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Plant pathology

A comparison of biological, molecular and enzymatic markers to investigate variability within *Microdochium nivale* (Fries) Samuels and Hallett

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Summary — The variation among isolates of *Microdochium nivale* obtained from a range of hosts and locations in Europe was investigated on the basis of conidial morphology, restriction fragment length polymorphisms (RFLPs) of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA), esterase profiles, aggressiveness on wheat seedlings and perithecial production. One homogeneous group was resolved on the basis of esterase profiles and RFLPs of the ITS region of rDNA. The isolates within this group possessed ITS profiles identical to reference isolates of var *majus* from other workers and therefore were assigned to var *majus*. The isolates within this homogeneous var *majus* grouping were obtained from a range of locations throughout Europe, demonstrating that it is not restricted to the United Kingdom. Nineteen esterase profiles and 2 ITS profiles were detected among the remaining isolates, suggesting that this grouping may be comprised of further sub-groups. The results demonstrated that several isolate types may exist at a single location and that pathogenicity to wheat seedlings was not related to any of the traits analysed.

***Microdochium nivale* / cereal / RFLP / internal transcribed spacer**

Résumé — Comparaison de marqueurs biologiques, moléculaires et enzymatiques pour l'analyse de la variabilité chez *Microdochium nivale*. *Les variations au sein d'une collection de souches européennes de* Microdochium nivale *isolées de plusieurs hôtes sont étudiées sur la base de la morphologie des conidies, des profils d'estérases et de RFLP de la région des ITS de l'ADN ribosomique, de l'agressivité sur des plantules de blé et de la capacité à produire des périthèces* in vitro. *Un groupe homogène est différencié à la fois par les profils d'estérases et les RFLP des ITS de l'ADN-r. Les isolats de ce groupe possèdent des profils d'ITS identiques aux isolats de référence de la variété* majus *utilisés par d'autres auteurs et sont, par conséquent, assignés à la variété* majus. *Les isolats à l'intérieur du groupe homogène* majus *ont été collectés à travers l'Europe, ce qui démontre que ce groupe n'est pas restreint au Royaume-Uni. Parallèlement, 19 profils d'estérases et 2 profils d'ITS sont observés parmi le reste des isolats, ce qui suggère que ce groupe pourrait être constitué de plusieurs autres sous-groupes. Les résultats démontrent que les 2 types d'isolats coexistent dans un même lieu et que l'agressivité sur plantules de blé n'est liée à aucun autre caractère étudié.*

Microdochium nivale *I céréales / RFLP / internal transcribed spacer*

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INTRODUCTION

Microdochium nivale (Fries) Samuels and Hallett (teleomorph *Monographella nivalis* (Schaffnit) Müller) is distributed worldwide and causes snow mould of cereals and turfgrasses (Cook, 1981), foot rot (Harris, 1986; Pettitt *et al,* 1993) and head blight diseases of cereals when high humidity and low temperature occur during anthesis (Cassini, 1981; Cook, 1981). It has been recorded as one of the dominant pathogen species identified from wheat samples in Western Europe (Duben and Fehrmann, 1979; Parry, 1990; Daamen *et al,* 1991; Maurin and Chénet, 1993), and resistance tests with artificial inoculation have been developed in order to assess levels of resistance of cereals to *M nivale* (Bruehl, 1982; Litschko *et al,* 1988; Miedaner *et al,* 1993). Two varieties have been distinguished, on the basis of conidial characteristics, within *M nivale* (Wollenweber and Reinking, 1935). These are *M nivale* var *nivale* (Fries) Samuels and Hallett and *M nivale* var *majus* (Wollenw) Samuels and Hallet (Gams and Müller, 1980; Gerlach and Nirenberg, 1982).

Despite its range of hosts (small grain cereals, lawn and turf grass) and the extent of its distribution (Australia, China, North America and Europe), little attention has been paid to the variability of the pathogen. Recently, Lees *et al* (1995) used random amplification of polymorphic DNA (RAPD) analysis to study variability within *M nivale* isolates. A distinct homogeneous subgroup, correlating broadly to *M nivale* var *majus* was identified along with a heterogeneous grouping which related to *M nivale* var *nivale.* Nevertheless, this work was restricted to British isolates originating from wheat only. The present work was undertaken to analyse the variation among isolates from a broader range of hosts and locations in order to test the variation of the fungus across Europe using a set of complementary traits. Esterase profiles, ITS-rDNA (internal transcribed spacer-ribosomal DNA) digestion profiles, conidial morphology and aggressiveness as well as perithecial production *in vitro* were considered in order to establish possible connections between morphological, biological and genetic variation within a European collection of *M nivale.*

MATERIALS AND METHODS

Origin and maintenance of fungal isolates

The isolates of *M nivale* used in this study were obtained from a number of European countries and a range of hosts (table I). All isolates were derived from single conidia and maintained on SNA medium (Nirenberg, 1981) slants at 4°C and subcultured each year for preservation.

Isolates N14 (*M nivale* var *nivale*) and N15 (*M nivale* var *majus*) were a kind gift from H Nirenberg (BBA collection, Berlin), and were used as taxonomic references of *nivale* and *majus* varieties. For the restriction enzyme digestion of ITS-rDNA, other isolates, B37, B38 and B101 (*M nivale* var *nivale*) and B02, B05, B10, B18 and B27 (*M nivale* var *majus*), were added in order to compare with the groupings identified using RAPDs in the study of Lees *et al* (1995).

Conidial morphology

All single conidial isolates were plated on potato dextrose agar (PDA) and incubated at 20°C for 3 d in the dark followed by 6 d at 8°C under a 18 h/6 h near ultraviolet/dark regime.

Twenty-five conidia of each isolate were examined and an average spore length, width and number of septa was calculated. Isolates were tentatively assigned to 1 of the 2 varieties on the basis of these characters as described by Gerlach and Nirenberg (1982).

Protein extraction and polyacrylamide gels

Mycelial explants of single conidial cultures were grown in a Tanaka liquid medium (Ou, 1972) containing 10 g glucose at 20°C in the dark. After 7 d incubation, the fungal mats were recovered by vacuum filtration using a No 1 Whatman filter paper, rinsed twice with distilled water and then freeze-dried. The proteins were extracted by the method of Somé and Tivoli (1993): 100 mg freeze-dried mycelium was added to 100 mg Fontainebleau sand and 100 mg insoluble polyvinylpyrrolidone (PVP) in 1 ml extraction buffer at 4°C and ground in a cool mortar for 15 min. The extraction buffer pH 7 contained 10 mM Tris, 1 mM EDTA, 3 mM 2-mercaptoethanol, and 1 mM cysteine. After centrifugation at 13 000 *g* for 10 min, glycerol was added to the supernatant to a total of 10% of the final volume and the protein-rich solution was stored at 20°C or was applied to a 9% polyacrylamide gel for electrophoresis using the Laemmli technique (Laemmli, 1970). Each well contained 20 μl of extracted solution and electrophoresis was carried out in a slab gel unit SE 280 (Hoefer Scientific Instruments) at 200 V at 4°C for 2 h.

Esterases were detected using a modified version of the method described by Siciliano and Shaw (1976). Fast blue RR salts (50 mg) were dissolved in 50 ml of 0.1 M phosphate buffer (pH 6) at 37°C. After filtration through a Whatman No 1 filter paper, 1 ml acetone containing 20 mg α-naphthyl acetate and 20 mg β-naphthyl acetate was added. After a few seconds of

Table I. Origin, conidial morphology, production of perithecia, esterase patterns and ITS groups of isolates of *Microdochium nivale.*

Isolate code	*Original code*	*Origin*	*Host*	*Perithecial production*	*Esterase profile*	*ITS* [a]	*Varietal designation* [b]
N2	Fn 2	France	Durum wheat	+	nd [c]	2b	*nivale*
N3	Fn 3	France	Durum wheat	–	D	2a	*nivale*
N5	F 75	Switzerland	Wheat	+	E	2a	*nivale*
N7	F 105	Switzerland	Rye	–	F	2a	*nivale*
N8	F 106	Switzerland	Wheat	–	G	2a	*nivale*
N10	F 129	Switzerland	Barley	–	A	1	*majus*
N12	131	Netherlands	Wheat	–	A	1	*majus*
N14	64624	Germany	Turf grass	–	B	2a	*nivale*
N15	64720	Germany	Durum wheat	+	A	1	*majus*
N18	RPA F 74	France	Turf grass	–	H	2a	*nivale*
N20	CBS 167.57	–	–	–	I	2b	*nivale*
N22	FN 22	France	Wheat	+	J	2b	*nivale*
N25	FN 25	France	Wheat	–	K	2b	*nivale*
N32	FN 32	France	Wheat	–	C	2b	*nivale*
N36	FN 36	France	Wheat	+	A	1	*majus*
N45	UPM/637	Belgium	Turf grass	–	L	2b	*nivale*
N48	FN 48	France	Durum wheat	+	A	1	*majus*
N58	FN 58	France	Durum wheat	+	M	2b	*nivale*
N62	FN 62	France	Durum wheat	+	A	1	*majus*
N68	15/85	Poland	Turf grass	–	N	2b	*nivale*
N71	2M3/86	Poland	Rye	–	O	2a	*nivale*
N74	1969/86	Poland	Rye	–	P	2b	*nivale*
N75	FN 75	France	Wheat	+	Q	2b	*nivale*
N81	FN 81	France	Wheat	+	R	2b	*nivale*
N88	FN 88	France	Wheat	–	S	2b	*nivale*
N90	FN 90	France	Wheat	–	C	2b	*nivale*
N99	FN 99	France	Wheat	+	A	1	*majus*
N101	FN 101	France	Wheat	+	T	2a	*nivale*
Additional isolates							
B 02	Mn 02	UK	Wheat	–	nd	1	*majus*
B 05	Mn 05	UK	Wheat	+	nd	1	*majus*
B 10	Mn 10	UK	Wheat	+	nd	1	*majus*
B 18	Mn 18	UK	Wheat	–	nd	1	*majus*
B 27	Mn 27	UK	Wheat	–	nd	1	*majus*
B 37	Mn 37	UK	Wheat	+	nd	2a	*nivale*
B 38	Mn 38	UK	Wheat	–	nd	2b	*nivale*
B 101	Mn 101	UK	Wheat	+	nd	2a	*nivale*

[a] Profile of amplified products of ITS rDNA after digestion by *Cfo* I and *Rsa* I; [b] varietal designation based upon ITS and esterase profiles; [c] not determined.

agitation, the gel was incubated in this mixture for 30 min at 37°C in the dark, and then rinsed with water and fixed with 10% acetic acid.

DNA extraction

Mycelium was scraped from the surface of 4-day-old colonies on PDA and used to inoculate 50 ml potato dextrose broth. Cultures were incubated at ambient temperature on an orbital shaker in the dark for 7 d. Mycelium, harvested by filtration onto Whatman No 1 filter paper disks, was freeze-dried and then ground in a mill to a fine powder using 2 steel balls in a 25 ml tube shaken vigorously for 4 min. DNA was extracted and purified by the method of O'Dell *et al* (1989), except for the initial incubation step, which was carried out at 65°C. DNA was diluted to 10 ng/μl for use in amplification reactions.

Primer and amplification conditions for PCR

Primers ITS4 and ITS5 (White *et al*, 1990) were used to amplify the (ITS) region between the 18 S and 25 S rDNA coding sequences. Amplification reactions were performed in a volume of 50 µl containing 10 ng genomic template DNA. The reaction buffer consisted of 100 µM each of dATP, dCTP, dGTP and dTTP, 200 nM each of ITS4 and ITS5, and 0.8 units of Taq polymerase (Boehringer Mannheim Ltd), in 10 nM Tris, HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 100 µg ml^{-1} gelatine, 0.05% Tween 20, and 0.05% Nonidet P-40. Reaction mixtures were overlaid with mineral oil prior to PCR.

Amplification was performed in a Perkin Elmer Cetus DNA Thermal Cycler programmed for 35 cycles of 30 s at 95°C, 1 min at 50°C and 2 min at 72°C using the fastest possible transitions between temperatures. A final extension step of 72°C for 5 min was included in the program followed by cooling to 4°C until recovery of the samples. Aliquots (10 µl) of amplification products were electrophoresed through 1.5% agarose gels and detected by staining with ethidium bromide and visualised on a UV transilluminator.

Restriction enzyme digestion

PCR products were used directly for restriction enzyme digestion. The digestions were performed in a volume of 30 µl containing 6 µl of PCR amplification product and 6 units of enzyme. Enzyme-digested DNA was run through 2% agarose gels stained with ethidium bromide, and visualized and photographed under UV light. Ten restriction enzymes (*Cla* I, *Cfo* I, *Dde* I, *Eco* R V, *Nde* I, *Pst* I, *Pvu* I, *Rsa* I, *Taq* I and *Xho* I) were used to digest the ITS region.

Aggressiveness

Fifty seedlings per isolate of the wheat cultivar Camp-Rémy were inoculated using a 5 mm agar disc from the margin of a 3-day-old colony, placed at the base of the seedlings at the 2-leaf stage and held in position by a ribbon of Parafilm (American National Can). After incubation for 1 month in a growth cabinet at 10°C day and 5°C night under a 16 h photoperiod, disease index (DI) was determined using the following 0–6 scale: 0 no symptoms; 1 slight lesion on 1 sheath; 2 lesion on 1 sheath only or 2 slight lesions on 2 sheaths; 3 one sheath entirely necrotic; 4 one sheath entirely necrotic and lesion on other sheath; 5 two sheaths entirely necrotic; 6 more than 2 sheaths entirely necrotic.

Production of perithecia in vitro

Straw segments (10 mm) of wheat were sterilized by autoclaving 3 times at 121°C for 20 min with 24 h between each sterilisation. Straws were placed with their lengths touching in Petri dishes containing PDA and a plug of mycelium from a single conidial isolate of *M nivale* was placed next to the straw. Plates were incubated at 15°C for 4 weeks and the presence of fertile perithecia recorded by examining the colony under a binocular microscope (x 40).

RESULTS

Conidial morphology

Conidial morphology was examined and found to differ among isolates in a manner similar to that described by Gerlach and Nirenberg (1982). According to these authors, *M nivale* var *nivale* is characterized by the presence of conidia mostly 1 (0–3), exceptionally 4–7 septate, measuring 16 µm long and 2.8 µm wide. *M nivale* var *majus* is distinguished by wider, predominantly 3 (1–7) septate conidia measuring 26 x 5.3 µm. Nevertheless, the conidial size varies considerably and ranges from 10 x 2.4 µm to 25 x 3.3 µm for *M nivale* var *nivale* and from 18 x 5.3 µm to 29 x 5.7 µm for *M nivale* var *majus*.

In this study, the average number of septa, conidial length and width were distributed continuously in the range 2.4–6.0 septa, 3.8–6.8 µm and 17.4–41.2 µm, respectively (table II). Statistical analysis of the data using PCA (principal component analysis) showed no distinct groups of isolates along the first axis, accounting for 75% of the total variance. Hierarchical clustering yielded 2 classes with low interclass inertia (68%), showing that the morphological characteristics used could not be used to assign isolates unambiguously to 1 group or the other. Because of the difficulty of prioritizing these characters, no further attempt was made to designate variety on the basis of morphological markers.

Restriction fragment length polymorphism in enzymatically amplified ribosomal DNA

The ITS region amplified by primer pair ITS4 and ITS5 was approximately 580 bp long for all the isolates of *M nivale*. The ITS-rDNA was not digested by the enzymes *Cla* I, *Dde* I, *Eco* R V, *Nde* I, *Pst* I, *Pvu* I, *Taq* I and *Xho* I, but length variations were detected after digestion with enzymes *Cfo* I and *Rsa* I (fig 1) and could be explained by the gain or loss, in some samples, of a single restriction site.

Table II. Conidial morphology (number of septa, conidial width and length) and aggressiveness (disease index) of 28 isolates of *Microdochium nivale*.

Isolate	*Number of septa* [a]	*Conidial width* [a] *(μm)*	*Conidial length* [a] *(μm)*	*Disease index* [b]
N2	2.45 ± 0.5	3.8 ± 0.6	18.8 ± 2.1	3.50 ± 0.50
N3	2.7 ± 0.4	4.1 ± 0.4	19.4 ± 2.0	2.39 ± 0.17
N5	2.6 ± 0.5	5.6 ± 0.6	20.0 ± 2.6	2.72 ± 0.53
N7	3.8 ± 0.6	5.0 ± 0.6	25.8 ± 2.8	1.96 ± 0.46
N8	4.2 ± 0.7	4.8 ± 0.6	21.8 ± 3.4	1.58 ± 0.15
N10	2.35 ± 0.4	4.1 ± 0.4	26.4 ± 1.2	2.04 ± 0.47
N12	6.0 ± 0.7	6.4 ± 0.8	41.2 ± 3.0	1.66 ± 0.27
N14	2.35 ± 0.6	5.0 ± 0.7	20.4 ± 2.9	2.14 ± 0.28
N15	5.1 ± 0.8	6.8 ± 0.9	31.6 ± 3.4	2.94 ± 0.49
N18	3.75 ± 0.7	4.8 ± 0.6	23.4 ± 1.8	2.28 ± 0.17
N20	2.8 ± 0.6	5.6 ± 0.6	23.6 ± 4.4	0.74 ± 0.21
N22	2.75 ± 0.5	5.2 ± 0.6	24.6 ± 2.6	2.04 ± 0.21
N25	3.15 ± 0.4	4.4 ± 0.4	17.4 ± 1.8	2.38 ± 0.33
N32	3.05 ± 0.3	4.2 ± 0.4	17.8 ± 1.8	2.18 ± 0.29
N36	4.0 ± 0.7	6.4 ± 0.8	33.4 ± 3.2	1.78 ± 0.33
N45	2.5 ± 0.4	5.6 ± 0.4	17.9 ± 1.6	1.48 ± 0.41
N48	3.5 ± 0.6	6.4 ± 0.6	36.6 ± 2.6	2.26 ± 0.39
N58	2.55 ± 0.3	5.2 ± 0.4	21.8 ± 3.0	2.70 ± 0.19
N62	3.0 ± 0.3	5.6 ± 0.2	26.8 ± 3.6	2.14 ± 0.39
N68	2.35 ± 0.5	4.0 ± 0.6	18.2 ± 2.4	2.58 ± 0.52
N71	3.3 ± 0.4	5.4 ± 0.4	22.8 ± 2.0	0.48 ± 0.23
N74	3.0 ± 0.6	5.8 ± 0.6	29.2 ± 4.4	2.24 ± 0.76
N75	3.5 ± 0.7	5.6 ± 0.8	22.6 ± 3.0	2.36 ± 0.31
N81	3.85 ± 0.6	5.2 ± 0.6	22.4 ± 2.2	3.31 ± 0.79
N88	3.9 ± 0.7	5.2 ± 0.6	19.0 ± 2.4	2.08 ± 0.21
N90	3.3 ± 0.4	5.0 ± 0.4	22.2 ± 3.2	2.04 ± 0.34
N99	2.65 ± 0.5	6.0 ± 0.4	32.6 ± 3.0	2.18 ± 0.27
N101	3.0 ± 0.7	4.6 ± 0.6	20.4 ± 1.8	2.46 ± 0.55

[a] Measurements for 25 conidia, means with standard error; [b] disease index scale of 0–6 (details of scale in text).

After digestion of the ITS-rDNAs with *Rsa* I, the isolates were split into 2 groups. Isolates N10, N12, N15, N36, N48, N62 and N99 constituted group 1 and were characterized by the presence of 2 DNA fragments (approximately 490 and 90 bp) resulting from the digestion by *Rsa* I at one restriction site. The ITS-rDNA of the remaining isolates (group 2) was not cut by *Rsa* I (fig 1a and 1b). Digestion of the ITS-rDNA with *Cfo* I produced 3 fragments (approximately 280, 170 and 130 bp) in several isolates (N3, N5, N7, N8, N14, N18, N71 and N101) within group 2 (sub-group 2a) (fig 1c and 1d) whereas amplification products from all group 1 isolates and the remaining group 2 (sub-group 2b) isolates were cut by *Cfo* I, at a single site to produce 2 fragments (approximately 300 and 280 bp) (fig 1c and 1d).

The var *majus* reference isolate (N15) and 5 British isolates (B02, B05, B10, B18 and B27) were assigned to group 1 whereas the var *nivale* reference (N14) and 3 British isolates (B37, B38 and B101) were assigned to sub-group 2a.

Electrophoresis protein patterns

In a preliminary study (results not shown), electrophoresis was carried out on 8 isolates, including the N14 and N15 reference isolates, using several enzyme systems (esterases, phosphoglucoisomerase, phosphoglucomutase, polyphenoloxidase and superoxidedismutase). The esterase enzymes revealed considerable variation among isolates of *M nivale* (fig 1) and was used on the full isolate set. Twenty clearly different esterase profiles were detected among the 27 isolates examined (table I). Only 2 profiles (A and C) were common to more than 1 isolate. Profile A was common to 7 isolates (N10, N12,

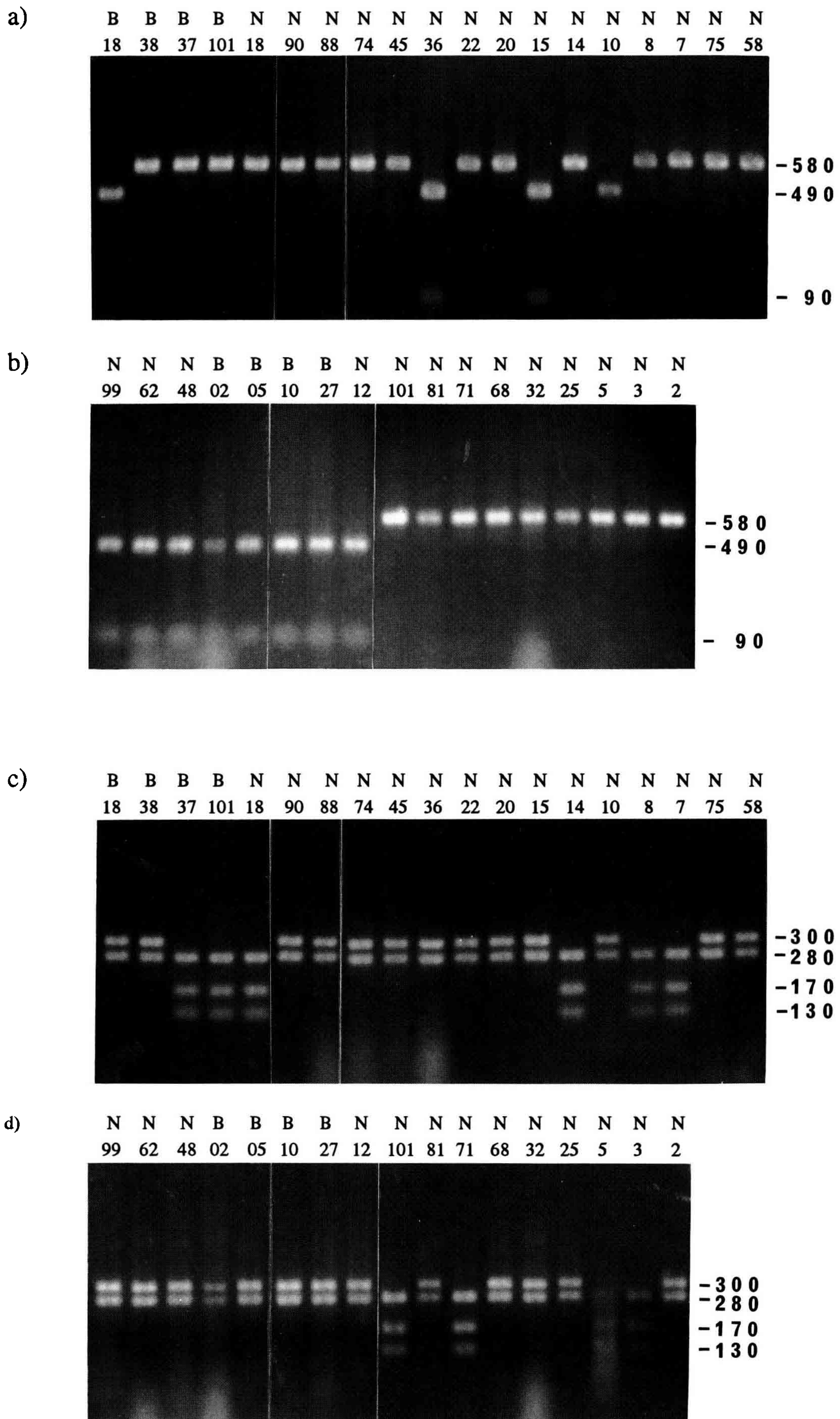

Fig 1. Agarose gel showing PCR-amplified ITS region of r-DNA of *Microdochium nivale* digested by restriction enzymes; a) and b) Rsa I: fragment sizes 490 and 90 bp, group 1; 580 bp (uncut) group 2; c) and d) Cfo I: fragment sizes 300 and 280 bp, groups 1 and 2b; 280, 170 and 130 bp, group 2a.

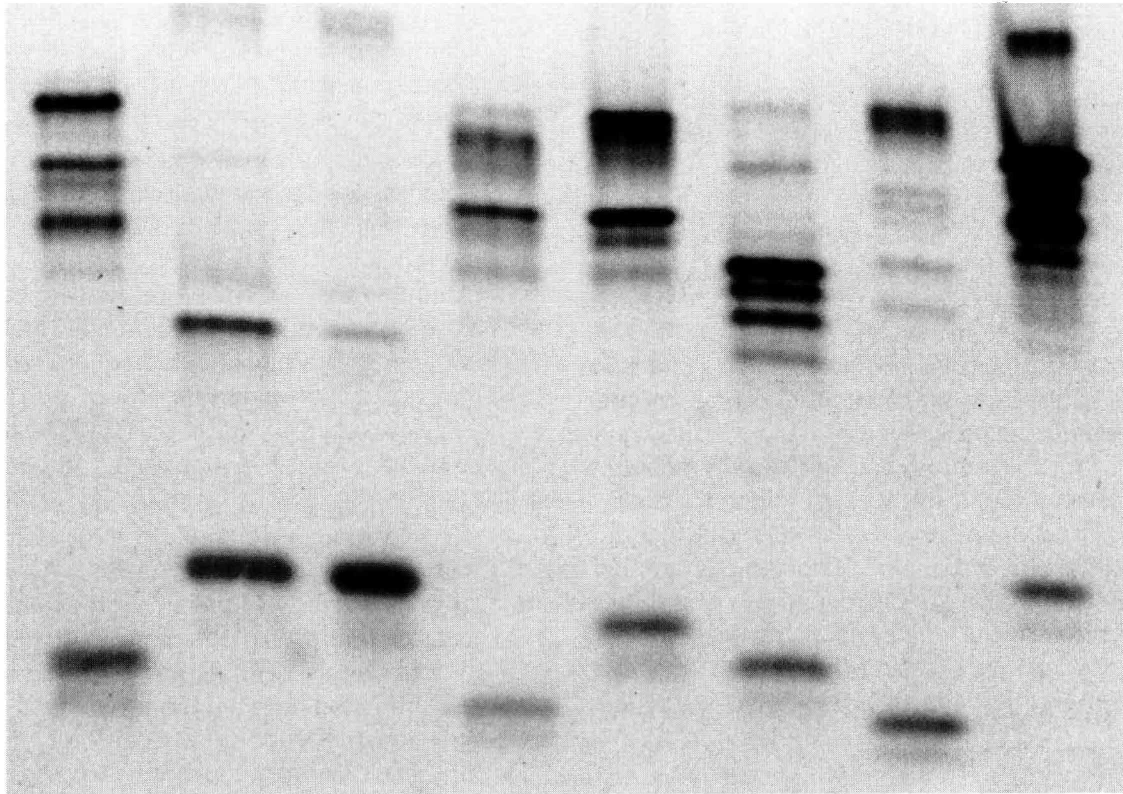

Fig 2. Examples of esterase profiles of isolates of *Microdochium nivale*.

N15, N36, N48, N62 and N99) isolated from 3 different cereal hosts and originating from a wide range of locations, including Switzerland, the Netherlands, Germany and France. Profile C was shared by 2 French isolates from wheat, N32 and N90 (table I).

Interestingly, all isolates which possessed the esterase profile A also had the group 1 ITS profile, whereas the remaining isolates showing various esterase profiles (B to T) possessed the group 2 ITS profile.

Aggressiveness

According to the seedling test carried out in greenhouse conditions, all isolates were virulent on cultivar Camp-Rémy. The disease index (DI) ranged from 0.5 to 3.3 (table II), depending on the isolate, and significant differences were observed between the isolates. Isolates N20 and N71 were only slightly aggressive with a DI of less than 1, while isolates N68, N58, N5, N15, N81 and N2 were more aggressive with DIs above 2.5.

No correlation appeared between pathogenicity and groupings whether according to esterase, ITS or conidial morphology. The isolates belonging to group 1 (esterase profile A) ranged from weakly aggressive (N12 and N36) to aggressive (N10, N62, N48 and N99). Isolate N15 was highly aggressive (DI 2.94). Isolates assigned to ITS sub-groups 2a and 2b were also distributed across the range of DI.

Production of perithecia in vitro

Twelve of the 28 isolates examined produced fertile perithecia on wheat straw *in vivo* (table I). Five of the 7 isolates with esterase profile A and ITS-group 1 produced mature perithecia *in vitro*. Conversely, only 2 of the 8 ITS-sub-group 2a isolates and 5 of the 13 ITS-sub-group 2b isolates produced perithecia *in vitro*. Thus, there was no clear correlation between the ability to produce perithecia *in vitro* and groupings.

DISCUSSION

The aim of this study was to examine variation in a collection of European isolates of *M nivale* from a number of countries and a range of hosts using different methods of characterization. The results indicate that *M nivale* is highly variable but discrete sub-groups may be identified on the basis of isozyme and restriction profiles of the ITS region. However, these subgroups can hardly be correlated with the varieties described in the literature. According to Gerlach and Nirenberg (1982) it is possible to distinguish 2 varieties on the basis of classical taxonomic criteria. In the present study, variation for conidial traits was almost continuous and showed no correlation with one another. Therefore, the assignment of individual isolates to 1 of the 2 varieties was not readily possible, because of the difficulty of prioritizing the characters. These data show the limitations of the traditional taxonomy based on morphology and indicate that the conidial characters do not provide a reliable means of identifying members of homogeneous sub-groups within *M nivale.*

RFLP (restriction fragment length polymorphism) analysis of the ITS indicated the presence of 3 groupings. One of them (group 1) included the var *majus* reference isolate from Germany N15, and the RAPD characterized var *majus* British isolates B02, B05, B10, B18 and B27. Esterase profiles appeared to be very polymorphic within *M nivale*, and only 2 profiles were observed in more than 1 isolate. One of these, profile A, was present in the 7 ITS group 1 isolates.

It is thus possible to characterize the members of a sub-group within a wide range of European isolates of *M nivale* using either RFLP ITS profiles or isozymes. Moreover, the ITS of all 48 RAPD-characterized isolates used by Lees *et al* (1995) have subsequently been examined in order to establish the correspondence of the groupings characterized by RAPD and RFLP of the ITS region. The results (not shown) indicated that the sub-group 1 as defined by ITS and isozyme profiles among European isolates correspond to the var *majus* as defined on the basis of RAPDs among British isolates. The *majus* group is widely distributed and is not restricted to the United Kingdom. It exists in all the European countries considered in this study, *ie* France, Germany, Poland and Switzerland.

The considerable polymorphism within the remaining isolates observed for esterase in the present work, and RAPDs in the work of Lees *et al* (1995), suggests that this may represent a diverse group, or may be comprised of further sub-groups. However, RFLP analysis of the ITS region revealed a single polymorphism for *Cfo* I among these isolates. In the absence of further information we have chosen to continue to treat this as a single heterogeneous group which we refer to as var *nivale.*

Variation for these characters was also detected at the field level. Two of 6 isolates (N48 and N62) from a single field were var *majus* while the remaining isolates (N58, N81, N88 and N90) differed in their esterase profiles (M, R, S and C) and aggressiveness on wheat seedlings. Thus it appears that a highly heterogeneous population may occur at the single field level.

All isolates from turf grass and rye were pathogenic on wheat and, in addition, all failed to produce perithecia *in vitro.* These observations concur with reports by Smith (1983), Harris (1986) and Litschko and Burpee (1987). All these isolates were assigned to var *nivale* on the basis of esterase and ITS rDNA profiles. It is interesting to note that, to date, no var *majus* isolates have been reported from turf grass, whereas both varieties may be found on wheat. Thus, the 2 forms may exist simultaneously in 1 ecological niche, the wheat crop, while only 1, var *nivale* appears to be present on turf grass and rye.

Despite the range of variation among the isolates of *M nivale* tested, their pathogenicity on seedlings was not related to any of the criteria used to discriminate groups among the population. The variation of DI was similar in both varieties, and no difference in aggressiveness according to the host of origin was observed. Because all isolates are pathogenic on wheat and exhibit variation for aggressiveness, isolates of either type may be used to screen variability of wheat for resistance against *M nivale*, provided that the isolate chosen is highly aggressive and produces the large numbers of conidia necessary to carry out artificial inoculation. It may be interesting to examine a larger number of turf grass and rye isolates in order to compare their pathogenicity on wheat and turf grass and to confirm that these isolates are all var *nivale* and are all heterothallic.

This study has provided further evidence of variation within *M nivale* and supports the presence of at least 2 groups among populations of *M nivale*. It has been demonstrated that considerable variation exists within the *nivale* group whereas the level of diversity is much lower within the *majus* group. A more detailed study of the *nivale* group is now required to determine the

mechanisms by which polymorphism is generated and maintained. Homothallic and apparently heterothallic isolates were present in both groups. Further work is required to determine whether mating groups exist within the heterothallic isolates, or whether isolates are merely unable to produce perithecia under the culture conditions provided.

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