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Mitochondrial DNA diversity and lineage determination of European isolates of *Fusarium graminearum* (*Gibberella zeae*)

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Abstract

Restriction fragment length polymorphisms (RFLPs) were used to assess genetic diversity of mitochondrial DNA (mtDNA) among standard isolates of seven lineages of *Fusarium graminearum*. The mtDNA patterns within each lineage were very similar (>89%), whereas significant differences were observed between the isolates belonging to different lineages, with the exception of lineages 1 and 4 where strong similarity was found between the RFLPs. Analysis of different band patterns resulted in characteristic *Hha*I and *Hae*III bands that were suitable for identification of members of lineages 7, 6, 5, 3 and 2. Investigation of lineage distribution of 144 European isolates revealed that 142 belong to lineage 7. These data, therefore, confirmed the hypothesis that members of lineage 7 are predominant in Europe. Further analysis of isolates belonging to lineage 7 resulted in five haplotypes. These haplotypes have arisen as different combinations of three RFLP patterns for both *Hae*III and *Hha*I restriction enzymes. Two isolates from Hungary, however, shared the same mtDNA RFLP profiles with a standard isolate of lineage 3, indicating that members of lineage 3, at a lower frequency, may also occur in Europe.

Introduction

Fusarium graminearum (teleomorph *Gibberella zeae*) causes serious foot rot and head blight diseases of small-grain cereals, grasses, and ear and stalk rot of maize (Snijders, 1990; Bottalico, 1998). Severe *Fusarium* head blight (FHB) epidemics have been reported in the United States (McMullen et al., 1997), Europe (Bottalico, 1998) and East Asia (Chen et al., 2000). Grain is often contaminated with trichothecene and oestrogenic mycotoxins, making it unsuitable for food and feed (Bottalico, 1998).

Investigation of the global diversity of *F. graminearum* based on the DNA sequence of six single-copy nuclear genes has revealed the existence of seven distinct phylogeographic lineages that appear to be reproductively isolated (O'Donnell et al., 2000). Because each lineage is represented by two to seven isolates only, additional data is needed to further characterise the geographic distribution of the seven *F. graminearum* lineages. In the work of Gale et al. (2002), 225 isolates of *F. graminearum* from wheat were investigated using restriction fragment length polymorphisms (RFLPs). Diagnostic RFLP markers established that all isolates belonged to *F. graminearum* lineage 6 ('Asian clade'). Lineage 7 is geographically more widespread and appears to be the dominant or sole lineage found in the Americas and in European countries ('Pan-Northern Hemisphere clade'), but to date there are limited data on lineage distribution of *F. graminearum* isolates.

The phylogeographic lineages of *F. graminearum* cannot be differentiated on the basis of morphological traits. Gale et al. (2002) identified nuclear DNA fragments that could be used for developing diagnostic RFLP markers for identification of *F. graminearum* lineages. Mitochondrial

DNA (mtDNA) is a compact circular DNA molecule of relatively small size (20–100 kb) that is well suited for RFLP analysis. Because of the rapid rate of evolution of mtDNA, which is estimated in animals to be 10 times faster than that of the nuclear DNA (Brown et al., 1979), analysis of mtDNA provides a sensitive measure of divergence. RFLP of mtDNA has been suggested as a molecular method suitable for analyses at the subspecies level (Avise, 1989). Whereas O'Donnell et al. (2000) indicated that the phylogeographic lineages of *F. graminearum* were reproductively isolated, RFLP analysis of their mtDNA may provide an excellent diagnostic marker for differentiation and identification of different lineages. Furthermore, mtDNA RFLP of *F. graminearum* isolates can be used to measure genetic diversity within populations as well as to compare different populations. In addition, the mtDNA RFLP using frequently cutting enzymes is a more time- and labour-efficient technique than other mtDNA and nuclear DNA RFLP methods used for *Fusaria*. The objectives were to develop mtDNA RFLP markers to distinguish between phylogenetic lineages within *F. graminearum* and to determine the lineage(s) that are predominant in Europe, especially in Hungary. In addition, to achieve differentiation and identification of lineages, the genetic diversity within the European population was also surveyed.

Materials and methods

Fungal strains and mycelium preparation

Standard isolates of the seven lineages of *F. graminearum* and the standard European isolates used are listed in Table 1. Other isolates (11 from Serbia-Montenegro, and 111 from Hungary obtained from various geographical areas) were isolated from barley, maize and wheat in 2000–2001. Wheat and barley seeds, and pieces of maize stalks were immersed for 30 s in 70% ethanol, surface sterilised in 10% commercial bleach (final concentration of 0.5% sodium hypochlorite), and briefly rinsed in sterile water before placing them on a *Fusarium*-selective agar containing pentachloronitrobenzene (Nelson et al., 1983); one *Fusarium* isolate from each plant sample was purified by single-spore isolation. For morphological identification, sporulation was promoted by carnation-leaf agar (CLA) plates (Nelson et al., 1983) at ambient temperature under neonlight/$UV_{360\ nm}$ with a photoperiod of 12 h until sporulation occurred. Perithecium production was determined (Windels et al., 1989). For DNA extraction, isolates were grown initially in 10 ml carboxymethyl cellulose (CMC) broth (15 g l^{-1} CMC, 0.1% NH_4NO_3, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.1% yeast extract) in a 50 ml Erlenmeyer flask inoculated with three mycelial discs (5 mm diam) from young colonies growing on potato dextrose agar (Nelson et al., 1983). Cultures were incubated for three days on an orbital shaker (120 rpm) at 25 °C, then the 10 ml of culture were transferred to 100 ml YM broth (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 2.0% D-glucose) in a 250 ml Erlenmeyer flask and incubated for two additional days on an orbital shaker (120 rpm) at 25 °C. Young mycelia were vacuum-filtered on Whatman No. 1 filter paper and washed three times with 50 ml of distilled water. The mycelial mats were lyophilised, ground with a mortar and pestle, and stored at −20 °C.

DNA isolation, restriction enzyme digestion and electrophoresis

DNA was isolated by the method of Leach et al. (1986). For total DNA isolation we used 30 mg lyophilised mycelium powder and diluted the extracted DNA to a final volume of 100 µl with TE (150–200 ng DNA μl^{-1}). Restriction enzymes (New England Biolabs Inc., Beverly, MA) were used to digest 15 µl of the DNA sample in a final volume of 100 µl overnight following manufacturer's instructions. We carried out gel electrophoresis in 0.8% horizontal agarose gels overnight (≅ 16 h). Gels were stained with ethidium bromide and visualised by UV transillumination.

Data analysis

The molecular weights of the DNA bands were estimated with a ChemiImager 4000 Low Light Imaging System (Alpha Innotech Corporation, CA, USA). The data were organised into a binary matrix, and a pairwise similarity matrix was generated with the Nei and Li coefficient (Nei and Li,

Table 1. Standard *F. graminearum* isolates used in this study

Isolate code	Lineage	Geographical origin	Host/substrate	Source
NRRL 2903	1	Brazil	Polypore	1
NRRL 28585	1	Venezuela	Herbaceous vine	1
NRRL 28718	1	Brazil	Corn	1
NRRL 28436	2	New Caledonia	Orange twig	1
NRRL 28723	2	Nepal	Corn	1
NRRL 29020	3	South Africa	Corn	1
NRRL 29011	3	South Africa	–	1
NRRL 25797	4	Honduras	Banana	1
NRRL 29148	4	Pennsylvania, USA	Grape ivy	1
NRRL 26752	5	South Africa	*Acacia mearnsii*	1
NRRL 26754	5	South Africa	*Acacia mearnsii*	1
NRRL 13818	6	Japan	Barley	1
NRRL 26156	6	China	Wheat	1
NRRL 5883	7	Ohio, USA	Corn	1
NRRL 13383	7	Iran	Corn	1
CBS 166.57	7	Netherlands	Corn	
CBS 389.62	7	Netherlands	Wheat	
70106	7	Finland	Barley	2
7137	7	Finland	Feed mix	2
72235	7	Finland	Oat	2
72323	7	Finland	Wheat	2
Fg 7.9	7	Germany	Corn	3
Fg 11	7	Germany	Wheat	3
Item 644	7	Italy	*Panicus crusgalli*, stalk	4
Item 3445	7	Slovakia	Corn	4
Item 2305	7	Italy	Corn	4
Item 2194	7	Italy	Corn	4

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1979) using PhylTools programme (version 1.32; Laboratory of Plant Breeding, Wageningen University (http://www.dpw.wau.nl/pv). Dendrograms were constructed using the software package PHYLIP 3.5 (J. Felseinstein, Washington University, Seattle). Cluster analysis (UPGMA) was carried out from the distance matrix using NEIGHBOR program with random input order. A data set containing only the value '0' was used as an outgroup. To determine the statistical significance of the dendrogram branches, the data were bootstrapped with 1000 replications using the programme PhylTools.

Results

*Hae*III and *Hha*I were used to generate mtDNA RFLP profiles. The smaller RFLP fragments were less visible due to the nuclear DNA smear; therefore only bands >2000 bp were scored. The mtDNA patterns within each lineage were very similar (>89%), while significant differences were observed between the isolates belonging to different lineages (Figure 1). Similarity values were between 35% and 82% with the exception of isolates belonging to lineages of 4 and 1, where similarity within a lineage was in some cases lower than that between isolates belonging to different lineages of 4 and 1.

Using the differences between *Hha*I RFLP patterns of the lineages, lineage 5 was differentiated using a 7200 bp band, while 5850 and 6400 bp bands were only found in lineage 3 isolates. Isolates of lineage 2 had a unique 4880 bp long *Hha*I band, while lineage 6 isolates were characterised by lack of the 4450 bp band that was found in all other *Hha*I mtDNA RFLP patterns

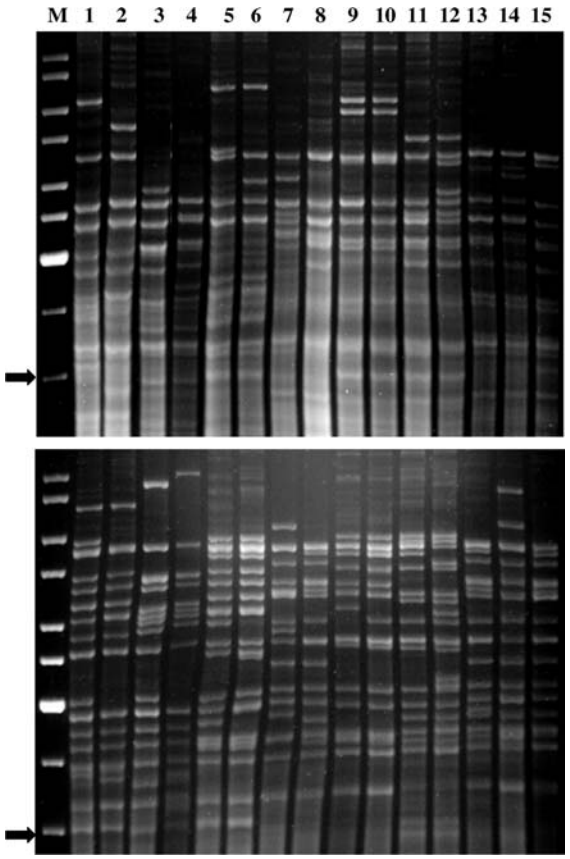

Figure 1. MtDNA RFLP patterns (1–15) detected after *Hha*I restriction enzyme (upper gel) and *Hae*III restriction enzyme (lower gel) digestions of standard isolates of each lineage of *F. graminearum*. Lanes 1 and 2, lineage 7 (NRRL 5883, NRRL 13383); lanes 3 and 4, lineage 6 (NRRL 13818, NRRL 26156); lanes 5 and 6, (NRRL 26752, NRRL 26754); lanes 7 and 8, (NRRL 25797, NRRL 29148); lanes 9 and 10, (NRRL 29020, NRRL 29011); lanes 11 and 12, (NRRL 28436, NRRL 28723); lanes 13–15, (NRRL 28718, NRRL 2903, NRRL 28585). M – 1-kb DNA ladder (Fermentas). The arrow indicates a 2000 bp band in the marker lane.

(Figure 1, upper gel). On the other hand, isolates of lineage 7 shared a unique 7500 bp band in the *Hae*III RFLP patterns (Figure 1, lower gel).

Analysis of European isolates revealed six haplotypes (I–VI). Of the 144 isolates, 142 belonged to five haplotypes (I–V), and all five contained the characteristic 7500 bp band in their *Hae*III pattern. Therefore, they were considered as members of lineage 7 of *F. graminearum*. These five haplotypes were detected as different combinations of three RFLP patterns for both *Hae*III (a1–a3) and *Hha*I (b1–b3) restriction enzymes (Figure 2). Haplotype I (a1 and b1 patterns for

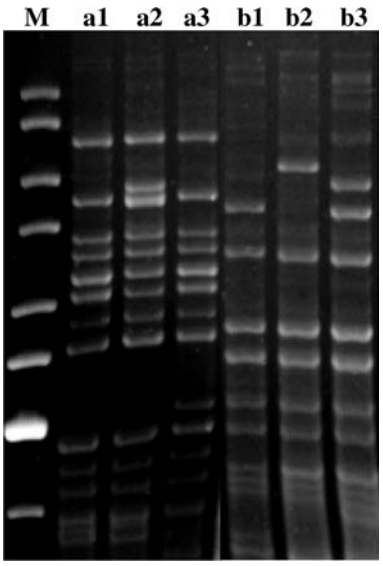

Figure 2. MtDNA RFLP patterns detected after *Hae*III restriction enzyme (a1–a3) and *Hha*I restriction enzyme (b1–b3) digestions in European isolates of *F. graminearum*. M indicates a 1 kb DNA ladder (Fermentas).

*Hae*III and *Hha*I, respectively) was the most common, containing 65 isolates (45%) from the total of 144 isolates. Haplotype II characterised by a2 and b2 patterns was also recovered frequently, accounting for 41 isolates (28%). Combinations of a3 and b1 (haplotype III), a1 and b3 (haplotype IV) and finally a2 and b3 (haplotype V) patterns were less frequent and made up only 16, 14 and 6 isolates for haplotypes III, IV, and V, respectively. The standard lineage 7 isolate NRRL 13383 represents a haplotype I isolate, while the NRRL 5883 lineage 7 isolate represents a unique *Hae*III pattern with a b2 type *Hha*I pattern (Figures 1 and 2). Similarity between each pair of haplotypes belonging to lineage 7 was high (89–98%), and did not differ from that obtained for isolates of lineage 6, 5, 3, and 2. Two isolates (haplotype VI), however, had the same pattern as that of lineage 3 isolate NRRL 29011 (Figure 1) and were, therefore, considered as members of this lineage.

The relatedness among RFLP haplotypes was analysed using UPGMA and Nei and Li's similarity matrix based on the presence and absence of mtDNA bands. The analysis clustered the haplotypes belonging to the same lineage into separate groups with the exception of lineages 4 and 1 (Figure 3). The resultant dendrogram confirmed the close relationship between the isolates

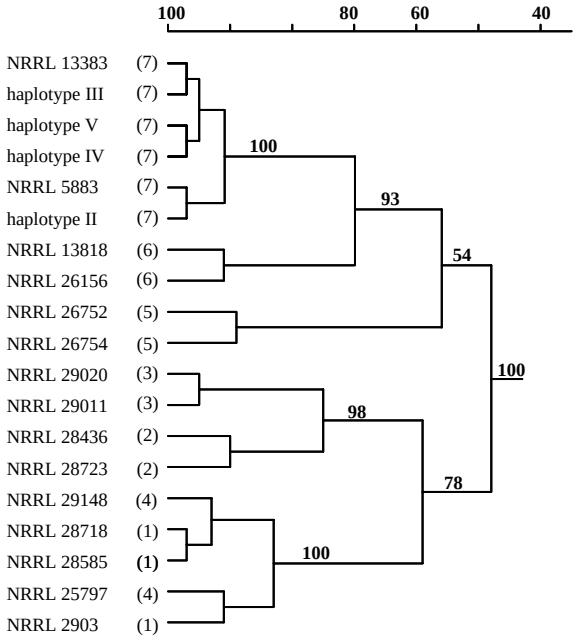

Figure 3. UPGMA cluster analysis dendrogram showing the genetic relationship of 19 mtDNA RFLP haplotypes. Numbers by nodes represent bootstrap support >50% from 1000 replications. In parentheses are the numbers of lineages of *F. graminearum*.

belonging to lineages 7, 6 and 5, but otherwise did not confirm the relationship among the seven lineages, which had been determined previously by sequence analysis (O'Donnell et al., 2000).

Discussion

Analysis of MtDNA RFLPs was used to differentiate recently described lineages of *F. graminearum*. One objective was to find RFLP bands that would reliably assign *F. graminearum* isolates into a lineage, especially lineage 7. Because the main interest was in lineage 7, the usefulness of the diagnostic probes in reliably identifying other lineages must be confirmed with larger sample sizes of remaining lineages.

The polymorphisms detected in mtDNA differed in the amount of variability depending on the restriction enzyme used. MtDNA RFLP analysis using *Hha*I restriction enzyme resulted in characteristic DNA fragments that were diagnostic for isolates of lineages 2, 3 and 5, while isolates of lineage 6 were differentiated by the absence of the 4450 bp long fragment in the *Hha*I restriction profile (Figure 1). Due to the observed diversity between *Hha*I digested mtDNAs of lineage 7 isolates (Figures 1 and 2), a specific 7500 bp long fragment in the *Hae*III restriction profile seemed to be more appropriate for identification of isolates belonging to lineage 7. The strong similarity between mtDNA RFLP patterns of lineage 4 and 1 isolates was unexpected (Figure 1). For example, *Hae*III digested mtDNA patterns of isolates NRRL 28718 and NRRL 29306 (lineage 1) and isolate NRRL 29148 (lineage 4) proved to be identical. Moreover, lineage 1 and 4 isolates were differentiated from other lineages using an easily detectable 3400 bp long band in the *Hae*III digested mtDNA pattern. In this respect, O'Donnell et al. (2000) found a relatively distant relationship between the isolates of lineages 1 and 4 in spite of their common geographic origin. The data from the present study, however, indicate a high similarity of mtDNAs of lineages 1 and 4.

Members of lineages of 2, 3, 5, 6 and 7 of *F. graminearum* were, therefore, differentiated by mtDNA RFLP using *Hae*III and *Hha*I restriction enzymes. The data confirmed the reproductive isolation of these lineages. As a diagnostic tool, this technique appears easier for identification of some lineages of *F. graminearum* compared to the RFLP method used by Gale et al. (2002), because lineage determination based on mtDNA RFLP can be obtained overnight and does not require pure DNA.

MtDNA RFLP profiles may also prove useful in verifying species identity, because neither of a selected set of isolates of *F. cerealis* or of *F. culmorum* shared the same mtDNA profile with any of the isolates belonging to any lineage of *F. graminearum* (unpublished data). Although several PCR and isozyme-based diagnostic assays have been implemented successfully for *F. graminearum* (Ouellet and Seifert, 1993; Schilling et al., 1996; Niessen and Vogel, 1997; Nicholson et al., 1998; Yoder and Christianson, 1998; Chelkowski et al., 1999; Láday and Szécsi, 2001), the usefulness of these methods could now be re-examined using standard isolates of each lineage of *F. graminearum*.

Analysis of European isolates has confirmed the applicability of the method for identifying isolates belonging to lineage 7. Of the 144 isolates of *F. graminearum*, 142 showed the characteristic

mtDNA RFLP patterns of standard isolates belonging to lineage 7. The data also confirmed the hypothesis that members of lineage 7 are dominant in Europe. Investigation of polymorphism within isolates of lineage 7 revealed five haplotypes. It would be interesting to investigate if this diversity corresponds to any phenotypical traits (aggressiveness, toxigenicity etc.).

Two isolates from Hungary shared a similar mtDNA RFLP profile with lineage 3 (isolate NRRL 29011), indicating that members of lineage 3 are present, at low frequencies, in the European populations. If isolates from these divergent populations interbreed, than there is the potential for the production of new genotypes that carry novel combinations of genes for pathogenicity, host range or toxin production (Brasier, 2000). Therefore, detection of additional isolates belonging to lineage 3 is especially important in the future. Analysis of populations, in which members of both lineages 3 and 7 co-exist, could also be a tool for examination of outcrossing and may provide a potential detection of sexual hybrids in the natural populations.

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