Genetic diversity of *Microdochium nivale* isolates from turfgrass

G. S. MAHUKU[1], TOM HSIANG[2]* AND L. YANG[2]

[1] *Agriculture and Agri-food Canada, Charlottetown Research Centre, P.O. Box 1210, Charlottetown, PEI, Canada C1A 7M8*
[2] *Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1*

Conserved primers were used in a polymerase chain reaction to amplify the ITS region of the rDNA of 100 *Microdochium nivale* isolates collected from different turfgrasses in southern Ontario. The profile of the restriction digestion of the amplified ITS region revealed that all the *M. nivale* isolates analysed belonged to var. *nivale*. RAPD profiling and RFLP analyses of the IGS regions of rDNA revealed extensive genetic diversity within var. *nivale*. With RAPD markers, the average similarity coefficient was 66% and the estimate of genotypic diversity was 0·179. Population subdivision analysis showed that 92·2% of the total genetic diversity was found among individuals within populations compared to 7·8% among populations. In dendrograms derived from genetic distances using RAPD and IGS-RFLP markers, there was some evidence for host specialization. Most RAPD markers were shared by individuals from different turfgrasses, and the populations were not highly differentiated. The high level of genotypic diversity detected within populations and the low level of genetic differentiation among populations show that recombination and migration are likely playing important roles in the population biology of *M. nivale* var. *nivale*.

Pink snow mould, caused by *Microdochium nivale* (Fr.) Samuels & I. C. Hallett (teleomorph: *Monographella nivalis* (Schaffnit) E. Müll.) is the most common low-temperature fungal disease of turfgrass (Smiley, Dernoeden & Clarke, 1992). The fungus is remarkably active at temperatures slightly above freezing and damages turf that is dormant or growing slowly due to low temperatures. It is favoured by low to moderate temperatures, plenty of moisture, long-lasting and deep snow cover, snow on unfrozen ground and alkaline soils (Smith, 1983; Smiley *et al*., 1992). On diseased turfgrass, *M. nivale* produces copious quantities of pink sporodochia, contributing to the pinkish appearance and hence the name pink snow mould. Conidia from sporodochia are considered a possible source of inoculum for the spread of pink snow mould. Sexual spores may be less important since sexual fruiting structures of this organism are seldom observed on turfgrass (Smith, 1983; Litschko & Burpee, 1987; Vargas, 1994).

Gams & Muller (1980) divided isolates of *M. nivale* from cereal hosts into two varieties: var. *majus* and var. *nivale*, the former having larger conidia. Other workers (Nelson, Toussoun & Marasas, 1983; Litschko & Burpee, 1987) could not discriminate isolates of var. *majus* from var. *nivale* using conidial morphology, conidiogenesis, response to fungicides or asexual compatibility among thalli, and therefore suggested that distinct varieties of *M. nivale* did not exist. Recently, Lees *et al*. (1995) used randomly amplified polymorphic DNA (RAPD) to confirm the existence of distinct varieties within *M. nivale* isolates collected from cereals. Parry *et al*. (1995) used restriction analysis of PCR amplified internal transcribed spacer (ITS) region of rDNA from cereal isolates to demonstrate the presence of two *M. nivale* varieties. One RFLP pattern correlated to the var. *majus* as determined by conidial morphology, and the other (without the *Rsa* I site) corresponded to the fairly heterogeneous *nivale* variety. There has, however, been no molecular characterization of *M. nivale* isolates from turfgrass.

Lees *et al*. (1995) observed a high level of uniformity in the RAPD profiles of isolates belonging to var. *majus* and concluded that, in general, this variety reproduced homothallically in nature. Smith (1983) also readily obtained perithecia of *M. nivale* when the fungus was cultured on sterilized cereal straw. Isolates from cereals produced perithecia but not those from perennial grasses (Smith, 1983). Lees *et al*. suggested that var. *majus* may colonize cereals more often than var. *nivale*. These results were confirmed by Parry *et al*. (1995), who showed that over 70% of isolates obtained from wheat stem bases and 93% of isolates recovered from contaminated grain were var. *majus*. Variety *majus* is homothallic because it produces perithecia freely when unpaired in culture, whereas the turfgrass isolates examined were mainly var. *nivale* because they produced perithecia only when paired (Litschko & Burpee, 1987; Lees *et al*., 1995). This characteristic led Smith (1983) to speculate that isolates of *M. nivale* from cereals may be genotypically, and possibly phenotypically, distinct from turfgrass isolates. Litschko & Burpee (1987) however, found sexual compatibility between isolates of *nivale* and *majus* that had been identified on the basis of conidial morphology,

* Corresponding author.

indicating that the distinction between the two varieties may be of questionable significance.

Several types of molecular markers could be used to answer the question of varietal specialization of *M. nivale* on turfgrasses, and to give insights into the population structure and genetic diversity of this organism. RAPD analysis has been used previously to examine genetic diversity in several fungi (e.g. Grajal-Martin, Simon & Muehlbauer, 1993; Huff, Bunting & Plumley, 1994). RFLP analysis of the ITS and intergenic spacer (IGS) regions of ribosomal DNA have also been used to detect genetic diversity in fungi (e.g. Woudt *et al*., 1995; Gac *et al*., 1996). The use of more than one type of molecular marker is able to provide supportive and complementary evidence in studies of genetic variability (Edel *et al*., 1995; Arora, Hirsch & Kerry, 1996).

The purpose of this study was to examine genetic variability in *M. nivale* collected from turfgrass using RAPD, ITS and IGS-RFLP markers. This study was designed to answer the following questions: (i) which varieties of *M. nivale* occur on turfgrasses in our area; (ii) how much genetic diversity is present within and between *M. nivale* populations from turfgrass; and (iii) is there host specialization of *M. nivale* on different turfgrass species?

MATERIALS AND METHODS

Collection of isolates

Samples of turfgrass exhibiting pink snow mould symptoms were collected in April 1996, from four sites hereon designated as populations. Population is used here loosely to refer to a collection site rather than to imply a local interbreeding unit since the local sexual habits of this fungus are uncertain. Populations 1–3 came from the Guelph Turfgrass Institute, Guelph, Ontario, Canada. At their closest sampling points, population 1 was 50 m from population 2, and 150 m from population 3. Populations 2 and 3 were a minimum of 200 m from each other. Population 4 came from a commercial golf course in Cambridge, 20 km from Guelph. Populations 1 and 4 were found on *Agrostis palustris* (creeping bentgrass). Population 2 was from *Lolium perenne* (perennial ryegrass), and population 3 was from *Poa pratensis* (Kentucky bluegrass). At each location, 32 spots were sampled 4 m apart along a transect. Leaf blade samples were brought back to the laboratory and stored at 4 °C. To isolate *M. nivale*, whole leaves of creeping bentgrass or 1 cm segments of Kentucky bluegrass or perennial ryegrass were surface sterilized in 1% hypochlorite for 20 s and rinsed three times for 10 s in autoclaved water. The samples were placed onto potato dextrose agar (PDA) and incubated for 2 wk at 15°. After characteristic sporodochia were produced (Smiley *et al*., 1992), a plug of mycelium was removed and subcultured onto fresh PDA for incubation at 15°. Sporodochia were removed from these subcultures and placed in 1·5 ml Eppendorf tubes containing 500 µl of sterile distilled water and suspended by vortexing briefly. Aliquots (100 µl) of this suspension were removed and spread on PDA with a sterile glass rod. Dishes were left open to dry under a laminar flow hood and then placed in a 15° incubators overnight. Single germinated conidia were located by microscopy under 40 × magnification, transferred to separate PDA plates, and incubated at 15°. Mycelial plugs were taken from these plates for permanent stock culture PDA slants and also to produce mycelium for DNA extraction. One hundred isolates of *M. nivale* were used in this study. There were 19 isolates in population 1, and 27 isolates each in populations 2, 3, and 4 (Table 1).

Genomic DNA extraction

Single spore isolates were cultured for 7–10 d at 15° on PDA overlaid with a cellophane membrane sheet (Flexel Inc., Atlanta, GA, U.S.A.). Mycelium was scraped from the surface of each membrane for each isolate, and DNA was extracted using the DNAzol™ genomic DNA isolation reagent (Life Technologies, Burlington, ON, Canada). The DNA concentration was estimated by comparison with a lambda DNA-*Hin*d III digest marker (Promega, Unionville, ON, Canada) on a 0·7% agarose gel stained with ethidium bromide. DNA solutions were then diluted in 0·1 × TE buffer to a final concentration of approximately 12·5–25 ng µl^{-1}.

ITS analysis

ITS analysis was conducted to determine the variety of *M. nivale* occurring locally on turfgrasses following Parry *et al*. (1995). The primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White *et al*., 1990), were used to amplify the region containing ITS1, 5·8S and ITS2 sequences between the 18S and 28S rDNA. Amplification reactions were conducted in a volume of 12·5 µl containing 1 µl of genomic template

Table 1. Population, sample size, host, location, and number of IGS and RAPD haplotypes of *Microdochium nivale* isolates in this study

Population*	Sample size	Host	Location†	IGS haplotypes‡	RAPD haplotypes§
1	19	*Agrostis palustris*	Guelph	12	17
2	27	*Lolium perenne*	Guelph	17	27
3	27	*Poa pratensis*	Guelph	14	27
4	27	*Agrostis palustris*	Cambridge	18	25

* Isolates were collected from diseased turfgrass.

† All locations were in Ontario, Canada.

‡ IGS haplotypes are unique banding patterns revealed for each isolate after PCR amplification of the IGS region followed by digestion with the restriction endonucleases, *Cfo* I, *Hae* III and *Mbo* I.

§ RAPD haplotypes are based on unique banding patterns produced by the RAPD primers, P-135, P-147, P-149, and P-159.

(DNA (12·5–25 ng), and 11·5 µl PCR mix containing 1 unit of *Taq* DNA polymerase (Sangon, Scarborough, ON, Canada), 200 µM of each dNTP, 1 × PCR buffer (50 mM Tris-HCl at pH 8·5, 1·5 mM $MgCl_2$, 50 mM KCl, 0·1% Triton X-100), 0·1 mg ml^{-1} gelatine, and 0·5 µM of each primer. The PCR reactions were overlaid with 10 µl of mineral oil. Amplifications were carried out in a BioOven Thermal cycler (Biotherm, Arlington, VA, U.S.A.) with an initial denaturation step of 94° for 5 min, followed by 35 amplification cycles of 94° for 1 min, 65° for 1 min, and 72° for 4 min, and a final extension at 72° for 10 min. Negative controls (no target DNA) were included to test for the presence of DNA contamination of reagents and reaction mixtures. Aliquots (5 µl) of amplification products were electrophoresed through 1·5% agarose gels and detected by staining with ethidium bromide and visualized on a UV transilluminator.

After fragments of the appropriate size were obtained, 5–10 µl of ITS-PCR product were digested with 10 units of the restriction endonuclease *Rsa* I (Promega, Unionville, ON, Canada) according to the manufacturer's instructions. Digested DNA was run through 1·5% agarose gels containing ethidium bromide and visualized and photographed under UV light. Absence or presence and size of bands were then scored.

IGS-RFLP analysis

IGS-RFLP analysis was performed to assess genetic distances between populations of *M. nivale*. The IGS region of rDNA was amplified using the primers CLN12 = 5′-CTGAACC-GCCTCTAAGTCAG-3′ (Anderson & Stasovski, 1992) and IGS2 = 5′-AATGAGCCATTCGCAGTTTC-3′ (sequence from Dr A. Lévesque, Agriculture Canada, Vancouver, Canada) with priming sites at the 3′ end of the 28S gene and the 5′ end of the 18S gene, respectively. The size of the amplified IGS fragment of *M. nivale* was estimated based on comparison with size markers separated on a 2% agarose gel. Amplification reactions contained 1·5 µl template DNA (12·5–25 ng) and 11 µl of PCR mix containing 200 µM of each dNTP, 1 × PCR buffer, 0·1 mg ml^{-1} gelatine, 1 unit of *Taq* DNA polymerase and 0·5 µM of each primer. Thermal cycling conditions were the same as for the ITS analysis.

When fragments of the appropriate size were obtained, the IGS PCR products were digested with *Cfo* I, *Hae* III and *Mbo* I according to the manufacturer's instructions. Digested DNA was run through 1·5% agarose gels containing ethidium bromide and visualized and photographed under UV light. Photographic negatives of the amplification patterns were scanned using the Bio-Image densitometer (Millipore Corporation, Ann Arbor, Michigan) with a whole band analysis computer program to determine band position. Absence or presence and size of bands were then scored.

RAPD analysis

To assess genotypic diversity and estimate genetic distances in *M. nivale* populations, RAPD fragments were generated for all isolates. RAPD primers were selected by screening genomic DNA from eight isolates (MN003, MN017, MN023, MN040, MN049, MN070, MN078, and MN097) with different IGS-RFLP patterns. Among the 35 primers (obtained from the Biotechnology Laboratory, University of British Columbia, Vancouver, BC, Canada), 21 gave reproducible amplifications. After additional testing to optimize reaction conditions, four of them [P-135 (5′-AAGCTGCGAG-3′), P-147 (5′-GTGCGT-CCTC-3′), P-149 (5′-AGCAGCGTGG-3′), and P-159 (5′-GAGCCCGTAG-3′)] were selected for further use on the basis of reproducible polymorphic banding patterns.

DNA amplification was performed in a BioOven Thermal Cycler with one cycle at 94° for 5 min, 36° for 2 min and 72° for 2 min, followed by 35 cycles at 94° for 1 min, 36° for 1 min and 72° for 2 min and a final cycle at 72° for 10 min. The most rapid transition between temperatures was chosen (maximum of 1° s^{-1}). Reactions were carried out in 12·5 µl volumes containing 1 × PCR buffer, 200 µM of each dNTP, 0·4 µM of primer, 1 unit of *Taq* DNA polymerase and 1 µl of genomic template DNA (12·5–25 ng). DNA amplification products were separated in 1·2% agarose gels. Fragments were visualized under 300 nm UV light after staining with ethidium bromide, and photographed with black and white film (Ilford EP4).

Photographic negatives of the amplification patterns were scanned using the Bio-Image densitometer with a whole band analysis computer program to determine band position. Bands included in the final analysis ranged in size from 0·2 to 3 kb, and were scored for presence or absence.

Analysis of genotypic diversity

To estimate genotypic diversity with RAPD data, the Shannon information statistic (h_O) was calculated where $h_O = -\Sigma\, p_i \log p_i$, and where p_i is the frequency of the ith multilocus genotype (Peever & Milgroom, 1994; Goodwin, Sujkowski & Fry, 1995). To correct for differences in sample sizes between populations, genotypic diversity values were normalized by the maximum diversity in each population so that $H_O = h_O/\ln k$, where k is the sample size. We partitioned the total diversity (H_{TOT}) into within and among population components (Goodwin *et al.*, 1992). The within-population component was the proportion of the total genotypic diversity that was due to variation within populations (H_{POP}/H_{TOT}), and the among-population component was the proportion of the total genotypic diversity that was due to differences between populations [(H_{TOT}-H_{POP})/H_{TOT}]. H_{TOT} is the average genotypic diversity within the species, and was calculated by treating all genotypes as if they belonged to a single population and using the formula above. H_{POP} is the average genotypic diversity for each population and was calculated separately for each population using the formula above.

Analysis of genetic distance

To estimate the genetic distance between populations, the presence or absence of bands was determined for all individuals with IGS-RFLP and RAPD data. Similarity coefficients (S) were calculated between isolates for each restriction enzyme or primer using eqn 1 (Nei & Li, 1979):

$$S = 2N_{xy}/(N_x + N_y). \quad (1)$$

N_x and N_y are the number of fragments amplified in isolates X and Y respectively, and N_{xy} is the number of fragments shared by the two isolates. Similarity coefficients were converted to genetic distances (D) using eqn 2 (Swofford & Olsen, 1990).

$$D = (1/S) - 1. \quad (2)$$

To estimate distances between populations, averages of the distances between individuals in the populations involved were calculated for all restriction enzymes and RAPD primers, and a genetic distance matrix of population by population was generated.

Similarly, a genetic distance matrix of isolate by isolate was generated by averaging distances across all primers. The population genetic distance matrices were used to construct dendrograms with the Neighbour UPGMA method of the software package PHYLIP (version 3.55c), and dendrograms were generated using the PHYLIP program drawgram.

RESULTS

Varietal typing with ITS analysis

Primers ITS4/ITS5 amplified the ITS regions in different *M. nivale* isolates (Fig. 1*a*), generating a single band of approximately 580 bp. This corresponded with the expected size as reported by Parry *et al.* (1995). After the ITS PCR products were digested with the restriction endonuclease *Rsa* I, we found that none of our turfgrass isolates contained an *Rsa* I restriction site (Fig. 1*b*). The lack of this site defined our isolates as var. *nivale* as described by Parry *et al.* (1995).

IGS-RFLP analysis

A single PCR amplification product of approximately 4 kb, representing the IGS, was amplified uniformly from all isolates of *M. nivale* var. *nivale*, using the primers CLN12 and IGS2. Digestion of the amplified IGS fragment using three restriction endonucleases (*Hae* III, *Cfo* I and *Mbo* I) revealed extensive polymorphisms among our isolates. IGS-RFLP patterns produced after digestion with the endonuclease *Mbo* I are presented in Fig. 2. The number of restriction fragments per isolate ranged from 2 for *Mbo* I to 10 when using *Hae* III. The size of IGS estimated by summing the sizes of constituent restriction fragments varied from 3·5 to 4·5 kb depending upon the endonuclease used. Differences are likely to be a reflection of a failure to detect very small fragments or co-migrating bands. Among the 100 isolates, the restriction enzyme *Hae* III identified 23 unique haplotypes, while 25 and 36 haplotypes were identified by the enzymes *Mbo* I and *Cfo* I respectively. The combined IGS-RFLP markers identified 60 unique genotypes.

Genetic diversity using RAPD markers

The four RAPD primers revealed polymorphisms among the *M. nivale* var. *nivale* isolates. RAPD primers gave 52 consistent markers. Examples of RAPD patterns generated by primer P-

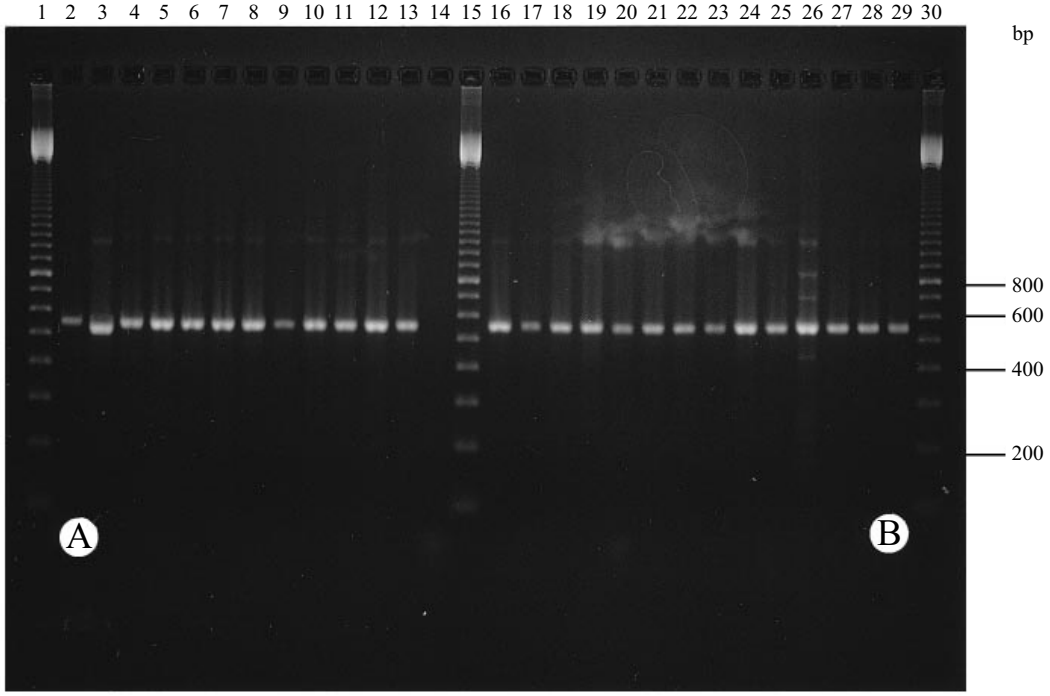

Fig. 1. (*a*) Ethidium bromide stained agarose gel of ITS-PCR products obtained with primers ITS5/ITS4 for selected *Microdochium nivale* isolates (*b*) Examples of patterns obtained following restriction digest of ITS-PCR products with the restriction endonuclease *Rsa* I. Lanes 1, 15 and 30 correspond to the 100 bp molecular weight marker. Lane 14 is control which lacked template DNA. Lanes 2–13 are *M. nivale* isolates MN001, MN003, MN004, MN024, MN025, MN028, MN051, MN058, MN059, MN074, MN088 and MN093 respectively. Lanes 16–27 contain the same *M. nivale* isolates as lanes 2–13, and lanes 28 and 29 correspond to isolates MN097 and MN098.

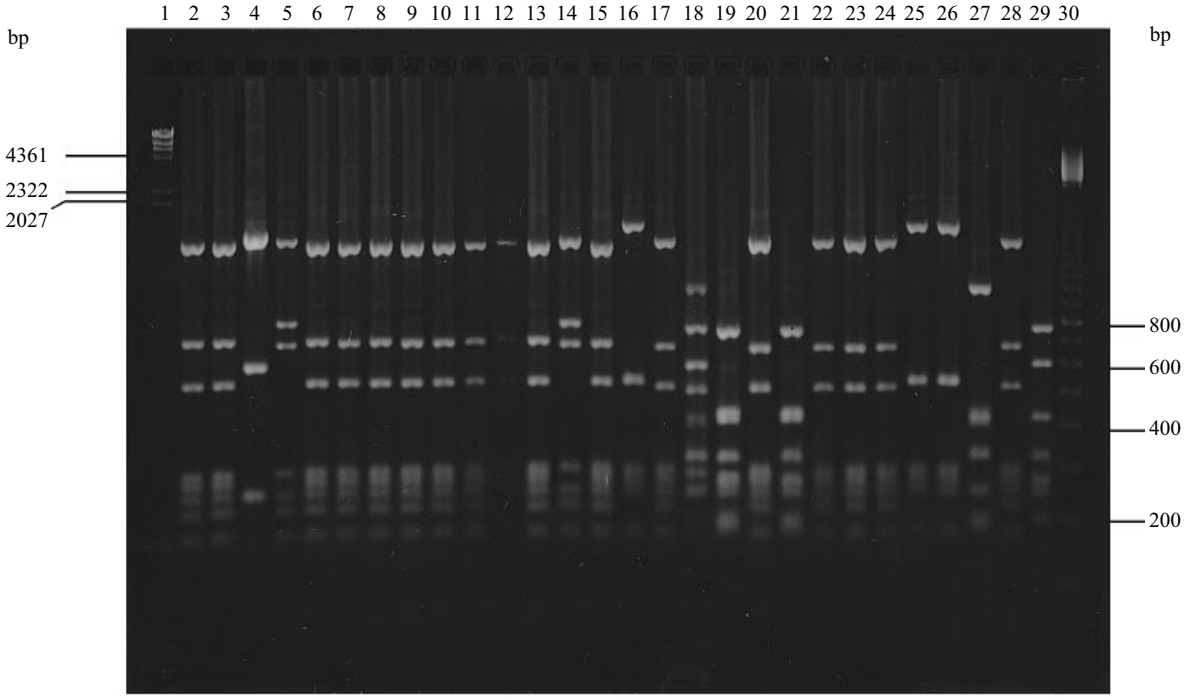

Fig. 2. Restriction fragment patterns of IGS-RFLP product amplified from selected *Microdochium nivale* isolates and digested with the restriction endonuclease, *Mbo* I. Lanes 1 and 30 correspond to the molecular weight markers, *Hin*d III digested Lambda DNA and 100 bp marker respectively. Lanes 2–29 correspond to *M. nivale* isolates MN002, MN004, MN005, MN006, MN007, MN008, MN009, MN010, MN011, MN012, MN013, MN014, MN015, MN016, MN017, MN018, MN020, MN025, MN026, MN027, MN028, MN029, MN030, MN032, MN033, MN034, MN035 and MN036 respectively.

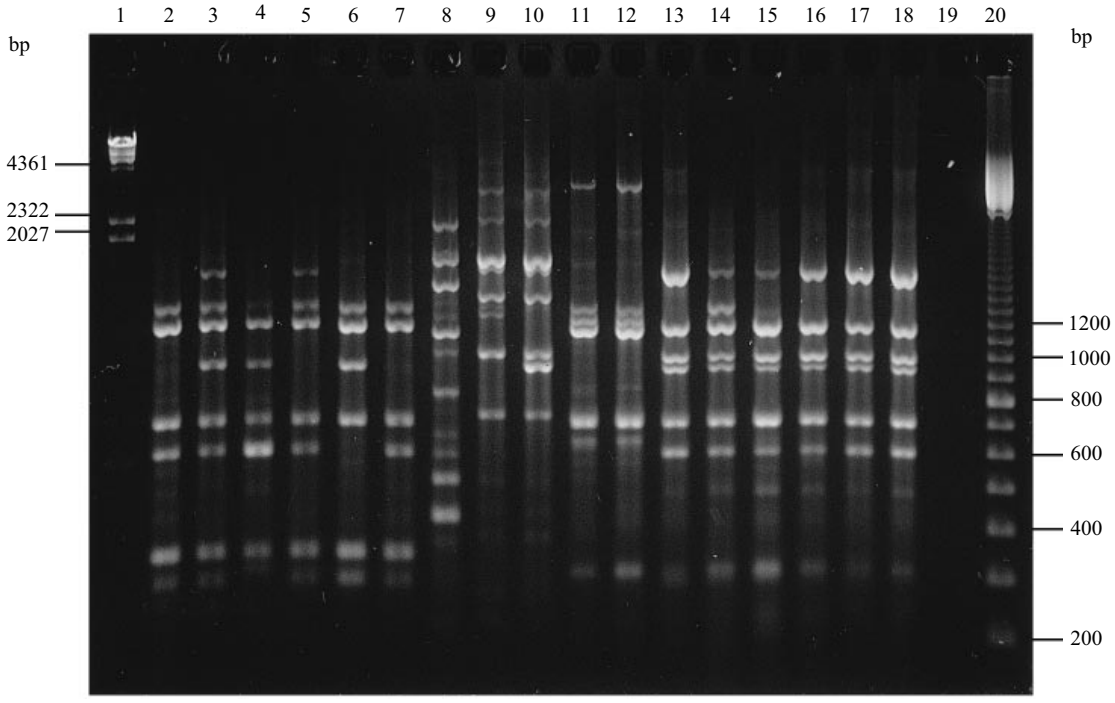

Fig. 3. RAPD patterns generated by primer P-159 for selected isolates of *Microdochium nivale*. Lanes 1 and 20 correspond to *Hin*d III digested Lambda DNA and 100 bp molecular weight marker respectively. Lanes 2–18 are *M. nivale* isolates MN001, MN003, MN004, MN011, MN024, MN025, MN028, MN029, MN030, MN036, MN071, MN078, MN083, MN094, MN095, MN098 and MN099 respectively. Lane 19 is the control which contained no template DNA.

159 are given in Fig. 3. The primers differed with P-149 providing a significantly higher estimate of genotypic diversity (Table 2). A dendrogram constructed from a matrix of isolate by isolate genetic distances (Swofford & Olsen, 1990) showed 96 distinct genotypes out of 100 isolates (Fig. 4). These genotypes could be clustered into four major groups, corresponding to their population of origin, although clusters were not always strongly separated. A small set of outliers

Table 2. Estimates of genotypic diversity (H_O) within populations of *Microdochium nivale* sampled from four populations near Guelph, Ontario, Canada

Primer	Population 1	Population 2	Population 3	Population 4	Mean*
P-135	0·192†	0·197	0·158	0·161	0·177 ab
P-147	0·162	0·165	0·200	0·148	0·169 b
P-149	0·239	0·214	0·185	0·208	0·212 a
P-159	0·184	0·140	0·176	0·128	0·157 b
Mean	0·194	0·179	0·180	0·161	

* Means in this column followed by the same letter are not significantly different from each other according to Duncan's Multiple Range Test ($P = 0{\cdot}05$). There were no significant differences between means of the different populations.

† Genotypic diversity was estimated for RAPD data based on the Shannon information statistic: $H_O = -\Sigma\, p_i \log p_i / \ln k$, where p_i is the frequency of the ith multilocus genotype and k is the sample size (Goodwin *et al.*, 1995).

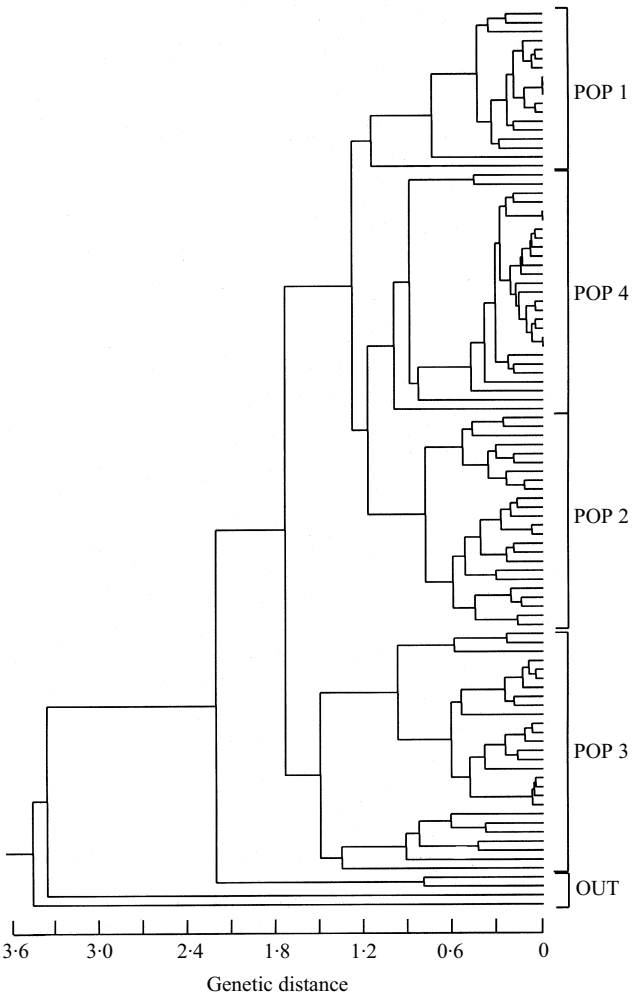

Fig. 4. Dendrogram of *Microdochium nivale* isolates based on UPGMA cluster analysis in the program PHYLIP version 3.55c using genetic distances calculated from RAPD-PCR analysis with four primers. POP refers to populations, and OUT consists of outlier genotypes from populations 1 and 2.

consisted of highly distinctive genotypes from populations 1 and 2 (Fig. 4).

The Shannon information statistic (Goodwin *et al.*, 1995) was used to quantify the genotypic diversity revealed by RAPD markers. Values of H_O (size-adjusted genotypic diversity within populations using data from all loci) ranged from 0·161 to 0·194 and were not statistically different (Table 2). Average genotypic diversity within the populations was 0·179, while average genotypic diversity in the species was 0·194 (Table 3). The Shannon diversity index showed that 92·2% of the total genotypic diversity identified was attributable to variation within populations of *M. nivale* var. *nivale*, as opposed to 7·8% among them (Table 3). These results indicate that there was little differentiation of local *M. nivale* var. *nivale* populations and that genotypic diversity was high both within the total sample and within individual populations.

Table 3. Partitioning of genotypic diversity of *Microdochium nivale* estimated from RAPD analysis into within- and among-population components

	Component of genotypic diversity			
Primer	H_{POP}*	H_{TOT}†	H_{POP}/H_{TOT}‡	$(H_{TOT}-H_{POP})/H_{TOT}$§
P-135	0·177	0·192	0·922	0·078
P-147	0·169	0·174	0·971	0·029
P-149	0·212	0·234	0·906	0·094
P-159	0·157	0·177	0·887	0·113
Mean	0·179	0·194	0·922	0·078

* H_{POP} is the average genotypic diversity within populations based on the Shannon information statistic following Goodwin *et al.* (1995).

† H_{TOT} is the average genotypic diversity across all samples.

‡ H_{POP}/H_{TOT} is the proportion of the total genotypic diversity that is ascribed to variation between individuals within a population.

§ $(H_{SP}-H_{POP})/H_{SP}$ is the proportion of total genotypic diversity due to differences among populations.

Genetic distance of populations

Genetic distances between populations were calculated based on the number of shared and exclusive IGS-RFLP or RAPD fragments (Swofford & Olsen, 1990). These were used to generate distance matrices (Table 4) and dendrograms (Fig. 5). With both types of markers, isolates in population 3 were most genetically distant from the other populations although these isolates were geographically close to populations 1 and 2. Populations 1 and 4 clustered together most closely. This could be an indication of host specialization since the two populations arose from creeping bentgrass while the remaining two populations were from two other turfgrass species (Table 1).

DISCUSSION

This is the first study of genetic variability at the molecular level of isolates of *M. nivale* from turfgrass. Restriction digestion of the amplified ITS segment with *Rsa* I resolved

Table 4. Genetic distances between *Microdochium nivale* populations estimated from IGS-RFLP and RAPD analyses

	Population 1		Population 2		Population 3		Population 4	
	IGS-RFLP	RAPD	IGS-RFLP	RAPD	IGS-RFLP	RAPD	IGS-RFLP	RAPD
Population 1	0·100*	0·467						
Population 2	0·173	0·656	0·191	0·462				
Population 3	0·368	0·754	0·424	0·729	0·141	0·430		
Population 4	0·152	0·549	0·190	0·604	0·407	0·640	0·160	0·243

* Distances were calculated based on the method described by Swofford & Olsen (1990). The variability within populations is represented by the leading diagonal. The remaining components are based on the means of distances between all individuals in the populations involved and are averaged across three restriction enzymes (IGS-RFLP) or four RAPD markers.

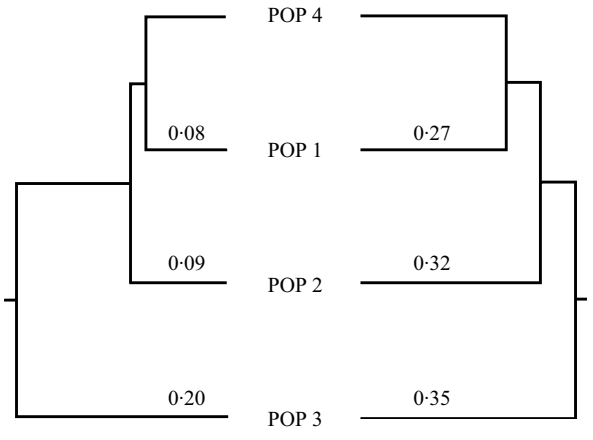

Fig. 5. Dendrogram of *Microdochium nivale* populations based on UPGMA cluster analysis in the program PHYLIP version 3.55c using genetic distances calculated from IGS-RFLP analysis (left side) or RAPD-PCR analysis (right side). The genetic distance data can be found in Table 4.

only one form that correlated to var. *nivale* described by Parry *et al*. (1995). The present study confirms that findings of Smith (1983) and Smiley *et al*. (1992) who contend that only var. *nivale* is found on perennial grass hosts.

Analysis of the IGS region revealed considerable genetic diversity between our isolates of *M. nivale* var. *nivale*. The IGS region is part of the rDNA repeat unit which occurs in a tandem array on one or more chromosomes (Appel & Gordon, 1995). This region separates the highly conserved rDNA genes and has been reported to be the most rapidly evolving spacer region (Hillis & Dixon, 1991). Variation within the IGS region has been used to reveal intra-specific variation and to characterize the population structure of several fungi including *Fusarium oxysporum* (Edel *et al*., 1995; Appel & Gordon, 1995; Woudt *et al*., 1995; Woo *et al*., 1996), *Pythium ultimum* (Buchko & Klassen, 1990), *Colletotrichum lindemuthianum* (Fabre *et al*., 1995), *Verticillium chlamydosporium* (Arora *et al*., 1996) and species of *Laccaria* (Henrion, Letacon & Martin, 1992).

In this study, RFLP analysis of the IGS region with three restriction enzymes demonstrated variation within and between populations and was able to distinguish 60 genotypes out of 100 isolates of *M. nivale* var. *nivale*. IGS-RFLP data are considered to represent only a single locus, and hence may not provide a proper estimate of genotypic diversity. They are however, useful for assessing genetic diversity at the population level and higher. Their value also lies in their ease of use and high reproducibility.

The presence of genetic diversity was confirmed by RAPD analysis. RAPD analysis identified a total of 96 genotypes out of the 100 isolates. In a dendrogram based on genetic distances, these 96 genotypes clustered according to their population of origin with just a few exceptions. When both types of markers were combined, all 100 genotypes were found to be unique. RAPD markers are more likely to be more informative in phylogenetic studies because they sample the entire genome compared to IGS-RFLP which only targets a highly variable region (Woo *et al*., 1996).

For both types of markers, there were indications of host specialization since the two populations arising from the same host turfgrass species (creeping bentgrass) clustered closely together even though they were 20 km apart. Furthermore, one of these two populations was within 500 m of the remaining populations on other turfgrass hosts, and yet was much more genetically distant from these. However, both IGS-RFLP and RAPD markers indicated that the populations were not highly differentiated and thus less likely to be highly specialized. Samples from a greater number of populations would be needed to answer this question of physiological specialization.

Calculated from RAPD data, Shannon's diversity index showed that 92·2% of the total genotypic diversity was accounted for by variation among individuals within populations. Furthermore, genetic diversity between populations was very low indicating that there is very little differentiation between populations. The presence of high genotypic diversity within populations (all isolates had unique genotypes) with low levels of genetic differentiation among geographical locations is consistent with the hypothesis that homogenizing forces such as migration result in low levels of differentiation between geographical locations, and that sexual recombination and dissemination result in high levels of local genetic diversity.

Most of the markers examined were shared among the four populations, indicating extensive mixing of genes, possibly through migration of isolates. Such movement of genotypes could have occurred by the utilization or maintenance of turfgrass at different locations (Landschoot, 1993), and the transport of contaminated maintenance equipment, or infected sod (Smith, Jackson & Woolhouse, 1989). Further evidence

supporting gene flow between populations is the low level of differentiation observed in this study. Similarly, low levels of genetic differentiation (ranging from 0 to 0·2) have been reported in fungi known to have extensive gene flow (Leung & Williams, 1986; Fry *t al*., 1991; Boeger, Chen & McDonald, 1993).

Genetic similarity between isolates ranged from 24 to 100%, with an average of 66%. A similar level of variation was reported among cereal isolates of this variety by Lees *et al*. (1995), who suggested that heterothallic reproduction may be common in this variety in nature. High levels of variability have also been reported for other sexually reproducing fungi but are absent in asexually reproducing populations of the same fungus (Burdon & Roelfs, 1985; Hoffman, 1986; Milgroom, 1996). On the other hand, Groth, McCain & Roelfs (1995) found that genetic polymorphisms were no less prevalent in asexual collections of *Uromyces appendiculatus* than sexual ones. They suggested that even a low level of sexual reproduction in a primarily asexually reproducing population is sufficient to maintain genetic variability.

Genotypic diversity in local populations may arise from different factors, depending on source and type of inoculum, migration, sexual recombination, genetic drift and selection (Huff *et al*., 1994; Morjane *et al*., 1994; Peever & Milgroom, 1994). Forces such as the founder effect and random genetic drift followed by selection may act to reduce genetic diversity (Kohn *et al*., 1988; McDonald *et al*., 1989; Milgroom, Lipari & Powell, 1992), while immigration of propagules from outside areas, or the presence of a sexual state would increase genetic diversity (Morjane *et al*, 1994). Selection over time might either amplify or reduce the presence of certain genotypes (Morjane *et al*., 1994). Although sexual reproduction in *M. nivale* var. *nivale* has been demonstrated under controlled conditions (Lees *et al*., 1995; parry *et al*., 1995), the extent and frequency of sexual reproduction of this variety in nature are unknown. The results of this study suggest that sexual reproduction and migration or dissemination of propagules are important in maintaining high genetic variability within populations of *M. nivale* var. *nivale*. Allelic markers and linkage disequilibrium analysis would, however, be required to demonstrate whether or not sexual recombination does occur.

In local populations of *M. nivale*, we found only var. *nivale* on turfgrass with very high levels of genetic diversity. We conclude that the sampled field populations of *M. nivale* var. *nivale* were genetically heterogeneous, and that this heterogeneity could be reliably determined by IGS-RFLP analysis or even more precisely by RAPD analysis. The sources of this genetic diversity and the full extent of host-specialization of this organism remain to be determined.

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