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Comparative study of morphological, cultural and molecular markers for the characterization of *Pseudocercosporella herpotrichoides* isolates

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Abstract

Nirenberg's classification system and the polymerase chain reaction (PCR) combined with restriction enzyme digestion of an amplified ribosomal DNA fragment, were compared for the characterization of sixty isolates of *Pseudocercosporella herpotrichoides*, from various geographical areas and with differing fungicide sensitivity. With Nirenberg's system, it was possible to identify most isolates as *P. herpotrichoides* var. *herpotrichoides* or *P. herpotrichoides* var. *acuformis*. However, identification was slow and sometimes inconclusive as overlap occurred between the two varieties for all criteria examined. Molecular markers identified two distinct types among the isolates tested and generally good correlation was found between the PCR-based assay and Nirenberg's system, but the molecular assay was more accurate and faster.

Introduction

Isolates of *Pseudocercosporella herpotrichoides* (Fron) Deighton, the cause of eyespot in cereal crops, vary considerably; two main types are generally distinguished. Nirenberg [1981] classified German isolates of the fungus into two varieties based on conidial and cultural characteristics: *P. herpotrichoides* var. *herpotrichoides*, with either curved or curved and straight conidia, fast-growing, even-edged colonies and squirrel grey or olive grey mycelia, and *P. herpotrichoides* var. *acuformis*, with only straight conidia, slow-growing, feathery or uneven-edged colonies and grey to brown grey mycelia. In the U.K., two main pathotypes called W-type and R-type are distinguished using cultural characteristics [Lange-de la Camp, 1966a, b; Scott et al., 1975; Hollins et al., 1985]. Originally these two types were also distinguished by their pathogenicity to wheat and rye [Lange-de la Camp, 1966a, b; Scott et al., 1975; Scott and Hollins, 1980], but pathogenicity varies within and between both types on both hosts [Mauler and Fehrmann, 1987; Creighton et al., 1989]. Moreover, although it has been reported [Hollins et al., 1985], pathogenicity is not always correlated with cultural characteristics [Nicholson et al., 1993]. King and Griffin [1985] and Sanders et al. [1986] suggested that W-type corresponded to *P. herpotrichoides* var. *herpotrichoides* and R-type to *P. herpotrichoides* var. *acuformis*, but recent studies showed that the two systems were not completely equivalent [Mauler and Fehrmann, 1987; Nicholson et al., 1991, 1993]. In France, criteria used to characterize isolates of *P. herpotrichoides* are daily growth rate on potato dextrose agar (PDA) at 20° C, and form of colony margin. N-type isolates have a daily growth rate greater than 2 mm and an even margin, whereas L-type isolates have a daily growth rate less than 1.5 mm and an irregular or feathery margin [Leroux and Gredt, 1985; Cavelier et al., 1987]. These criteria are not always reliable because fast-growing isolates with an irregular or feathery margin and slow-growing isolates with an even margin have been observed [Hollins *et al.*, 1985; Maraite et al., 1985; Gallimore et al., 1987; Creighton, 1989]. To simplify the classification of atypical isolates and homogenize the different nomenclatures, a more reliable and rapid method of identification is

needed. As W- and R-types differ epidemiologically [Goulds and Fitt, 1988] and in sensitivity to fungicides [King and Griffin, 1985; Leroux and Gredt, 1988], such a method would also be of great importance for epidemiological studies and disease-monitoring programmes.

Molecular markers have been reported to give clear discrimination of W- and R-type isolates [Julian and Lucas, 1990; Nicholson et al., 1991; Priestley et al., 1992; Thomas et al., 1992; Frei and Wenzel, 1993; Nicholson et al., 1993; Nicholson and Rezanoor, 1994], but the techniques used were labour-intensive (analysis of isozyme polymorphism and detection of DNA restriction fragment length polymorphisms by Southern blot hybridization) or suffered from lack of reproducibility of the patterns (random amplification of polymorphic DNA (RAPD)). A rapid and reproducible method is the amplification of DNA sequences specific to each type of the fungus using the polymerase chain reaction (PCR). Sequencing the internal transcribed spacer (ITS) region of the ribosomal RNA genes (rDNA) of N- and L-type isolates, Gac [1991] and Poupard et al. [1993] revealed sequences characteristic of each fungus type. Cluster of nucleotide differences in ITS1 permitted the synthesis of oligonucleotide primers that hybridized differentially with DNA of N- and L-types, thus allowing clear identification of isolates [Poupard et al., 1993]. However, the specificity of the primers was tested on a limited number of isolates (four of each type) all originating from France. Moreover, the PCR test required different amplification conditions according to the primer set used. A simplified procedure would allow the rapid analysis of large numbers of isolates or plant samples.

In this paper, Nirenberg's [1981] classification system and a PCR test combined with restriction enzyme digestion of an amplified rDNA fragment were compared for the characterization of *P. herpotrichoides* isolates. For this purpose, a large number of isolates of various geographical origins and with differing fungicide sensitivity were examined. The study included the development of a PCR procedure which permitted the differentiation of the two fungus types using the same amplification conditions.

Materials and methods

Fungal isolates

Sixty isolates of *P. herpotrichoides* were used (Table 1). Thirty-eight were from single-spore cultures. Isolates were previously classified as N-type/L-type, W-type/R-type, var. *herpotrichoides*/var. *acuformis* and fast-growing type/slow-growing type depending on origin. Two groups were discernible: group 1, N-/W-/*herpotrichoides* and fast-growing types (thirty isolates); group 2, L-/R-/*acuformis* and slow-growing types (thirty isolates). All isolates were subcultured on 3.9% (w/v) PDA (Difco) to assess morphological and cultural characteristics. The sensitivity of the isolates to methyl benzimidazole carbamate (MBC) fungicides and inhibitors of sterol biosynthesis (triazole and imidazole fungicides) was determined as described by Leroux and Gredt [1988] and Leroux and Marchegay [1991].

Two isolates of *Pseudocercosporella anguioides* Nirenberg [Nirenberg, 1981] (a fungus related to *P. herpotrichoides* but less damaging on cereals) were used to check the specificity of the PCR test (Table 2). The other pathogens tested were *Fusarium* spp., *Microdochium nivale* var. *nivale* and *M. nivale* var. *majus* (responsible for *Fusarium* foot rots), *Rhizoctonia cerealis* (responsible for sharp eyespot) and *Gaeumannomyces graminis* var. *tritici* (responsible for take-all).

Morphological and cultural characteristics of isolates

Colony growth and morphology. Petri dishes containing PDA were inoculated centrally with 4-mm-diameter disks cut from the margin of colonies growing actively on PDA. There were three replicates for each isolate. After incubation in the dark at 20°C for 2 weeks, growth of colonies was measured and daily growth rate determined. Colour of colonies was described. Morphology of margin and elevation of hump were assessed using two arbitrary scales: feathery margin, irregular margin, even margin; pronounced hump, slight hump, no hump.

Spore shape. Colonies were made to sporulate [Cavelier and Le Page, 1985] and spore shape was determined according to Nirenberg [1981].

DNA extraction

Conical flasks containing liquid medium [Belkhiri and Dick, 1988] were inoculated with mycelial plugs taken from the margin of colonies. Cultures were incubated at 20°C on an orbital shaker in darkness for 3–4 weeks. Total DNA from freeze-dried mycelia was extracted as described by Lee et al. [1988].

Primer selection

Primers spN 5′-TCATTAATAGAGCAATGAAC-3′ and spL 5′-TCATTAATAGAGCAATGGAT-3′ were designed from the ITS1 sequences of N- and L-type isolates determined by Gac [1991] and Poupard et al. [1993]. The non-specific primer ITS4 was described by White et al. [1990] (Figure 1). Using primers spN or spL paired with primer ITS4, we expected to specifically amplify DNA from each type of the fungus under identical PCR conditions. Indeed, primers spN and spL have the same melting temperature as a result of their similar A + T and G + C contents. Oligonucleotide synthesis was carried out by Bioprobe Systems.

DNA amplification

Amplification reactions were performed under the standard conditions given by Perkin-Elmer Cetus using the Perkin-Elmer Cetus thermal cycler 480. Cycling parameters were an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 60 s, annealing at 49°C for 30 s, and extension at 72°C for 45 s, with a final extension at 72°C for 10 min. To achieve the specific amplification of each type of the fungus, a series of PCR experiments was conducted by raising the annealing temperature. Negative controls (reactions without added template DNA) were included in each experiment. After amplification, the PCR products were checked by electrophoresis in 1.5% agarose gels.

Restriction enzyme digestion of the amplified products

Ava II and *Stu* I sites specific to each type of the fungus are located in the ITS1 region of the ribosomal genes [Gac, 1991] (Figure 1). To check the identity of the amplified fragments, PCR products were digested with the appropriate restriction enzymes following the manufacturer's instructions (Boehringer Mannheim GmbH). Digested DNA fragments were separated by electrophoresis in 4% agarose gels (agarose Biogel, Bio 101).

Results

Characterization of isolates on the basis of morphological and cultural criteria

Morphological and cultural characteristics of the sixty isolates of *P. herpotrichoides* are listed in Table 3. A synthesis of the results for each of the group previously defined is presented in Table 4. Most group 1 isolates had characteristics typical of *P. herpotrichoides* var. *herpotrichoides* [Nirenberg, 1981], i.e. fast-growing colonies with even margins and a hump, squirrel grey or olive grey mycelia and curved or curved and straight conidia. However, isolates varied greatly in mycelial colour with only half having the typical squirrel grey or olive grey colours (Tables 3 and 4). The mean daily growth rate of group 1 isolates was greater than 2 mm (Table 4). This agrees with Leroux and Gredt [1985] and Cavelier et al. [1987] for N-type. Nevertheless, six isolates (190, 328 and 489 from France; PHH5 and PHH12 from Germany and PH90.153.1 from the U.S.A.) had a daily growth rate less than 2 mm, and one isolate (487 from France) grew at a rate characteristic of L-type, i.e. less than 1.5 mm day^{-1} [Leroux and Gredt, 1985; Cavelier et al., 1987] (Table 3). Thus, isolate 487 had a growth rate that did not correlate with its colony morphology and spore shape, whereas isolate BY151 from the U.K. had a conidial morphology (only straight conidia) which did not correspond to its colony morphology and growth rate. However, the thirty group 1 isolates can be classified as *P. herpotrichoides* var. *herpotrichoides* because they possess characteristics closer to var. *herpotrichoides* than var. *acuformis*.

Only eleven group 2 isolates had characteristics typical of *P. herpotrichoides* var. *acuformis* [Nirenberg, 1981], i.e. slow-growing colonies with irregular or feathery margins, grey to brown grey mycelia and straight conidia. Much variation in morphology of margin, elevation of hump, colour of colony and growth rate was observed in this group, but all isolates produced straight conidia (Tables 3 and 4). The mean daily growth rate of group 2 isolates was less than 1.5 mm (Table 4). This agrees with Leroux and Gredt [1985] and Cavelier et al. [1987] for L-type. However, two isolates (183 from France and PHA52 from Germany) had a daily growth rate greater than 1.5 mm, although they formed feathery or uneven-edged colonies and straight conidia (Table 3). Three isolates (247, 300 and 399 from France) exhibited characteristics typical of both varieties, i.e. even-edged colonies

Table 1. Characteristics of *P. herpotrichoides* isolates: code, origin, host, year of isolation, sensitivity *in vitro* to fungicides

Code	Origin	Host	Year of isolation	Sensitivity to fungicides MBC[a]	triazoles[b]	imidazoles[c]
Group 1						
	France (N-type)					
99	Somme	Wheat	1986	R	S	
117	Ille et Vilaine	Wheat	1987	S	S	
118	Ille et Vilaine	Wheat	1987	R	S	
147	Oise	Wheat	1987	S	S	
148	Oise	Wheat	1987	S	R	
159*	Loiret	Wheat	1988	R	S	
165	Marne	Wheat	1988	R	R	
168	Côte d'Or	Wheat	1988	R	R	
171	Marne	Wheat	1988	S	R	
189	Marne	Wheat	1988	S	S	
190*	Marne	Wheat	1988	R	S	
193*	Marne	Wheat	1988	R	R	
228*	Ille et Vilaine	Wheat	1989	S	R	
233*	Indre et Loire	Wheat	1989	R	S	
251*	Somme	Wheat	1990	R	R	
253*	Somme	Wheat	1990	R	S	S
256*	Somme	Wheat	1990	R	S	S
328*	Vienne	Wheat	1990	R	R	S
487*	Calvados	Wheat	1992	R	R	R
489*	Calvados	Wheat	1992	R	R	R
	U.K. (W-type)					
BK51	Rothamsted	Wheat	1983	S	S	
BK58	Rothamsted	Wheat	1983	S	S	
BY151	Rothamsted	Wheat	1983	R	S	
15.2	Suffolk	Wheat	1984	S	S	
15.4	Kilham	Wheat	1984	R	S	
	Germany (var. *herpotrichoides*)					
PHHB	Grünbach	Wheat	1987	S	S	
PHH5	Grünbach	Wheat	1988	S	S	
PHH12	Grünbach	Wheat	1988	S	S	
	U.S.A. (fast-growing type)					
PH90.70.1	North-west	Wheat	1990	S	S	
PH90.153.1	North-west	Wheat	1990	S	S	

[a] Sensitivity to carbendazim at 2 μg ml^{-1},
[b] Sensitivity to triadimenol at 30 μg ml^{-1},
[c] Sensitivity to prochloraz at 0.5 μg ml^{-1} and at 2 μg ml^{-1}; S = sensitive, R = resistant.
[d] Isolate 386 grew normally on PDA amended with prochloraz at 0.5 μg ml^{-1}, but it was not tested on PDA amended with prochloraz at 2 μg ml^{-1}.
* Isolate not derived from a single spore.

Table 1. Continued

Code	Origin	Host	Year of isolation	Sensitivity to fungicides MBC[a]	triazoles[b]	imidazoles[c]
Group 2						
	France (L-type)					
97	Somme	Wheat	1986	R	R	
98	Somme	Wheat	1986	R	R	
107	Somme	Wheat	1986	R	R	
108	Somme	Wheat	1986	R	R	
115*	Ille et Vilaine	Wheat	1987	S	R	
125	Ille et Vilaine	Wheat	1987	R	R	
135*	Somme	Wheat	1987	R	R	
141	Somme	Wheat	1987	R	R	
172	Marne	Wheat	1988	R	R	
181	Marne	Wheat	1988	R	R	
183	Pas de Calais	Barley	1988	S	R	
210*	Loir et Cher	Wheat	1989	R	R	
247*	Somme	Wheat	1990	R	R	
293*	Côtes d'Armor	Wheat	1990	S	R	
297*	Côtes d'Armor	Wheat	1990	S	R	
300*	Côtes d'Armor	Wheat	1990	S	R	
304*	Côtes d'Armor	Wheat	1990	S	R	
325*	Vienne	Wheat	1990	R	R	S
386*	Oise	Wheat	1991	R	R	R[d]
399*	Marne	Wheat	1992	R	R	R
	U.K. (R-type)					
M4	Rothamsted	Wheat	1984	S	R	
M9	Rothamsted	Wheat	1984	S	R	
11.2	Kilham	Wheat	1984	S	R	
15.7	Suffolk	Wheat	1984	R	R	
LP11	Bristol	Wheat	1985	R	R	
	Germany (var. *acuformis*)					
PHA52	Grünbach	Wheat	1988	S	R	
PHAIII	Bad Schönborn	Rye	1988	S	R	
PHA3/10	Stuttgart	Rye	1988	S	R	
	U.S.A. (slow-growing type)					
PH90.88.5	North-west	Wheat	1990	S	R	
PH90.90.7	North-west	Wheat	1990	S	R	

Table 2. Characteristics of the other cereal stem-base pathogenic fungi: code, origin, host, year of isolation

	Code	Origin	Host	Year of isolation
Pseudocercosporella anguioides	863020	Germany	–	–
	64003	Germany	–	–
Fusarium culmorum	F68	France	Wheat	1989
	F72	France	Wheat	1989
Fusarium graminearum	F67	France	Wheat	1988
	F99	Poland	Wheat	1988
Fusarium avenaceum	F52	Poland	Wheat	–
Microdochium nivale var. *nivale*	FN14	Germany	–	–
Microdochium nivale var. *majus*	FN15	Germany	–	–
Rhizoctonia cerealis	–	France	Wheat	1983
Gaeumannomyces graminis var. *tritici*	GGT885	France	Wheat	1991

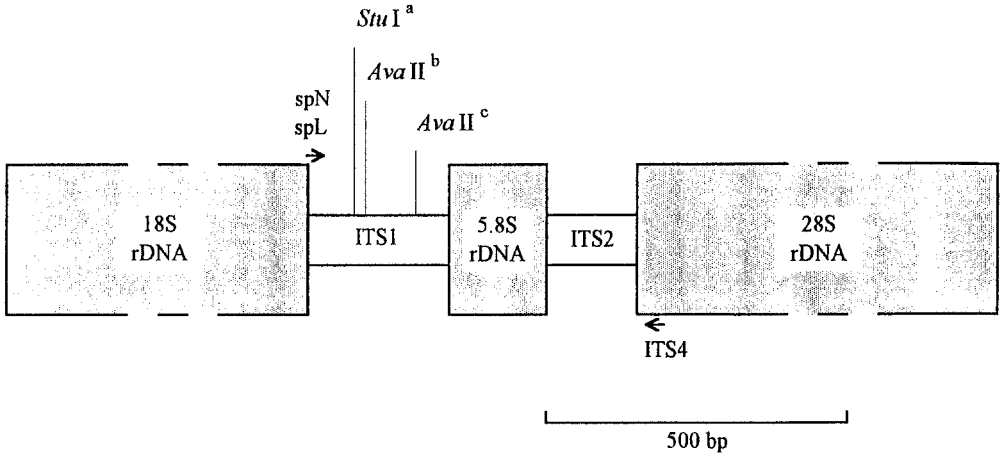

Figure 1. Locations of PCR primers and specific restriction sites on the ribosomal RNA genes of N- and L-type isolates of *P. herpotrichoides* originating from France. [a] *Stu* I site located in the ITS1 region of N-type. [b] *Ava* II site located in the ITS1 region of L-type. [c] *Ava* II site located in the ITS1 region of N- and L-types. The sequences of primers spN and spL are indicated in the text.

with a daily growth rate less than 1.5 mm and straight conidia. Nevertheless, these isolates can be identified as *P. herpotrichoides* var. *acuformis* because they are more typical of this variety. By considering all criteria, it is thus possible to classify most group 2 isolates as *P. herpotrichoides* var. *acuformis*. There is uncertainty concerning nine isolates coded 107, 125, 141, 181, 386 (isolates from France), M4, M9, 11.2 (isolates from the

U.K.) and PHAIII (isolate from Germany) which possess characteristics close to both varieties (Table 3). However, these isolates were previously identified as L-type, R-type and var. *acuformis*. In France, spore shape was used to classify intermediate isolates and it is likely that isolates from the U.K. (M4, M9 and 11.2) and the isolate from Germany (PHAIII) were similarly classified, but the risk of error existed because some isolates of var. *herpotrichoides* have also straight conidia only (see isolate BY151 from the U.K., Table 3).

Molecular characterization of isolates

DNA from four N-type isolates (N 99, N 117, N 118 and N 147) and three L-type isolates (L 97, L 98 and L 108) from France (Table 3) was tested for amplification using both primer pairs: spN/ITS4 and spL/ITS4. The amplified products were approximately 600 bp in length, which was the predicted size based on the rDNA sequences [Gac, 1991; Poupard et al., 1993]. At an annealing temperature of 49°C, specific amplification of N- and L-type isolates was not observed: isolates of each type tested produced a 600-bp DNA fragment with both primer sets (data not shown). By raising the annealing temperature, specificity of the signal was increased; specific amplification of N-type isolates using primers spN and ITS4 and of L-type isolates using primers spL and ITS4 was achieved at 60°C (Figure 2). No amplified product was observed when either primer pair was used with DNA of *P. anguioides* or other cereal stem-base pathogenic fungi (Figure 3).

The four amplified products obtained using primers spN and ITS4 had the same *Ava* II and *Stu* I patterns (Figure 4). These consisted of two bands the sizes of which agreed with those anticipated based on the rDNA sequences (410, 186 bp and 515, 81 bp, respectively) [Gac, 1991]. The three amplified products obtained using primers spL and ITS4 exhibited the same *Ava* II pattern (Figure 5). Three fragments of 411, 100 and 88 bp were expected from the rDNA sequences [Gac, 1991]. Only two bands were visible on the gel, but considering the size of the digested fragment (approximately 600 bp) and the intensity of the small-sized band, the latter is a doublet (100 and 88 bp). No digestion was observed with *Stu* I (Figure 5). This agreed with the rDNA sequences: L-type isolates had no *Stu* I site within the amplified region [Gac, 1991].

DNA from the remaining twenty-six group 1 isolates and twenty-seven group 2 isolates was tested for amplification and restriction analysis. Results were identical to those obtained with the four N-type and three L-type French isolates, respectively (Table 3). Thus, the nine isolates considered ambiguous on the basis of morphological and cultural criteria were classified unequivocally in group 2 using molecular markers.

Discussion

Nirenberg's [1981] classification system identified positively most isolates as *P. herpotrichoides* var. *herpotrichoides* or *P. herpotrichoides* var. *acuformis*, but determination of morphological and cultural characteristics was time-consuming. Moreover, overlap occurred between the two varieties for all criteria examined, thus leading to nine intermediate isolates which could not be classified.

Poupard et al. [1993] demonstrated the efficacy of a PCR test using primers selected in a variable region of the ribosomal genes to differentiate the two types of *P. herpotrichoides*, but the procedure developed was not suited for rapid analysis of large numbers of isolates or plant samples. Using two new oligonucleotide primers located in the ITS1 region of N- and L-type isolates, we were able to specifically amplify DNA from four N-type isolates and three L-type isolates from France. Moreover, specific amplification of each fungus type could be achieved using one PCR protocol. The test was accurate for the fifty-three remaining isolates, regardless of geographical origin and fungicide sensitivity, and no cross-reaction was observed with DNA of *P. anguioides* or other cereal stem-base pathogenic fungi. The thirty group 1 isolates exhibited identical *Ava* II and *Stu* I patterns. All group 2 isolates had a unique *Ava* II pattern, distinct from that of group 1. No cutting site was observed for *Stu* I in the amplified region of group 2 isolates. Molecular markers therefore identified two distinct types among the isolates tested. Classification obtained correlated with that of Nirenberg's [1981] system, but isolates were easily characterized using molecular criteria with no intermediate types observed. Our results therefore demonstrate the limitations of criteria relying upon characteristics expressed in culture, even when combined, to identify isolates of *P. herpotrichoides*. Takeuchi and Kuninaga [1994] divided isolates of the fungus into two genomic species on the basis of nuclear DNA reassociation kinetics. From our study [Gac, 1991] and that of Poupard et al. [1993], it is not possible to determine whether the two types of *P. herpotrichoides* diverge at

Table 3. Characteristics of *P. herpotrichoides* isolates: origin, code, colony morphology, daily growth rate, spore shape, signal with primers spN/ITS4, signal with primers spL/ITS4, restriction patterns after digestion of the amplified rDNA fragment with *Ava* II or *Stu* I

Origin and code	Colony morphology			Daily growth rate (mm day^{-1})	Spore shape[c]	Signal with primers spN/ITS4	Signal with primers spL/ITS4	*Ava* II[d]	*Stu* I[e]
	Margin[a]	Hump[b]	Colour of top surface						
Group 1									
France (N-type)									
99	3	+	Squirrel grey	2.65	cv	Yes	No	1	1
117	3	+	White	2.43	cv & st	Yes	No	1	1
118	3	+	Squirrel grey	2.71	cv	Yes	No	1	1
147	3	+	Squirrel grey	2.39	cv	Yes	No	1	1
148	3	+	Beige grey	2.71	cv	Yes	No	1	1
159	3	+	Brown grey	2.28	cv	Yes	No	1	1
165	3	sh	Squirrel grey	2.50	cv	Yes	No	1	1
168	3	+	White grey	2.40	cv	Yes	No	1	1
171	3	+	Squirrel grey	2.18	cv	Yes	No	1	1
189	3	+	Squirrel grey	2.25	cv & st	Yes	No	1	1
190	3	+	Beige grey	1.86	cv	Yes	No	1	1
193	3	+	Brown grey	2.16	cv	Yes	No	1	1
228	3	+	White brown	2.02	cv	Yes	No	1	1
233	3	+	Olive grey	2.50	cv	Yes	No	1	1
251	3	sh	White	2.03	cv	Yes	No	1	1
253	3	+	White grey	2.12	cv & st	Yes	No	1	1
256	3	+	Beige grey	2.34	cv & st	Yes	No	1	1
328	3	+	Squirrel grey	1.85	cv & st	Yes	No	1	1
487	3	+	Squirrel grey	1.49	cv & st	Yes	No	1	1
489	3	+	Squirrel grey	1.85	cv	Yes	No	1	1
U.K. (W-type)									
BK51	3	+	Squirrel grey	2.68	cv	Yes	No	1	1
BK58	3	sh	Brown	2.33	cv & st	Yes	No	1	1
BY151	3	+	Squirrel grey	2.60	st	Yes	No	1	1
15.2	3	+	White brown	2.20	cv	Yes	No	1	1
15.4	3	+	Squirrel grey	2.50	cv	Yes	No	1	1
Germany (var. *herpotrichoides*)									
PHHB	3	+	Squirrel grey	2.07	cv	Yes	No	1	1
PHH5	3	+	Beige pink	1.78	cv	Yes	No	1	1
PHH12	3	+	Beige pink	1.89	cv	Yes	No	1	1
U.S.A. (fast-growing type)									
PH90.70.1	3	sh	Squirrel grey	2.64	cv	Yes	No	1	1
PH90.153.1	3	+	Squirrel grey	1.93	cv	Yes	No	1	1

[a] 1 = feathery margin, 2 = irregular margin, 3 = even margin.
[b] + = pronounced hump, sh = slight hump, – = no hump.
[c] cv = curved conidia, st = straight conidia, cv & st = curved and straight conidia.
[d] 1 = restriction pattern with 410- and 186-bp DNA fragments, 2 = restriction pattern with 411-, 100- and 88-bp DNA fragments.
[e] 1 = restriction pattern with 515- and 81-bp DNA fragments, 2 = amplified rDNA fragment not digested.

Table 3. Continued

Origin and code	Colony morphology			Daily growth rate (mm day^{-1})	Spore shape[c]	Signal with primers spN/ITS4	Signal with primers spL/ITS4	*Ava* II[d]	*Stu* I[e]
	Margin[a]	Hump[b]	Colour of top surface						
Group 2									
France (L-type)									
97	2	–	Beige grey	1.49	st	No	Yes	2	2
98	1	–	Beige pink	1.22	st	No	Yes	2	2
107	3	sh	Brown	1.96	st	No	Yes	2	2
108	1	+	Beige pink	1.11	st	No	Yes	2	2
115	2	sh	Squirrel grey	1.00	st	No	Yes	2	2
125	3	+	Brown	2.07	st	No	Yes	2	2
135	2	sh	Beige	0.86	st	No	Yes	2	2
141	3	sh	Squirrel grey	1.96	st	No	Yes	2	2
172	1	sh	Beige grey	1.32	st	No	Yes	2	2
181	3	+	Squirrel grey	1.93	st	No	Yes	2	2
183	2	–	Beige grey	1.73	st	No	Yes	2	2
210	2	–	White	1.11	st	No	Yes	2	2
247	3	sh	Brown grey	1.28	st	No	Yes	2	2
293	1	–	White brown	1.30	st	No	Yes	2	2
297	1	–	Olive grey	1.13	st	No	Yes	2	2
300	3	sh	Beige	1.21	st	No	Yes	2	2
304	1	–	White grey	1.16	st	No	Yes	2	2
325	1	sh	Beige grey	1.12	st	No	Yes	2	2
386	3	+	Beige grey	1.86	st	No	Yes	2	2
399	3	+	Brown grey	0.92	st	No	Yes	2	2
U.K. (R-type)									
M4	3	sh	Beige grey	1.71	st	No	Yes	2	2
M9	3	sh	Beige grey	2.64	st	No	Yes	2	2
11.2	3	sh	Beige pink	2.16	st	No	Yes	2	2
15.7	1	–	Brown grey	0.83	st	No	Yes	2	2
LP11	2	+	Squirrel grey	1.48	st	No	Yes	2	2
Germany (var. *acuformis*)									
PHA52	1	–	Beige grey	1.86	st	No	Yes	2	2
PHAIII	3	sh	Beige grey	2.05	st	No	Yes	2	2
PHA3/10	1	sh	Beige grey	1.33	st	No	Yes	2	2
U.S.A. (slow-growing type)									
PH90.88.5	1	–	Squirrel grey	0.89	st	No	Yes	2	2
PH90.90.7	1	–	Squirrel grey	0.86	st	No	Yes	2	2

Table 4. Morphological and cultural characteristics of *P. herpotrichoides* isolates: synthesis of results listed in *Table 3*

	Colony morphology(*)									
	Margin[a]			Hump[b]						
	1	2	3	+	sh	–				
Group 1	0	0	30 (100.0)	26 (86.7)	4 (13.3)	0				
Group 2	12 (40.0)	6 (20.0)	12 (40.0)	6 (20.0)	13 (43.3)	11 (36.7)				
	Colour of top surface									
	Squirrel grey	Olive grey	Beige grey	White grey	Brown grey	Brown	White	White brown	Beige	Beige pink
Group 1	15	1	3	2	2	1	2	2	0	2
Group 2	6	1	10	1	3	2	1	1	2	3
	Mean daily growth rate (mm day^{-1} with standard error of mean)									
Group 1	2.2 ± 0.3									
Group 2	1.4 ± 0.5									
	Spore shape[c](*)									
	cv	cv & st	st							
Group 1	22 (73.3)	7 (23.3)	1 (3.3)							
Group 2	0	0	30 (100.0)							

[a] 1 = feathery margin, 2 = irregular margin, 3 = even margin.
[b] + = pronounced hump, sh = slight hump, – = no hump.
[c] cv = curved conidia, cv & st = curved and straight conidia, st = straight conidia.
(*) Results represent the number of isolates in each class of a given character. Values in brackets represent the percentage of isolates in each class.

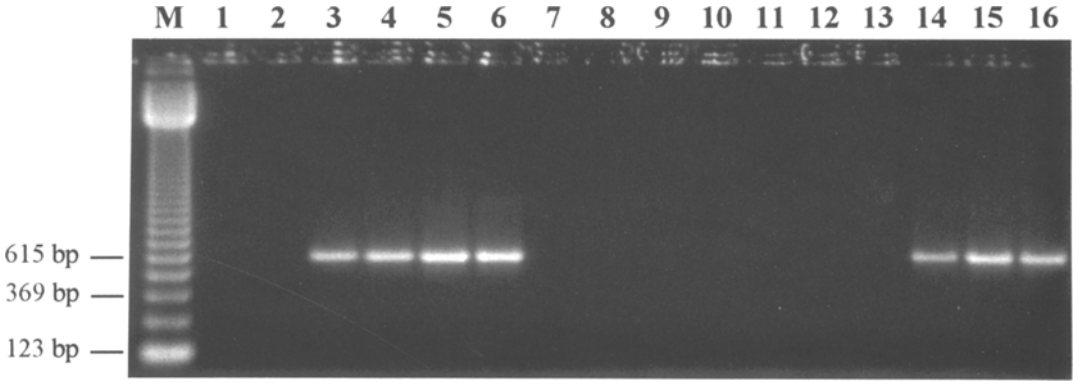

Figure 2. Specific PCR amplification of N-type isolates with primers spN and ITS4 and of L-type isolates with primers spL and ITS4. Lanes: M, Molecular weight marker (123-bp DNA ladder); 1, Negative control (no DNA template) for primers spN and ITS4; 2, Negative control (no DNA template) for primers spL and ITS4; 3–10, PCR products of four N-type isolates with primers spN and ITS4 (3: N 99, 4: N 117, 5: N 118, 6: N 147) and primers spL and ITS4 (7: N 99, 8: N 117, 9: N 118, 10: N 147); 11–16, PCR products of three L-type isolates with primers spN and ITS4 (11: L 97, 12: L 98, 13: L 108) and primers spL and ITS4 (14: L 97, 15: L 98, 16: L 108).

the species level without comparisons with other *Pseudocercosporella* spp.. However, our results agree with those of other researchers in showing major differences between the genomes of the two types of the fungus [Julian and Lucas, 1990; Nicholson et al., 1991; Priestley et al., 1992; Thomas et al., 1992; Frei and Wenzel, 1993; Nicholson et al., 1993; Nicholson and Rezanoor, 1994; Takeuchi and Kuninaga, 1994]. Moreover, since N-/W-/*herpotrichoides* and fast-growing types were classified in the same group as was the case for L-

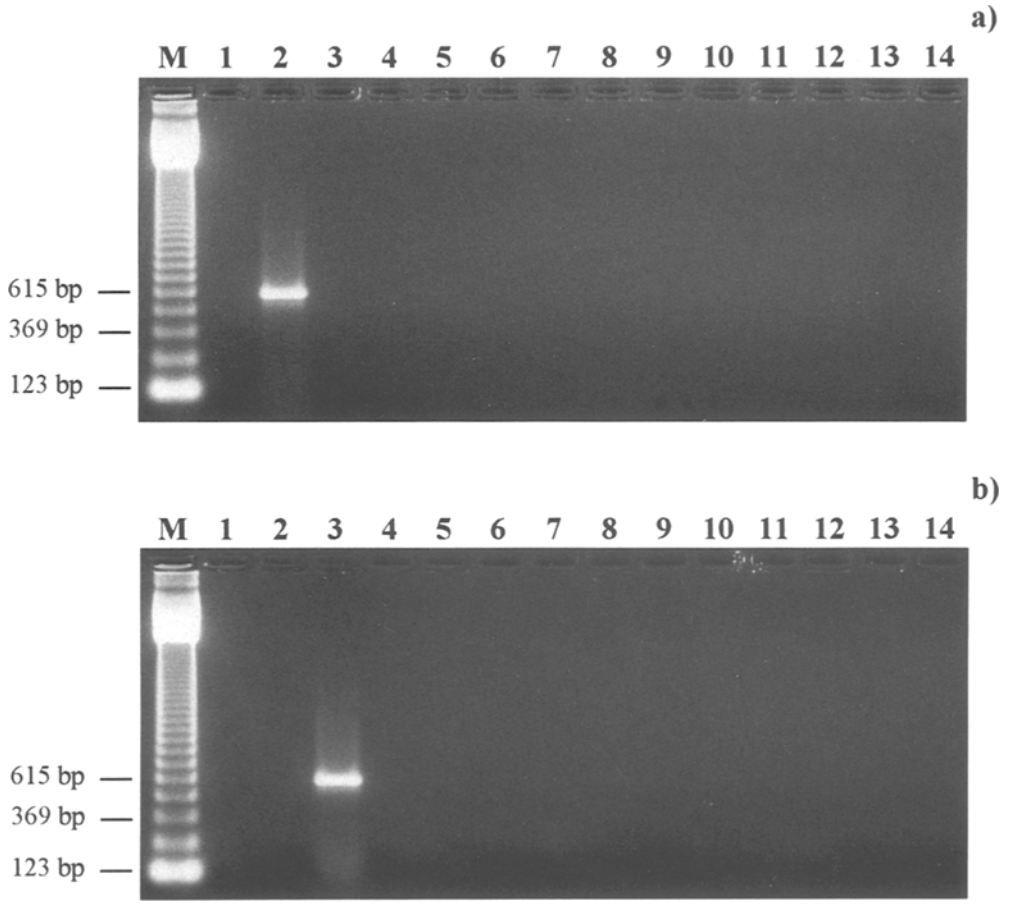

Figure 3. Specific PCR amplification of (a) N-type with primers spN and ITS4 and of (b) L-type with primers spL and ITS4. Lanes: M, Molecular weight marker (123-bp DNA ladder); 1, Negative control (no DNA template); 2, PCR product of one N-type isolate (N 99); 3, PCR product of one L-type isolate (L 97); 4–14, PCR products of the other cereal stem-base pathogenic fungi (4–5: isolates of *Pseudocercosporella anguioides*, 6–7: isolates of *Fusarium culmorum*, 8–9: isolates of *F. graminearum*, 10: *F. avenaceum*, 11: *Microdochium nivale* var. *nivale*, 12: *M. nivale* var. *majus*, 13: *Rhizoctonia cerealis*, 14: *Gaeumannomyces graminis* var. *tritici*).

/R-/*acuformis* and slow-growing types, we propose the use of a unique nomenclature which could be that of Nirenberg [1981]: *P. herpotrichoides* var. *herpotrichoides* and *P. herpotrichoides* var. *acuformis*. Indeed, Nirenberg's [1981] system is that which takes into account the greatest number of criteria to characterize isolates of *P. herpotrichoides*. Furthermore, Nirenberg [1984] showed that hyphal anastomosis occurred only between isolates of the same variety which supports our study and those of the authors cited above suggesting that the two types present in *P. herpotrichoides* are genetically isolated.

PCR provides a reliable and rapid method for identifying isolates of *P. herpotrichoides* without the difficulties associated with conventional methods (e.g. intermediate types) and some techniques of molecular biology (e.g. laborious Southern blot hybridization; hazards associated with the use of radioactive probes; lack of reproducibility of the RAPD patterns). Restriction analysis of PCR-amplified DNA is a simple and rapid way of identifying organisms. The method has been used successfully with several fungi [Cubeta et al., 1991; Chen et al., 1992; Ward and Akrofi, 1994]. Our results indicate that restriction analysis of the PCR products is useful to check the identity of the amplified fragments and verify that specific amplification conditions were used. Recently, the PCR method has permitted the detection of fungal pathogens in infected plants [Nazar et al., 1991; Schesser et al., 1991; Xue et al., 1992; Henson et al., 1993; Johanson and Jeger,

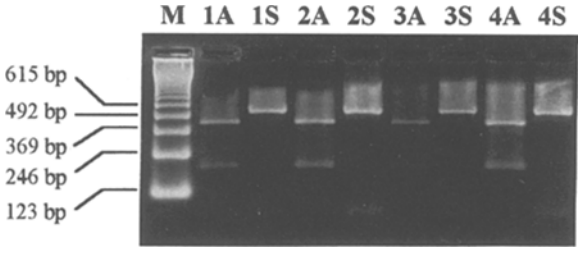

Figure 4. Restriction patterns after digestion of N-type-specific amplified products with *Ava* II or *Stu* I. Lanes: M, Molecular weight marker (123-bp DNA ladder); 1–4, *Ava* II (A) and *Stu* I (S) restriction patterns of amplified products specific to four N-type isolates (1: N 99, 2: N 117, 3: N 118, 4: N 147).

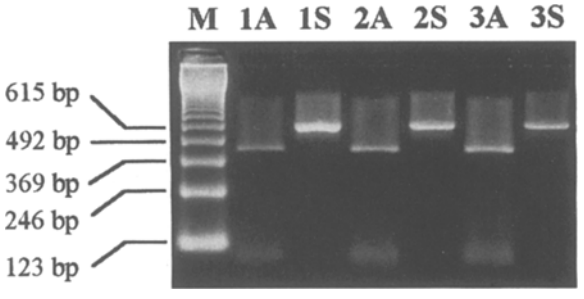

Figure 5. Restriction patterns after digestion of L-type-specific amplified products with *Ava* II or *Stu* I. Lanes: M, Molecular weight marker (123-bp DNA ladder); 1–3, *Ava* II (A) and *Stu* I (S) restriction patterns of amplified products specific to three L-type isolates (1: L 97, 2: L 98, 3: L 108).

1993; Stammler and Seemüller, 1993]. Such an application should be examined for *P. herpotrichoides* as precise information on the *in vivo* development of each type of the fungus is needed.

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