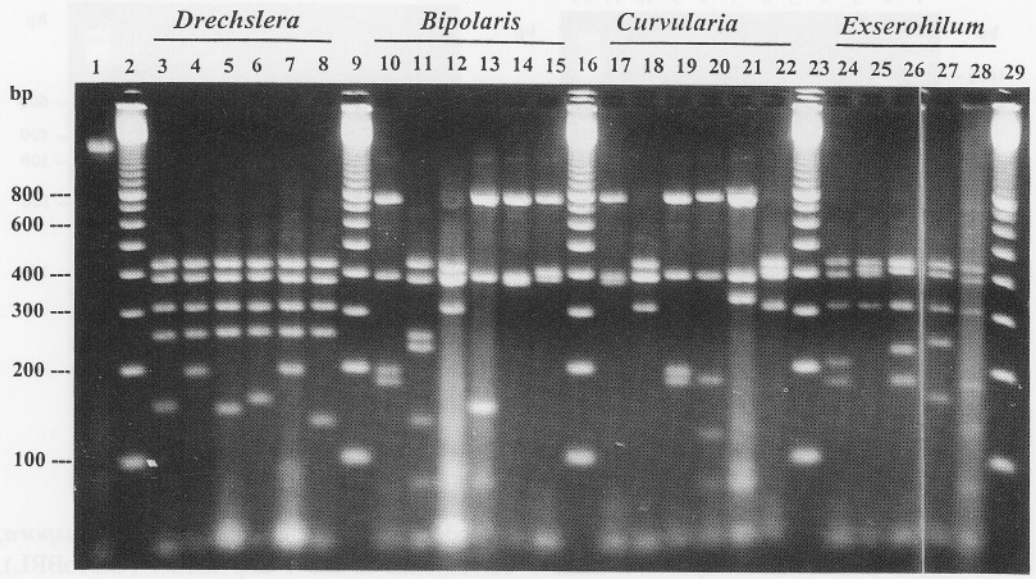

Fig. 4. Restriction patterns of ITS/28S-PCR products following digestion with *Rsa* I. Lane 1 is uncut product of BEl. Lanes 2, 9, 16 and 29 correspond to the molecular weight marker (100 bp ladder, Pharmacia). Lanes 3-8 correspond to *Drechslera* species, DAv, DBi, DBr, DCa, DTr and DTR, respectively; Lanes 10-15 correspond to *Bipolaris* species, BEl, BIr, BMy, BOv, BPp and BRv, respectively; Lanes 17-22 correspond to *Curvularia* species, CCy, CEr, CHe, CIq, CLu and COr, respectively; Lanes 24-28 correspond to *Exserohilum* species, EFu, EGe, EHo, EMi and ETu.

Digestion with *Rsa* I of the ITS-PCR products of the 28 helminthosporioid taxa resulted in 13 phenotypes (Fig. 3; Table 3). A total of at least eleven unique bands were resolved within these taxa. Three distinct restriction patterns were exhibited by the seven *Drechslera* species, in which two common fragments (i.e. of approximately 260 and 210 bp, respectively) were shared amongst these taxa. *Bipolaris iridis* has a phenotype very similar to those of the *Drechslera* group. The other ten phenotypes were exhibited by species of *Bipolaris*, *Curvularia* and *Exserohilum*, in which a fragment of *ca* 400 bp long was common in most species of these three genera. Unique phenotypes were expressed in four taxa, viz. *B. cynodontis*, *B. ovariicola*, *C. lunata*, and *E. fusiforme*.

Digestion with *Rsa* I of the ITS/28S-PCR products of the selected 23 helminthosporioid taxa resulted in fourteen phenotypes (Fig. 4; Table 4). A total of at least 15 unique bands were resolved within these taxa. Again, three distinct patterns were exhibited by the six *Drechslera* species, in which four common fragments (i.e. of approximately 440, 390, 320 and 260 bp, respectively) were shared amongst them. In *Exserohilum* species, four phenotypes were evident,

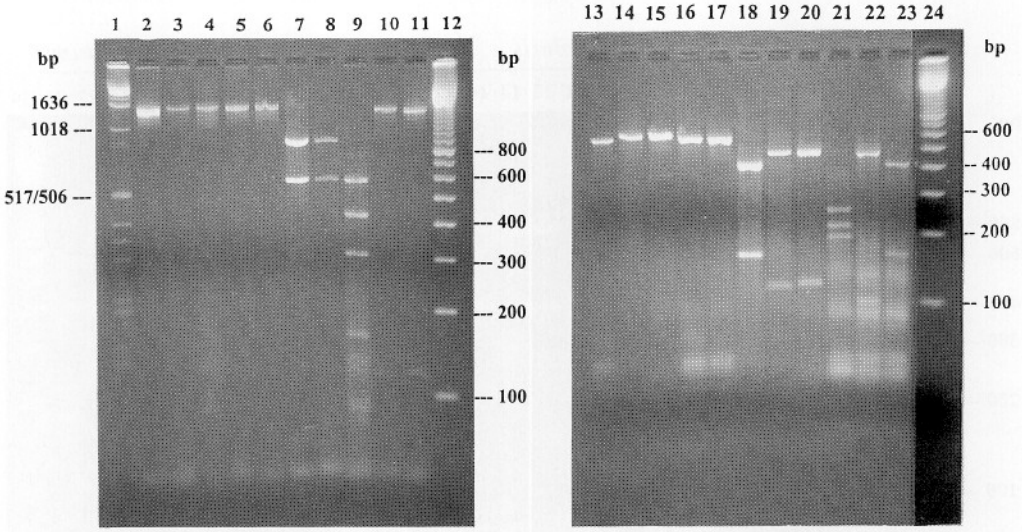

Fig. 5. Restriction analysis with *Rsa* I of PCR products for selected species of *Cercospora*, *Corynespora* and *Helminthosporium*. Lane 1 corresponds to the 1 kbp ladder (GibcoBRL). Lanes 12 and 24 correspond to the 100 bp ladder (Pharmacia). Lanes 2-6 correspond to uncut ITS/28S-PCR products obtained from amplification with primers *ITS5/LR5*, whereas lanes 7-11 correspond to restriction fragment patterns of the products. Lanes: 2, 7 = CEap; 3, 8 = CEcn; 4, 9 = COcs; 5, 10 = COol; 6, 11 = H1. Lanes 13-15 correspond to uncut ITS-PCR products obtained from amplification with primers *ITS5/ITS4*, whereas lanes 16-23 correspond to restriction fragment patterns of the products. Lanes: 13, 16 = CEap; 14, 18 = COcs; 15, 20 = H1; 17 = COcn; 19 = COol; 21 = H2; 22 = H3; 23 = H4.

but three common fragments (i.e. of approximately 460, 400 and 320 bp, respectively) were shared amongst these taxa. Although at least seven different phenotypes could be resolved for species of *Bipolaris* and *Curvularia*, two fragments of *ca* 800 and 400 bp appeared to be common in most of these taxa. In *Bipolaris/Curvularia*, unique phenotypes were expressed in four taxa, viz. *B. iridis*, *B. ovariicola*, *C. inaequalis* and *C. lunata*.

The restriction patterns generated by digestions with *Rsa* I of the ITS-PCR and ITS/28S-PCR products (of *ca* 600 and 1600 bp, respectively) of representative species of *Cercospora*, *Corynespora* and *Helminthosporium* species (*sensu stricto*) (Fig. 5) were distinctly different from those of the four helminthosporioid genera described above (see Figs. 3, 4). None of the *Cercospora* species contained an *Rsa* I restriction site in the region amplified by the primers *ITS5* and *ITS4*, but there was a single site beyond the ITS region, which yielded two fragments (of *ca* 600 and 950 bp, respectively) with *Rsa* I digestion of the ITS/28S product. It was obvious that *Corynespora cassiicola* and *C. olivacea* differed from each other based on *Rsa* I restriction patterns of both their ITS-PCR and ITS/28S-PCR products. When comparing the patterns generated by digestion of the ITS/28S-PCR-product, that of *C. cassiicola* yielded four distinct bands (of *ca* 600, 430, 320 and 170 bp, respectively), whereas that of *C. olivacea* yielded only two bands (of *ca* 1470 and 130 bp, respectively). Digestion of the ITS-PCR products yielded two bands for each of the two *Corynespora* species. These fragments, however, differed in size: those of *C. cassiicola* were *ca* 430 and 170 bp respectively, whereas those in *C. olivacea* were *ca* 470 and 130 bp. When comparing fragments generated by the digestion of the ITS-PCR products in the four *Helminthosporium* species, heterogeneity in phenotypes was observed. *Helminthosporium solani* and two other unnamed *Helminthosporium* species (i.e. H3 and H4) showed similar restriction patterns, each with two distinct bands of comparable sizes, one of *ca* 420-470 bp and the other of *ca* 130-170 bp. It was noted that *H. solani* and *C. olivacea* exhibited the same phenotype, whereas an unnamed *Helminthosporium* (H4) also share a common phenotype with *C. cassiicola*. A unique phenotype was exhibited by *H. velutinum*, which comprised three distinct fragments (of *ca* 260, 220 and 190 bp, respectively).

Cluster analysis

The cluster analysis derived from restriction phenotypes of ITS/28S-PCR products divided the helminthosporioid taxa into two major clusters (Fig. 6). One major cluster comprised the majority of the species in *Bipolaris* and *Curvularia*, whereas the other comprised mainly all species of *Drechslera* and

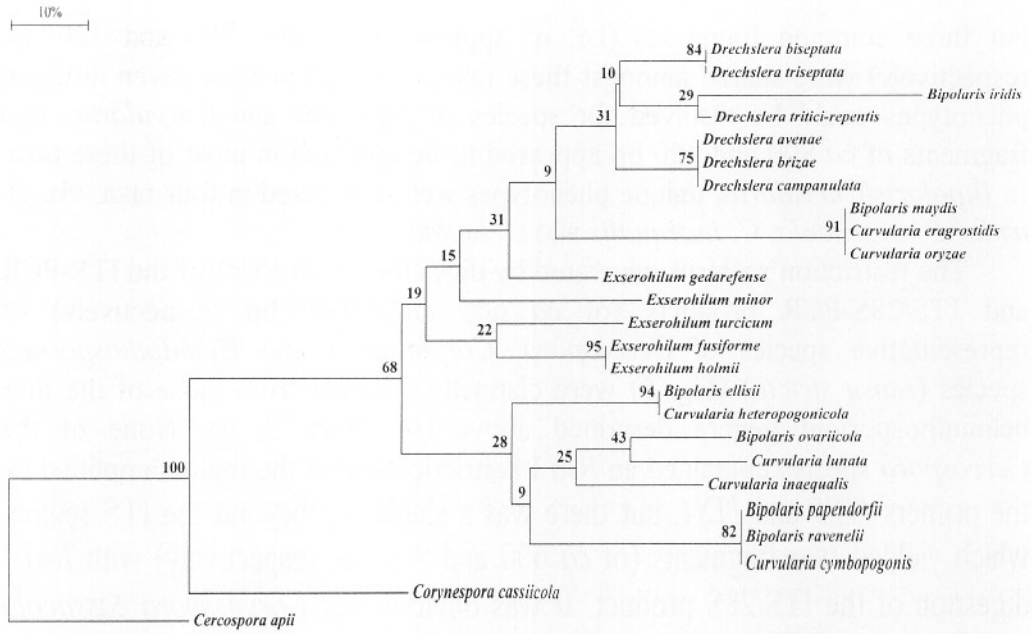

Fig. 6. Neighbour-joining tree showing relationships amongst 23 helminthosporioid taxa, *Cercospora apii* and *Corynespora cassiicola*, based on restriction phenotypes of ITS/28S-PCR products analyzed with UPGMA. Bootstrap values are calculated from 100 replications. Scale bar = percentage dissimilarity.

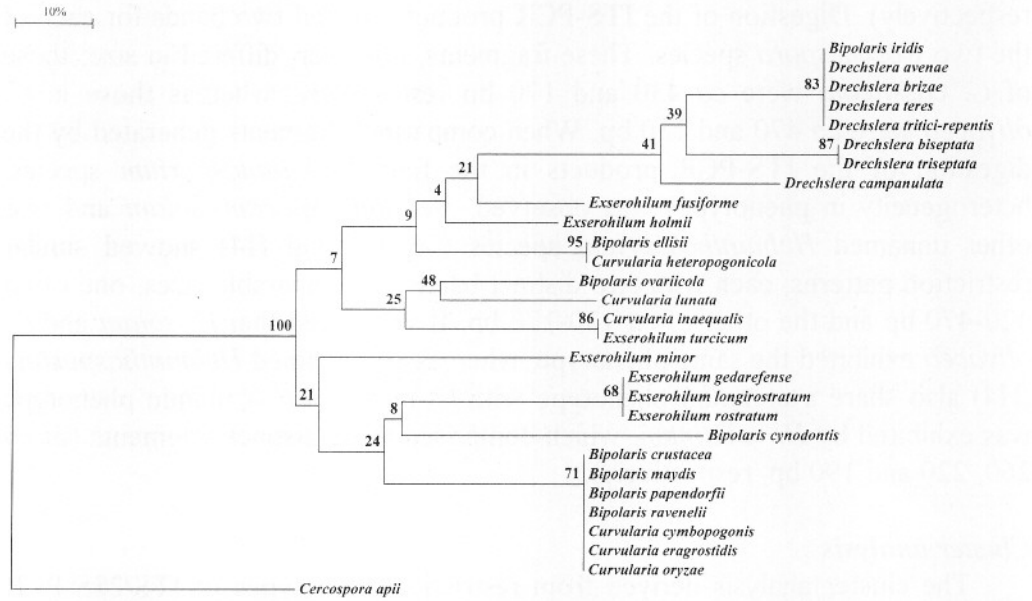

Fig. 7. Neighbour-joining tree showing relationships amongst 28 helminthosporioid taxa and *Cercospora apii*, based on restriction phenotypes of the ITS-PCR products analyzed with UPGMA. Bootstrap values are calculated from 100 replications. Scale bar = percentage dissimilarity.

Exserohilum. The grouping of *Drechslera* species was unequivocal, distinct from groupings of *Exserohilum* species and clearly distant from the major cluster of *Bipolaris /Curvularia*. *Bipolaris iridis* clustered with the *Drechslera* group but appeared distantly from *D. tritici-repentis* (D = 31 %). Three species which shared the same restriction phenotype (Table 4), namely *B. maydis*, *C. eragrostidis* and *C. oryzae*, clustered together, and formed a small subgroup amongst the *Drechslera* group and the groupings of *Exserohilum* species. This 3-membered subgroup was surprisingly distant from the major cluster of the *Bipolaris* and *Curvularia* species (D = 73 %). The *Bipolaria/Curvularia* cluster formed a major branch which was distant from the cluster of *Drechslera/Exserohiulm* species, but was subdivided into several subgroups. Each of these subgroups consists of a mixture of one or two species of *Bipolaris* and *Curvularia*. When restriction phenotypes were analysed together, *Cercospora apii* and *Corynespora cassiicola* were positioned outside the groupings of the helminthosporioid taxa. *Corynespora cassiicola*, however, was closer to the helminthosporioid groups (D = 57 %) than *Cercospora apii* (D = 72 %).

The restriction analysis of the ITS-PCR products (Fig. 7) appeared to be less informative than that of the ITS/28S-PCR products (Fig. 6). On the neighbour-joining tree (dendrogram) obtained by UPGMA based on restriction phenotypes of the ITS-PCR products, *Drechslera* species were grouped together and separated from other helminthosporioid genera. Within this cluster, *D. biseptata* and *D. triseptata* formed a subgroup, whereas *D. campanulata* appeared to be slightly distant from other members of the group. *Bipolaris iridis* was positioned amongst four *Drechslera* species with a bootstrap value of 83. Other than the distinct grouping of *Drechslera* species, the dendrogram based solely on restriction phenotypes of the ITS-PCR products failed to distinguish species of *Bipolaris*, *Curvularia* and *Exserohilum*.

The UPGMA-based cluster analysis on the combination of restriction phenotypes of the 28S-PCR and ITS-PCR products yielded a dendrogram (Fig. 8) which was similar to that derived from the ITS/28S-PCR products alone (see Fig. 6). In this combined form, the dendrogram showed two major groupings of the helminthosporioid taxa, one comprising *Drechslera* and *Exserohilum* species, and the other comprisng the majority of *Bipolaris* and *Curvularia* species. *Bipolaris iridis* in this case, however, did not cluster together with the *Drechslera* species, but was positioned distant from the rest of the helminthosporiod taxa (D = 66 %). *Bipolaris maydis*, *Curvularia eragrostidis* and *C. oryzae* again clustered as a small group which was distant from the major cluster comprising the *Bipolaris* and *Curvularia* species (D = 59 %). This group

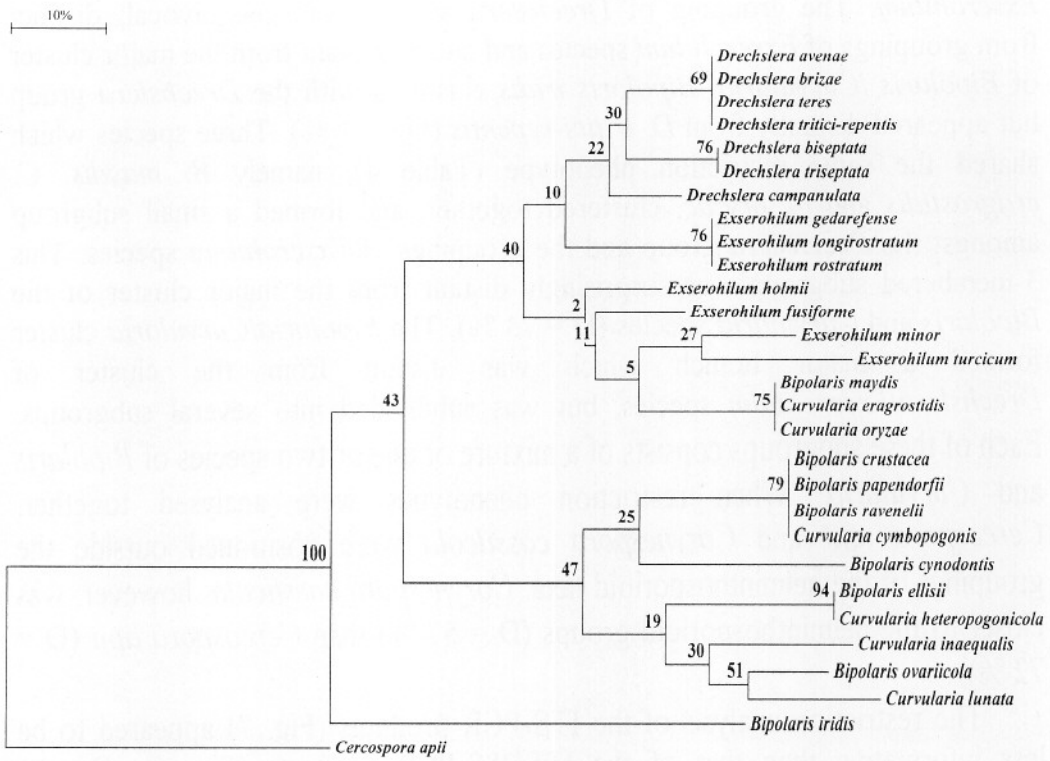

Fig. 8. Neighbour-joining tree showing relationships amongst 28 helminthosporioid taxa and *Cercospora apii*, based on restriction phenotypes of the ITS-PCR as well as the 28S-PCR products, which were analyzed in combined format with UPGMA. Bootstrap values are calculated from 100 replications. Scale bar = percentage dissimilarity.

occurred as a cluster within a clade comprising mainly the *Exserohilum* species. Similarly, within the major cluster of the *Bipolaris* and *Curvularia* species there were subgroups, each of which also contained a mixture of *Bipolaris* and *Curvularia* species.

Discussion

Separation of genera based on restriction patterns and cluster analysis

The fungal ribosomal gene comprises both phylogenically conserved as well as variable regions, and therefore contains useful information in fungal systematics (White *et al.*, 1990). The results presented in this paper indicate that restriction patterns generated by the digestion of PCR-amplified ribosomal DNAs with the endonuclease *Rsa* I are useful in discriminating helminthosporioid taxa from the true *Helminthosporium* species. In addition to using *Rsa* I, other endonucleases, e.g. *Alu* I, *Hae* III, *Mbo* I, *Mse* I, *Msp* I and *Sau3A* I, have also been used in preliminary experiments to digest different PCR-amplified segments of the rDNA. The results using these other

endonucleases were not useful in the discrimination of the helminthosporioid taxa because the PCR poducts were either uncut (perhaps due to the lack of specific restriction sites) or cut, but the restriction patterns were not informative.

The usefulness of *Rsa* I restriction patterns also depended on which PCR-amplified region of the rDNA was analysed. For example, the patterns generated by digestion of the 28S-PCR products basically separated the 28 helminthosporioid taxa into two major groups: one that comprised *Drechslera* and *Exserohilum* species, and the other comprising the majority of *Bipolaris* and *Curvularia* species. The patterns failed to distinguish *Drechslera* from *Exserohilum*, and could not explain why *B. cynodontis*, *C. eragrostidis* and *C. oryzae* exhibited the same phenotype as the *Drechslera/Exserohilum*. *Bipolaris iridis* however, based on its unique restriction phenotype of the 28S-PCR product alone, could be distinguished from other helminthosporioid species.

The restriction patterns derived from *Rsa* I digestion of the ITS-PCR product appeared to separate the *Drechslera* from other genera in the helminthosporioid complex. Heterogeneity in phenotypes of species of *Bipolaris* and *Curvularia* was evident, although most taxa exhibited a similar restriction pattern comprising two bands. Most *Exserohilum* species exhibited restriction phenotypes which were hardly discernible from those of the *Bipolaris/Curvularia* species. Restriction analysis based on *Rsa* I digestion of the ITS-rDNA alone failed to distinguish between the graminicolous helminthosporioid genera with the exception of *Drechslera* species. Nevertheless, the results demonstrated that the *Exserohilum* was more similar to *Bipolaris/Curvularia* than to *Drechslera*.

Restriction phenotypes and cluster analysis of the patterns derived from *Rsa* I digestion of ITS/28S-PCR products (i.e. with primers *ITS5* and *LR5*, 1600 bp) proved to be more meaningful in separating genera in the helminthosporioid complex. The results were equally informative when restriction data of both the 28S-PCR and the ITS-PCR products were pooled together and analysed through UPGMA, rather than analysed separately. Based on restriction patterns of the ITS/28S-PCR products alone, both the genera *Drechslera* and *Exserohilum* exhibited unique restriction phenotypes that were distinguishable from those of *Bipolaris* and *Curvularia*. Such unique phenotypes could be regarded as fingerprints for the taxa. For example, when the ITS/28S-PCR products were digested with *Rsa* I, all *Drechslera* species gave a restriction pattern containing a minimum of the four common fragments (i.e. of *ca* 440, 390, 320 and 260 bp, respectively). Similarly, all *Exserohilum* species yielded a pattern containing three common fragments (i.e. of *ca* 460, 400 and 320 bp, respectively). *Bipolaris* and *Curvularia*, however, appear to be heterogeneous,

and perhaps most of the taxa could be combined in a single genus based on the common phenotype with the two common fragments (i.e. of *ca* 800 and 400 bp, respectively).

When comparing different dendrograms based on restriction phenotypes generated by digestion with *Rsa* I, the results were hard to interpret and sometimes contradictory. This, to a great extent, was due to the fact that the bootstrap values were very low and that all the trees were essentially generated based on very few nucleotide differences. While unambiguous conclusions could not be drawn solely from the present molecular data, conclusions made regarding the major groupings are still valid. Morphological data are indispensable and therefore need to be taken into consideration. It is also worth noting that the dendrograms do not necessarily indicate phylogenetic relationships between taxa.

Taxonomy based on restriction analysis and traditional criteria

Helminthosporium

The restriction patterns of *Helminthosporium* species (*sensu stricto*) generated from *Rsa* I digestion of both the ITS-PCR and ITS/28S-PCR products have been shown to be sufficiently different from those of the graminicolous helminthosporioid taxa. The genus *Helminthosporium sensu stricto* therefore appears to be different from *Bipolaris*, *Curvularia*, *Drechslera* and *Exserohilum*, based on comparison of both morphological and molecular data. The restriction patterns of *H. velutinum*, however, were surprisingly different from those of the other three *Helminthosporium* species used in this study (Fig. 5). It may be that the culture under study was not *H. velutinum*, but that of another fungus. Its identity could not be verified because the fungus failed to sporulate.

Cercospora and *Corynespora*

In the cluster analysis, *Cercospora apii* and *Corynespora cassiicola* were excluded from the clusters of the helminthosporioid taxa, with a D value of over 57 %. *Cercospora* was at a position more distant from the helminthosporioid group than was *Corynespora*. The results supplement the current generic separation of the two genera with comparison to the helminthosporioid taxa (Ellis, 1971, 1976). *Cercospora* species (*sensu stricto*) are similar to the helminthosporioid taxa in having dematiaceous, geniculate conidiophores bearing sympodially proliferating, polyblastic conidiogenous cells with distinctly thickened conidial scars, but differ in their hyaline, acicular, euseptate conidia (Hsieh and Goh, 1990). *Corynespora* species resemble the helminthosporioid

taxa in having dematiaceous conidiophores with conspicuous conidial scars and pseudoseptate conidia, but differ in their monotretic, percurrently proliferating, cylindrical or doliiform conidiogenous cells (Ellis, 1957; Luttrell, 1963).

Drechslera

When comparing the *Rsa* I restriction phenotypes, *Drechslera* species exhibited the most pronounced dissimilarity from the other three helminthosporioid genera. The history of *Drechslera* has been reviewed by Alcorn (1983b) and Sivanesan (1987). Before the generic concepts of *Bipolaris* and *Exserohilum* were established, many taxa have been included in the genus *Drechslera* (see synonyms listed in Sivanesan, 1987). As currently circumscribed, *Drechslera* species are characterized as having cylindrical (not curved) conidia which germinate from every cell, and a teleomorph in the genus *Pyrenophora* (Luttrell, 1977). During septum ontogeny in conidia of *Drechslera*, the first formed septum is basal, followed by median and finally distal septation (Alcorn, 1988). The combination of these characteristics is significant in differentiating *Drechslera* from *Exserohilum*, *Bipolaris* and *Curvularia* which have comparable criteria (Alcorn, 1983b; Sivanesan, 1987). The results of restriction analysis in this paper further support generic distinction of *Drechslera*. Interspecific differences were noted in *Drechslera* species. For example, in neighbor-joining trees generated with *Rsa* I digestion, *D. biseptata* and *D. triseptata* were always clustered together as a separate subgroup (Figs. 6-8). This agrees with morphological observations of the two species. *Drechslera biseptata* is morphologically somewhat anomalous in having small conidia resembling a straight-spored *Curvularia* (Sivanesan, 1987). *Drechslera triseptata*, however, is somewhat atypical in having 2-3-septate conidia with a minute, often slightly protruding (papillate) hilum, and germinating only from the basal cell (Shoemaker, 1959).

Bipolaris iridis clustered together with *Drechslera* species based on the restriction phenotype generated by digestion of the ITS-PCR-product (Fig. 6). In another cluster analysis based on combined restriction phenotypes of the ITS-PCR and 28S-PCR products (Fig. 8), it occurred distant from the four graminicolous helminthosporioid taxa. *Bipolaris iridis* is similar to other *Bipolaris* species because of the bipolar germination of its conidia. It has been placed in the genus *Drechslera* probably because of its cylindrical conidia (Ellis, 1971). This species, however, has been considered atypical of *Bipolaris* in septum ontogeny of its conidia (Alcorn, 1983b). Moreover, the hilum structure of the conidia is sometimes not unequivocal of the *Bipolaris* type (Alcorn, 1983b). In addition the species is not graminicolous (Ellis, 1971). The present

cluster analysis indicates that *B. iridis* represents a dubious taxon in the helminthosporioid group and probably belongs to a separate genus other than *Drechslera* or *Bipolaris*.

Exserohilum

The genus *Exserohilum* as currently circumscribed comprises species in which the conidial hilum is strongly protuberant (Alcorn, 1988), and has a connection with the ascomycete *Setosphaeria* (Luttrell, 1977; Alcorn, 1978; Sivanesan, 1987). The results of the present restriction analysis based on *Rsa* I digestion of the ITS/28S-PCR product also support generic distinction of *Exserohilum*. Cluster analysis of species based on restriction patterns of this amplified rDNA segment proved to be reasonable. *Exserohilum fusiforme* and *E. holmii* are regarded typical of the genus (Alcorn, 1991) and they were grouped together as a cluster closer to the type species *E. turcicum* (Fig. 6). *Exserohilum rostratum* and *E. longirostratum* have conidia with a rostrate apex. Their identities of being distinct species have been questioned by Alcorn (pers. comm.), because the conidial length in *E. rostratum* has been found to be extremely variable (Leonard, 1976). It is uncertain that the conidia of these two *Exserohilum* species differ in shape or just part of a continuum with respect to conidial length. The results of the present restriction analysis indicated that these two *Exserohilum* could be identical (Figs. 3, 7, 8), but it is not conclusive. Further investigation comparing their DNA sequences in future work might resolve this taxonomic problem.

Curvularia heteropogonicola was previously accommodated in *Exserohilum* based on its conidia having a protruding hilum (Sivanesan, 1987). Alcorn (1991) noted that the protrusion of its hilum is not typical of the type exhibited by the true *Exserohilum* species. The present cluster analysis based on *Rsa* I digestion of the ITS/28S-PCR product gives support to this relocation because *C. heteropogonicola* did not cluster with *Exserohilum* species, but did cluster with the main *Bipolaris/Curvularia* group (Fig. 6).

Bipolaris* and *Curvularia

The results of present study demonstrate that species in *Bipolaris* and *Curvularia* are heterogeneous when considering restriction phenotypes. Many of the species from both genera shared a similar restriction phenotype and thus they grouped together in cluster analysis. The restriction patterns could not distinguish *Bipolaris* from *Curvularia*. The grouping of *B. maydis*, *C. eragrostidis* and *C. oryzae* in a cluster distant from the major *Bipolaris/Curvularia* cluster was puzzling. The fact that *Bipolaris maydis*, the

type species of the genus, which has fusoid, slightly curved conidia and a known *Cochliobolus* state, clustered with two *Curvularia* species in which conidia are straight and with paler end cells, is unexplainable and needs further investigation.

The separation of *Bipolaris* and *Curvularia* as distinct genera has long been controversial (see Sivanesan, 1987; Alcorn, 1988). *Bipolaris* and *Curvularia* species are identical in their morphological characters and separation is only based on conidial morphology. These include size of conidia, number of septa in conidia, accentuation of septa in conidia, whether conidia are straight or curved, whether the conidial hilum is flush or protrudes, and presence or absence of a disproportionately swollen cell in the conidium. In fact classification based on these characters is sometimes artificial. Many species in *Bipolaris* and *Curvularia* have conidia that are morphologically intermediate between the two genera. Most *Curvularia* species have curved conidia that are 3- or 4-septate, and the septa are often accentuated by a dark band. In some *Bipolaris* species, e.g. *B. ellisii* and *B. papendorfii*, conidia are slightly curved and have small numbers of septa, and thus are *Curvularia*-like. Sometimes conidial septa in *Bipolaris* species are accentuated, e.g. *B. crustacea* and *B. ravenelii*, but a swollen, thicker-walled, more pigmented penultimate cell is absent in these conidia. Conversely, *Curvularia* species, e.g. *C. inaequalis*, may have straight conidia which lack a conspicuous swollen penultimate cell, and thus are *Bipolaris*-like. A more reliable criterion of generic separation would probably be anamorph-teleomorph connections (Luttrell, 1979). *Cochliobolus* teleomorphs are common to both *Bipolaris* and *Curvularia* anamorphs (Luttrell, 1977). Another ascomycete genus, *Pseudocochliobolus* Tsuda, Ueyama and Nishih., has been connected with both *Bipolaris* and *Curvularia* species (Tsuda and Ueyama, 1981, 1983), but this genus was regarded as synonymous with *Cochliobolus* (Alcorn, 1983a; Sivanesan, 1984). It is evident that from the many overlapping characters found in these two large groups of hyphomycetes that they are probably congeneric, and the results of restriction analysis gives further support to their synonymy. If the two genera were to be combined, *Bipolaris* should be treated as a synonym of *Curvularia* with regard to priority in nomenclature. Further investigation comparing the DNA sequences of these and other representative species of *Bipolaris* and *Curvularia* in future work might help to resolve natural relationships in these fungi.

Acknowledgements

We would like to thank Dr. J.L. Alcorn of DPI, Queensland, Australia, for supplying the cultures of fungi used in the present study. We are also grateful to Dr. Alcorn for his precious input into the taxonomic interpretation of the taxa studied in this paper. Dr. E.C.Y. Liew is thanked for his valuable comments on the manuscript. The University of Hong Kong is

thanked for the award of a postdoctoral fellowship to Dr. T.K. Goh. Thanks are extended to Dr. J.C. Kang, Mr. C.Y. Cheng, Mr. A.Y.P. Lee, Ms. Helen Leung, Ms. Sarah Leung, Ms. Angela Lo, and Mr. John H.K. Lum for technical and photographic assistance.

References

Alcorn, J.L. (1978). *Setosphaeria monoceras* sp. nov., ascigerous state of *Exserohilum monoceras*. Mycotaxon 7: 411-414.

Alcorn, J.L. (1983a). On the genera *Cochliobolus* and *Pseudocochliobolus*. Mycotaxon 16: 353-379.

Alcorn, J.L. (1983b). Generic concepts in *Drechslera*, *Bipolaris* and *Exserohilum*. Mycotaxon 17: 1-86.

Alcorn, J.L. (1988). The taxonomy of *Helminthosporium* species. Annual Review of Phytopathology 26: 37-56.

Alcorn, J.L. (1991). New combinations and synonymy in *Bipolaris* and *Curvularia*, and a new species of *Exserohilum*. Mycotaxon 41: 329-343.

Bruns, T.D., White, T.J. and Taylor, J.W. (1991). Fungal molecular systematics. Annual Review of Systematics 22: 525-564.

Drechsler, C. (1923). Some graminicolous species of *Helminthosporium*. I. Journal of Agricultural Research 24: 641-739.

Ellis, M.B. (1957). Some species of *Corynespora*. Mycological Papers 65: 1-15.

Ellis, M.B. (1971). Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, Kew, England.

Ellis, M.B. (1976). More Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, Kew, England.

Hsieh, W.H. and Goh, T.K. (1990). *Cercospora* and similar fungi from Taiwan. Maw Chang Book Company, R.O.C.

Lee, S.B. and Taylor, J.W. (1990). Isolation of DNA from fungal mycelia and single spores. In: *PCR Protocols: A Guide to Methods and Applications* (eds. M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White). Academic Press, Inc.: 282-287.

Leonard, K.J. (1976). Synonymy of *Exserohilum halodes* with *E. rostratum*, and induction of the ascigerous state, *Setosphaeria rostrata*. Mycologia 68: 402-411.

Luttrell, E.S. (1963). Taxonomic criteria in *Helminthosporium*. Mycologia 55: 643-674.

Luttrell, E.S. (1964). Systematics of *Helminthosporium* and related genera. Mycologia 56: 119-132.

Luttrell, E.S. (1977). Correlations between conidial and ascigerous state characters in *Pyrenophora*, *Cochliobolus* and *Setosphaeria*. Review in Mycology 41: 271-279.

Luttrell, E.S. (1979). Deuteromycetes and their relationships. In: *The Whole Fungus. Vol. 1* (ed. B.W. Kendrick). National Museum of Natural Sciences, Ottawa, Canada: 241-264.

Misra, A.P. (1973). *Helminthosporium* species occurring on cereals and other Gramineae. Tirhut College of Agriculture (Dholi), The Catholic Press, Ranchi, India.

Nei, M. and Li, W.H. (1979). Mathematical models for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academia of Science USA 76: 5269-5273.

Nicholson, M.S., Bunyard, B.A. and Royse, D.J. (1997). Phylogeny of the genus *Lentinula* based on ribosomal DNA restriction fragment length polymorphism analysis. Mycologia 89: 400-407.

Persson, Y., Erland, S. and Jansson, H. (1996). Identification of nematode-trapping fungi using RFLP analysis of the PCR-amplified ITS region of ribosomal DNA. Mycological Research 100: 531-534.

Shoemaker, R.A. (1959). Nomenclature of *Drechslera* and *Bipolaris*, grass parasites segregated from '*Helminthosporium*'. Canadian Journal of Botany 37: 879-887.

Sivanesan, A. (1984). The Bitunicate Ascomycetes and their Anamorphs. J. Cramer, Vaduz, Liechtenstein.

Sivanesan, A. (1987). Graminicolous species of *Bipolaris*, *Curvularia*, *Drechslera*, *Exserohilum* and their teleomorphs. Mycological Papers 158: 1-261.

Tsuda, M. and Ueyama, A. (1981). *Pseudocochliobolus australiensis*, the ascigerous state of *Bipolaris australiensis*. Mycologia 73: 88-96.

Tsuda, M. and Ueyama, A. (1983). *Pseudocochliobolus pallescens* and variability of conidium morphology. Memoirs of the College of Agriculture, Kyoto University 122: 85-91.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990). Amplification and direct sequencing of the fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications* (eds. M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White). Academic Press, Inc.: 315-322.

Van de Peer, Y. and De Wachter, R. (1997). Construction of evolutionary distance trees with TREECON for Windows: accounting for variation in nucleotide substitution rate among sites. Computer Application in Bioscience 13: 227-230.