

HETEROGENEITY IN *PYRENOPHORA GRAMINEA* AS REVEALED BY ITS-RFLP

M.I.E. Arabi and M. Jawhar

Department of Molecular Biology and Biotechnology, AECS, P. O. Box 6091, Damascus, Syria

SUMMARY

Fifty-six isolates of *Pyrenophora graminea*, the causal agent of barley leaf stripe disease, were collected from different sites in Syria and analyzed using restriction digestion of PCR-amplified internal transcribed spacers (ITS) of ribosomal DNA. A total of 594 bands were scored of which 453 (76.3%) were polymorphic. The fingerprints generated from the six restriction digestions of the nrDNA ITS region showed high levels of intraspecific variation within the *P. graminea* population. A neighbour-joining diagram, based on Nei's genetic distances, showed that isolates formed two phylogenetic groups, and did not fall into clusters or clades specific to the origin or colour of the isolate, which suggests a regional dispersal of *P. graminea*. The molecular parameter used in this study provides crucial information for studying genetic variation in *P. graminea* and provides important information for future selection of isolates to develop durable barley leaf stripe resistance.

Key words: *Pyrenophora graminea*, leaf stripe, barley, genetic diversity, ITS-RFLP marker.

INTRODUCTION

Pyrenophora graminea [anamorph *Drechslera graminea* (Rabenh. ex Schltdl.) Ito] is a seed-borne pathogen which causes leaf stripe of barley (*Hordeum vulgare* L.), a disease responsible for heavy crop losses (Porta-Puglia *et al.*, 1986; Arabi *et al.*, 2004). The variability of the fungus is well known and has been confirmed by many reports on its virulence and morphological and physiological traits (Zriba and Harrabi, 1995; Gatti *et al.*, 1992; Jawhar *et al.*, 2000). However, despite the substantial importance of the fungus, there is a lack of appropriate and adequate information regarding population structure and genetic diversity at the molecular level.

Pathogenicity phenotypes can be useful for assessing genetic variation in fungal pathogens; however, pathogenicity markers are often limited in number and subject to host selection (Leung *et al.*, 1993). Molecular methods involving the use of the polymerase chain reaction (PCR) have recently been proposed to resolve genetic variation in various organisms. The internal transcribed spacer (ITS) region of the nuclear rRNA repeat units, evolve faster, and may vary among species within a genus or among populations (Redecker *et al.*, 1997; Hsiang and Wu, 2000; Martin and Rygielwicz, 2005).

P. graminea is capable of reproducing both sexually and asexually. This mixed reproductive system, as well as the capacity for long distance dispersal, has stimulated the study of genetic variability in *P. graminea* populations. Such a study was conducted on 56 *P. graminea* isolates collected from different regions of Syria using data on the restriction fragment length polymorphism (RFLP) of the ITS region.

MATERIALS AND METHODS

Fungal isolates. During the period 1995-2006, more than 93 isolates of *P. graminea* were obtained from barley leaves showing leaf stripe symptoms in different regions of Syria (in average ~ 400km among regions). Leaf tissues with necrotic lesions were cut into pieces (5 × 10 mm) and sterilized in 5% sodium hypochlorite (NaOCl) for 5 min. After three washings with sterile distilled water, the pieces were transferred onto Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI, USA) with 13 mg/l kanamycin sulphate added after autoclaving and incubated for 10 days, at 21 ± 1°C in the dark to allow mycelial growth and sporulation. In preliminary studies, different barley genotypes were inoculated with 93 isolates and evaluated for host-pathogen reaction and lesion formation. Subsequently, 56 monosporic isolates were selected for this study. Colony colour was recorded using an empirical scale from 1 (white) to 5 (black) (Table 1).

DNA extraction. Fifty-six isolates were grown on PDA medium for 2 weeks at 20 ± 1°C and stored at 4°C

Table 1. Location sites, ITS-RFLP polymorphism and colour of 56 isolates of Syrian *Pyrenophora graminea*.

Isolate	Location	Year of collection	Mycelia colour	Total number of bands	Polymorphic bands	Percentage polymorphism
PgSy1	ICARDA (north)*	1996	5 ^y	7	4	57.1
2	ICARDA (north)	2002	3	10	7	70
3 ^z	ICARDA (north)	1997	4	11	8	72.7
4	ICARDA (north)	2002	4	10	7	70
5	ICARDA (north)	2002	5	15	11	73.3
6	ICARDA (north)	2006	5	10	7	70
7	ICARDA (north)	1997	5	8	5	62.5
8	ICARDA (north)	2003	2	10	7	70
9	ICARDA (north)	1997	1	17	14	82.4
10	ICARDA (north)	2006	3	16	13	81.3
11	ICARDA (north)	1997	3	10	7	70
12	Daraa (south)	1999	5	15	12	80
13	Daraa (south)	1997	5	10	7	70
14	Daraa (south)	2006	2	9	6	66.7
15	Daraa (south)	1997	3	9	6	66.7
16	Daraa (south)	2002	5	12	9	75
17	Daraa (south)	2002	5	10	7	70
18	Daraa (south)	1997	1	6	4	66.7
19	Daraa (south)	2001	1	14	11	78.6
20	Daraa (south)	2003	3	9	6	66.7
21	Hassaka (north-east)	2001	1	9	6	66.7
22	Hassaka (north-east)	2006	1	9	6	66.7
23	Hassaka (north-east)	2001	1	16	13	81.3
24	Hassaka (north-east)	2001	1	7	4	57.1
25	Hassaka (north-east)	2006	2	10	7	70
26	Hassaka (north-east)	2006	3	7	4	57.1
27	Hassaka (north-east)	1997	1	8	5	62.5
28	Hassaka (north-east)	1997	1	16	13	81.3
29	Hassaka (north-east)	1997	1	13	11	84.6
30	Hassaka (north-east)	2006	5	13	11	84.6
31	Raqa (north-east)	2006	2	15	13	86.7
32	Raqa (north)	2006	2	17	15	88.2
33	Raqa (north)	2003	1	9	7	77.8
34	Raqa (north)	2003	2	10	8	80
35	Raqa (north)	2003	2	12	10	83.3
36	Raqa (north)	2002	5	13	11	84.6
37	Raqa (north)	1997	4	14	12	85.7
38	Raqa (north)	2003	5	17	15	88.2
39	Raqa (north)	2002	3	17	15	88.2
40	Raqa (north)	2001	4	7	5	71.4
41	Raqa (north)	2003	5	5	4	80
42	Raqa (north)	1999	5	9	8	88.9
43	Aleppo (north)	1996	1	8	5	62.5
44	Aleppo (north)	2003	2	6	4	66.7
45	Aleppo (north)	2003	4	11	9	81.81
46	Aleppo (north)	1995	5	10	8	80
47	Aleppo (north)	2006	3	11	9	81.81
48	Aleppo (north)	2006	1	11	9	81.81
49	Aleppo (north)	2006	5	8	5	62.5
50	Aleppo (north)	1997	5	11	9	81.81
51	Aleppo (north)	1997	5	6	4	66.7
52	Aleppo (north)	1997	4	6	4	66.7
53	Aleppo (north)	2003	4	10	8	80
54	Aleppo (north)	2006	4	7	5	71.4
55	Aleppo (north)	2006	5	9	7	77.8
56	Aleppo (north)	2006	5	9	6	66.7
Total				594	453	76.30%

* ICARDA, International Center for Agricultural Research in Dry Areas, Syria.

^y(1): white, (2): white gray, (3): gray, (4):gray black and (5): black.^zThe PgSy3 was the most virulence isolate as reported by Arabi *et al.*, (2005).

for further study. Mycelium was harvested and DNA was extracted according to standard protocols (Leach *et al.*, 1986), resuspended in TE buffer (10 mM Tris-HCl, pH 8.0; 1mM EDTA) and stored at -20°C.

ITS-RFLP. The ITS regions and the 5.8S rDNA were amplified for all isolates using the primers ITS 4R (5' TCCGTAGGTGAACCTGCGG 3') and ITS 5F (5' TCCTCCGCTTATTGATATGC 3') designed by White *et al.* (1990). Amplification reactions (25µl) contained 1x PCR buffer, 1 U *Taq* polymerase (MBI Fermentas, York, UK), 0.2 mM dNTPs, 1.5 mM MgCl₂, 0.5 µM of each primer and 10 ng of genomic DNA per reaction mixture. PCR was performed in a Gene Amp 9700 Thermocycler (Applied Biosystems, USA). Initial denaturation of 95°C for 2 min was followed by 36 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min. A final extension of 72°C for 10 min was, followed by cooling to 4°C until recovery of the samples. PCR products were separated on a 1.5% agarose gel and visualized using UV light (302 nm) after staining with ethidium bromide.

In separate reactions, 10 µl of PCR product was digested for 3 h with six different endonucleases (*AluI*, *EcoR1*, *BsuI*, *BamHI*, *RsaI* and *HindIII*) following the manufacturer's recommendations (MBI Fermentas, York, UK). The DNA fragments were size-fractionated by electrophoresis through 1.5% agarose gels. The sizes were estimated by comparison with a DNA ladder (Q.BIOgene, Heidelberg, Germany).

RFLP banding profiles for each restriction enzyme were scored for the presence (1) or absence (0) of bands. The experiments were repeated twice for each isolate to confirm the repeatability and the monomorphic bands were removed from the analysis). Neighbour-Joining diagrams and bootstrap analysis were constructed on genetic distances among populations using the Nei's distance (Saitou and Nei, 1987) by the PHYLIP package ver 3.5c (Felsenstein, 1993).

RESULTS AND DISCUSSION

PCR amplification with specific primers ITS 4R and ITS 5F yielded single DNA fragments of ~ 650 bp, present in all isolates. Table 1 shows that 594 bands were scored of which 453 (76.3%) were polymorphic and the number of DNA bands varied between 5 and 17.

The fingerprints generated from the six restriction digestions of the nrDNA ITS region demonstrated high levels of intraspecific variation within the *P. graminea* population. For *RsaI* and *BsuI*, three distinct patterns were recognized, one for each of *EcoR1*, *BamHI* and *AluI*, and five for *HindIII* (Fig. 1). The six enzymes were sufficient to distinguish between the isolates examined, when in fact only three enzymes were used to generate the polymorphic data.

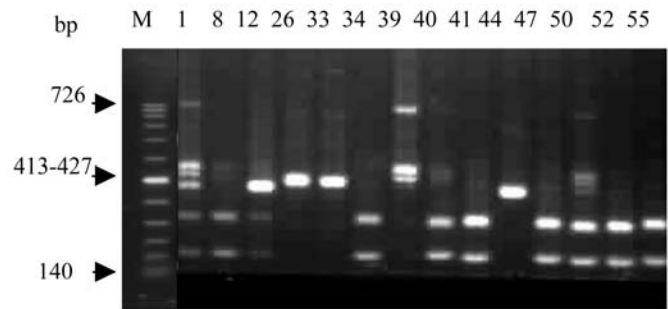


Fig. 1. Agarose gel electrophoresis of restriction fragments of the ITS-RFLP amplification products of 14 *P. graminea* isolates digested by the restriction enzyme *HindIII*. M, molecular weight marker (*HinfI*; MBI Fermentas, York, UK).

Different colony colours were observed among isolates after 10 days of growth on PDA media, but these were not correlated with geographical origin within the country. However, morphological characters such as colony colour may be subject to environmental variation and so a molecular approach was used to help identify isolates (Table 1).

Distance analysis of ITS-RFLP data clearly divided isolates of *P. graminea* into two major clades (Fig. 2), strongly supported by high bootstrap values. Clade 1 supported by a bootstrap score of 85 %, contained two major sub-clusters consisting of 39 isolates, whereas the remaining 17 isolates were placed in clade 2 that had a bootstrap value of 90. No relationship was observed between the genetic profiles and geographic origin (Fig. 2). This can be attributed to the fact that *P. graminea* has frequently been reported to be seed-borne (Zriba and Harrabi, 1995), so populations of *P. graminea* in Syria may not remain geographically isolated for long due to the possible exchange of infected seeds. This can also occur through transportation of infected seeds by birds, or spore dispersal by wind (O'Donnell *et al.*, 1998). Thus, population divergence is not always proportional to distance following the model of Wright (1943), as it is easy to find greater genetic differences among adjacent populations rather than those far apart. According to Platenkamp (1976) cultural practices as well as transport of seeds, soil and plant materials have contributed to the genetic diversity observed within geographic areas. This could also account for the lack of correlation between genetic distance and origin found among the Syrian isolates.

The patterns generated from ITS-RFLPs demonstrated variability among *P. graminea* isolates (Table 1). While this variability may have arisen through point mutations, gene flow and/or recombination (Parry *et al.*, 1995), we were not able to determine which, if any, of these particular mechanisms was responsible for the high degree of genetic diversity observed.

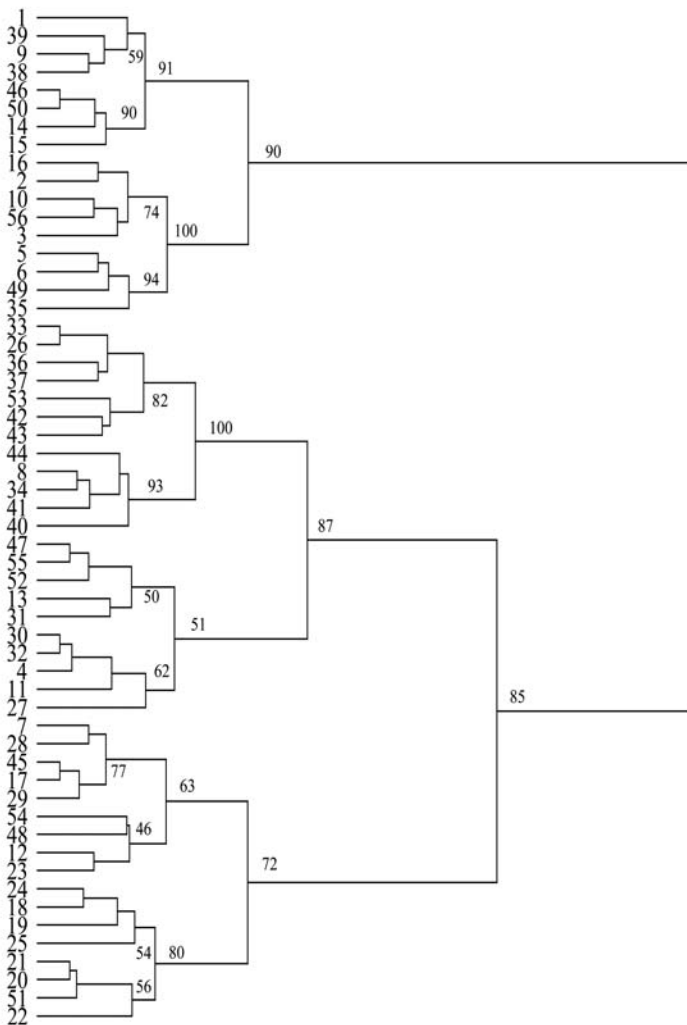


Fig. 2. Neighbour-joining tree of 56 *Pyrenophora graminea* isolates based on RFLP data from the ITS region. Percentages from 1000 bootstrap replications are given on tree branches.

The present results confirm the findings of Arabi *et al.* (2006) on the genetic diversity of Syrian *P. graminea*. Although different polypeptide patterns were observed among the fungal isolates, the authors suggested the use of other markers to better clarify genetic diversity in Syrian *P. graminea* populations. In this study, ITS-RFLP was applied and new types of polymorphisms were identified. This method reveals variation within a small region of the genome but the banding patterns obtained are very stable and consistent (Farmer and Sylvia, 1998). In contrast, other markers such as RAPD and AFLP reveal variation within a whole genome (Meunier and Grimont, 1993). ITS-RFLP markers have been suggested by several authors for investigating genetic variation in different fungal populations (Hsiang and Wu, 2000; Geln *et al.*, 2001).

The high polymorphism (76.3%) found among isolates of *P. graminea* and the distinct grouping of these isolates was unexpected. Using different techniques, the

genetic variability was relatively high when compared to previous studies (Gatti *et al.*, 1992; Johansen *et al.*, 1992; Taylor *et al.*, 2004). However, the presence of the sexual stage of this pathogen in the field suggests that genetic recombination may be responsible for the variation (Smedegard-Petersen, 1972). It is possible that the sexual stage of *P. graminea* occurs on stubble or debris between growing seasons and plays some role in the establishment of a founder population, followed by exclusively asexual propagation. This hypothesis will be tested in further studies by monitoring changes in the genetic structure of field populations over the growing season. Additional data, especially from sequencing of the ITS region, could provide useful information on the variation and dispersal of *P. graminea*.

ACKNOWLEDGMENTS

The authors thank the Director General of AECS for his support and Dr. N. MirAli the Head of the Biotechnology Department for critical readings of the manuscript.

REFERENCES

- Arabi M.I.E., MirAli N., Jawhar M., Al-Safadi B., 2002. Differentiation of *Drechslera graminea* isolates by cultural characters and SDS-PAGE. *Journal of Plant Pathology* **84**: 153-156.
- Arabi M.I.E., Jawhar M., Al-Safadi B., MirAli N., 2004. Yield responses of barley to leaf stripe (*Pyrenophora graminea*) under experimental conditions in southern Syria. *Journal of Phytopathology* **152**: 519-523.
- Arabi M.I.E., Jawhar M., MirAli N., 2005. Storage protein (hordein) patterns of barley-*Pyrenophora graminea* interaction. *Seed Science and Technology* **33**: 409-418.
- Arabi M.I.E., Jawhar M., MirAli N., 2006. Polypeptide patterns of Syrian isolates of *Pyrenophora graminea*. *Journal of Plant Pathology* **88**:129-132.
- Gatti A., Rizza F., Delogu G., Terzi V., Porta-Puglia A., Vannacci G., 1992. Physiological and biochemical variability in a population of *Drechslera graminea*. *Journal of Genetics and Breeding* **46**: 179-186.
- Farmer D.J., Sylvia D.M., 1998. Variation in the ribosomal DNA internal transcribed spacer of a diverse collection of ectomycorrhizal fungi. *Mycological Research* **74**: **102**: 859-865.
- Felsenstein J., 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Department of Genetics, University of Washington, Seattle, USA.
- Geln M., Tommerup I.C., Bougher N.L., O'Brien P.A., 2001. Interspecific and intraspecific variation of ectomycorrhizal fungi associated with Eucalyptus ecosystems as revealed by ribosomal DNA PCR-RFLP. *Mycological Research* **105**: 843-858.

- Hsiang T., Wu C., 2000. Genetic relationships of pathogenic Typhula species assessed by RAPD, ITS-RFLP and ITS sequencing. *Mycological Research* **104**: 16-22.
- Jawhar M., Sangawn R. S., Arabi M. I. E., 2000. Identification of *Drechslera graminea* isolates by cultural characters and RAPD analysis. *Cereal Research Communication* **28**: 89-93.
- Johansen, L.H., Husted K.H., Olson L.W., Heide M., 1992. Differentiation of *Pyrenophora graminea* and *Pyrenophora teres*. 1. Gel electrophoresis and isozyme analysis of soluble mycelial proteins. *Tidsskrift for Planteavl* **96**: 391-397.
- Leach J., Finkelstein D.B., Rambosek J.A., 1986. Rapid miniprep of DNA from filamentous fungi. *Fungal Genetics Newsletter* **33**: 32-33.
- Leung H., Nelson R.J., Leach J.E., 1993. Population structure of plant pathogenic fungi and bacteria. *Advances in Plant Pathology* **10**: 157-205.
- Martin K.J., Rygiewicz P.T., 2005. Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. *BMC Microbiology* **5**: 21-28.
- Meunier J.R., Grimont P.A., 1993. Factors affecting reproducibility of random amplified polymorphic DNA fingerprinting. *Research Microbiology* **144**: 373-379.
- O'Donnell K., Gigelink E., Nirenberg H.I., 1998. Molecular systematics and phylogeography of *Gibberella fugikurori* species complex. *Mycologia* **90**: 465-493.
- Parry D.W., Jenkinsen P., McLeod L., 1995. Fusarium ear blight (scab) in small grain cereals – a review. *Plant Pathology* **44**: 207-238.
- Platenkamp R., 1976. Investigations on the infection pathway of *Drechslera graminea* in germinating barley. *Review in Plant Pathology* **46**: 319-320.
- Porta-Puglia A., Delogu G., Vannacci G., 1986. *Pyrenophora graminea* on winter barley seed: effect on disease incidence and yield losses. *Phytopathology* **117**: 26-33.
- Redecker D., Thierfelder H., Walker C., Werner D., 1997. Restriction analysis of PCR-amplified internal transcribed spacers of ribosomal DNA as a tool for species identification in different genera of the order Glomales. *Applied and Environmental Microbiology* **63**: 1756-1761.
- Saitou N., Nei, M., 1987. The Neighbour-Joining method: a new method for reconstructing phylogenetics trees. *Molecular Biology and Evolution* **4**: 406-425.
- Smedegard-Petersen V., 1972. The perithecial and pycnidial stages of *Pyrenophora teres* and *P. graminea* in Denmark. *Friesia* **10**: 61-85.
- White T.J., Bruns T., Lee S., Taylor J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand D.H., Sninsky J.J. White T.J. (eds), PCR protocol, a guide to methods and applications, pp. 315-322. Academic Press, San Diego, CA, USA.
- Wright S., 1943. Isolation by distance. *Genetics* **28**: 114-138.
- Taylor E.J.A., Konstantinova P., Leigh F., Bates J.A., Lee D., 2004. Gypsy-like retrotransposons in *Pyrenophora* species: an abundant and informative class of molecular markers. *Genome* **47**: 519-525
- Zriba W., Harrabi M., 1995. Cultural and pathogenic variability in *Pyrenophora graminea* isolates. *Rachis* **14**: 99.

Received May 18, 2007

Accepted July 9, 2007

