Molecular and Pathogenic Variation Within Iranian *Pyrenophora graminea* Population; More Polymorphism in IGS Region

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**Abstract:** *Pyrenophora graminea* is the causal agent of leaf stripe disease of barley which occurs in all barley growing areas and causes severe yield reductions. Pathogenic diversity among *P. graminea* isolates in Iran is well established. Hereby, for the first time genetic diversity in Iranian population of *P. graminea* is described with new characteristics compared to previous reports. Several Iranian isolates were studied in relation to pathogenicity and variation within the ribosomal intergenic spacer (IGS). The data demonstrated that variation occurred in the pathogenicity to isolates. Successful amplification of the whole IGS in *P. graminea* was obtained with a two temperature PCR protocol. Single amplification products showed length differences. The restriction patterns obtained with HindIII and Aval enzymes were polymorphic among isolates. In accordance to previous reports, results showed that *P. graminea* isolates are hypervariable not only in the length of IGS, but also in RFLP patterns. However, our results demonstrated that Iranian population of *P. graminea* displays variation in RFLP analysis using HindIII enzyme not only among isolates with different IGS length but also among isolates with the same IGS length. As this variation has not been reported yet, this is not only considered as a new type of RFLP pattern in IGS region, but also it is concluded that Iranian population of *P. graminea* is variable from previously studied isolates. Moreover, the present study further confirmed that the described PCR-RFLP analysis of the IGS could be employed as a powerful tool to resolve genetic variation among various *P. graminea* isolates.

**Key words:** Barley leaf stripe • Length variation • Pathogenicity • PCR-RFLP • rDNA

**INTRODUCTION**

*Pyrenophora graminea* Ito & Kuribayashi [anamorph *Drechslera graminea* (Rabenh. ex. Schlech.) Shoemaker] is the causal agent of leaf stripe disease of barley (*Hordeum vulgare* L.). The fungus is a seed-borne pathogen which occurs in all barley growing areas, causing severe yield reductions [1]. The variability of the fungus is well known and has been confirmed by many reports on its virulence [2-6], morphological and physiological traits [7] and restriction fragment length polymorphism (RFLP) patterns [8-10]. It is well recognized that pathogenic variability poses difficulty in development and deployment of effective host resistance, which is a dependable and economic means of disease management. Therefore, in breeding for stable resistance it is necessary to take into consideration the genetic and pathogenic variability of the pathogen. Pathogenicity phenotypes are used for assessing genetic variation in fungal pathogens; however, pathogenicity markers are often limited in number and subjected to host selection [11].

A rapid and reproducible tool for characterizing the pathogen genotypes would help researchers to follow the shift in genetic make-up of the pathogen population and thus providing a dynamic picture of the interactions between the host and pathogen genotypes. This would, in turn, help devising strategies for management of this disease. Careful study of pathogen variability could thus be useful for evaluation of intraspecific genetic diversity.

Direct analysis of DNA polymorphisms is a more general approach to establish genetic variation in fungi. Molecular methods involving the use of the polymerase chain reaction (PCR) have been proposed to resolve
genetic variation among *P. graminea* isolates [12]. PCR amplification of targeted genomic sequences followed by RFLP or direct sequencing is increasingly used to detect and characterize fungal pathogens [13]. IGS-RFLP [8, 9] and ITS-RFLP [14] markers have been used to determine the genetic structure of *P. graminea*.

Ribosomal DNA (rDNA) is sequences of nuclear DNA encoding ribosomal RNA, includes both highly conserved genes and more variable spacer regions. The conserved sequences found in large and small subunit genes have been widely exploited in the study of relationships among distantly related fungi [15]. In fungi as well as other organisms, the noncoding spacer regions of rDNA, which evolve more rapidly, have been utilized in inferring phylogeny among more closely related taxa. Both the internal transcribed spacer (ITS) [14, 16, 17] and the intergenic spacer (IGS) [18-20] have been examined in the course of evolutionary and taxonomic studies of fungi. ITS can display variation within genera and is used in differentiation of species [21]. At the intraspecific level, variation in ITS sequences is generally very low or undetectable [16]. IGS is located between the 28s and the 18s genes and separates the ribosomal repeat units. PCR primers based on highly conserved regions of rDNA have been designed to amplify intergenic spacer. It is well established that the intergenic spacer of the rDNA is the most rapidly evolving spacer region and is highly polymorphic, providing a useful tool for taxonomic and phylogenetic studies [13, 22]. IGS is known to show differences at the intraspecific level either as variation in the restriction patterns or as variation in the length of the PCR products [20, 22-28].

The aim of this work was to characterize and assess the extent of variability in pathogenicity and IGS region by using PCR-RFLP marker among Iranian *P. graminea* isolates.

**MATERIALS AND METHODS**

**Fungal Isolates and Culture Conditions:** A total of 22 isolates of *Pyrenophora graminea* used in this study were obtained from lesions on naturally infected barley leaves collected in 2003 from different regions in north-west of Iran (Table 1). Barley leaf tissues showing necrosis and chlorosis were cut into pieces (5×10 mm) and sterilized in a 0.5% sodium hypochlorite solution (NaOCl) for 3 min. After immersion in sterile distilled water (three times for 3 min), leaf fragments were transferred to Petri dishes containing potato dextrose agar (PDA) and incubated for 7 days at 25°C in dark. Each isolate was grown on a filter paper overlaid on PDA, dried and stored at 4°C. To obtain mycelium for DNA extraction, the isolates were grown in potato dextrose broth (PDB) at 25°C with shaking at 180 rpm for 5 days, filtered and lyophilized or were grown on Petri dishes on complete agar medium (CM), at 25°C [29]. After two weeks mycelium was scraped from the agar surface and lyophilized.

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Host plant</th>
<th>Barley variety</th>
<th>Location</th>
<th>Year of collection</th>
<th>Percentage of infected plants</th>
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Pathogenicity Test: Pathogenicity test was conducted under growth room conditions using susceptible “Zarjo” cultivar and a randomized complete block design with four replications. Seeds were inoculated by sandwich technique [30] and disease severity was assessed 6 weeks after inoculation. Pathogenicity test was performed using the Mathur and Bhatnagar method [31].

DNA Extraction: Fungal DNA of 7 selected isolates was extracted from lyophilized mycelium using rapid mini-preparation method [32]. To a 1.5-ml Eppendorf tube containing 500 µl of lysis buffer (400 mM Tris-HCl [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaCl, 1% Sodium dodecyl sulfate), a small lump of mycelia was added by using a sterile toothpick, with which the lump of mycelia was disrupted. The tube was then left at room temperature for 10 min. Following addition of 150 µl potassium acetate (pH 4.8; which is made of 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of distilled water), the tube was vortexed briefly and spun at 12,000 × g for 2 min. The supernatant was transferred to another 1.5-ml Eppendorf tube and centrifuged again as described above. After transferring the supernatant to a new 1.5-ml Eppendorf tube, an equal volume of isopropyl alcohol was added. The tube was mixed by inversion briefly, spun at 12,000 × g for 2 min and the supernatant was discarded. The resultant DNA pellet was washed in 300 µl of 70% ethanol, spun at 10,000 rpm for 1 min and the supernatant was discarded. The DNA pellet was air dried and dissolved in 50 µl of 1× TE (Tris-HCl/EDTA) and stored at -20 °C until use.

PCR Amplification of the IGS Region of P. graminea rDNA: The whole IGS region of the ribosomal DNA was amplified using primers CNL12 (5′-CTG AAC GCC TCT AAG TCA G-3′) and CNS1 (5′-GAG ACA AGC ATA TGA CTA CTG-3′) which were deseribed to amplify the IGS region of Fusarium oxysporum [26]. The primers were synthesized and supplied by MWG-Biotech (Ebersberg, Germany).

PCR reaction mixtures were made up in a total volume of 50 µl containing 25-50 ng of genomic DNA, 200 mM of each dNTPs, 3 mM MgCl2, 0.5 mM of each oligonucleotide primers, 1×PCR buffer, 1.5 units of Taq DNA-polymerase (MBI Fermentas).

Amplification was performed in a GP001 Thermostyler (Corbett Research, Australia) using a two-temperature PCR protocol [9] with some modifications. An initial denaturation step at 94°C for 3 min was followed by 35 amplification cycles of a denaturation step at 94°C for 30 s and a primer annealing/extension step at 68°C for 5 min. Once the cycles were completed, samples were incubated for 10 min at 72°C as final extension step. Reagent mixtures lacking target DNA were used as negative controls. In these experiments two isolates of F. oxysporum f. sp. phaseoli were included as positive controls.

PCR products were analyzed by electrophoresis on 1% agarose gels in 1× TBE buffer (89.1 mM Tris base, 88.9 mM Boric acid, 50 mM EDTA, pH 8.0). The 1 kb DNA ladder (MBI Fermentas) was used as a molecular size marker. The gels were stained with ethidium bromide (0.5 µg ml−1 in H2O) and visualized under UV light.

RFLP Analysis of the IGS-PCR Products: Aliquots (5 µl) of PCR products were digested with 5 units of Aul and HindIII (MBI Fermentas) restriction endonucleases for 3 h at 37°C in a 10 µl total reaction mixture according to the manufacturer’s instructions. The restriction fragments were separated by electrophoresis on 1.5% agarose gel using 1× TBE as running buffer and detected by UV light after ethidium bromide staining. The 1 kb and 100 bp DNA ladders (MBI Fermentas) were used as molecular size markers. Photographic images of all gels were recorded using an image processing system [Gel Documentation System-Imago (B&L System)], stored as JPEG files.

RESULTS

Pathogenicity Test: The analysis of isolates’ pathogenicity variance showed considerable variation among the percentages of infected plants (P<0.001) (Table 1). The isolates showed different levels of pathogenicity on barley plants according to the Mathur and Bhatnagar (1992) virulence index. However, the pathogenic and weakly pathogenic isolates were not concentrated in one geographical region.

A dendrogram was constructed by UPGMA cluster analysis method on the basis of the percentages of infected plants (Fig. 1). The set of isolates under study was divided into four clusters at 90% similarity level. The first cluster included the highest virulent isolates. The second, third and fourth clusters contained, the high, medium and low virulent isolates, respectively. However, the pathogenic and weakly pathogenic isolates were not concentrated in one geographical region.

PCR Amplification of the IGS Region: A single PCR amplification product was amplified with CNS1 and CNL12 primers from each P. graminea isolate. For any given isolate, the size of IGS was estimated both by measuring the uncut IGS-PCR product band and by
Fig. 1: Dendrogram of virulence for 22 *P. graminea* isolates showing the results of UPGMA cluster analysis.

Fig. 2: a, b) Agarose gel electrophoresis of the IGS-PCR amplification products of *P. graminea* isolates using primers CNL12/CNS1. Lane 1: Kaledar1; lanes 2-3: Mianeh; lane 4: Bonab1; lane 5: Mianeh; lanes 6-7: Maragheh; lane 8: Ajabshir1; lane 9: Shabestar1; lane 10: Oskoo2; lane 11: Negative control without template DNA. c) Agarose gel electrophoresis of the IGS-PCR amplification products of two *F. oxysporum* f. sp. *phaseoli* isolates using primers CNL12/CNS1. Lane 12: isolate A; lane 13: isolate B.

Lane M: DNA size marker, 1 kb DNA ladder (fragment size indicated on the left).

Summing the sizes of fragments generated by restriction endonucleases. Based upon the length of the unique amplified fragment, the isolates were divided in two groups. The first group includes isolates whose IGS-PCR products were approximately 4.4 kb (isolates Kaledar1, Mianeh and Shabestar1) and the second group includes isolates with IGS length of 3.8 kb (isolates Bonab1, Maragheh, Oskoo2 and Ajabshir1) (Fig. 2a, b). These sizes correspond to the estimated size of the amplified IGS fragment of *P. graminea* reported by Pecchia and colleagues (1998).
Using the above-described PCR protocol a single product of approximately 2.6 kb, representing IGS, was amplified from the two isolates of *F. oxysporum* f. sp. *phaseoli* included in the experiments as internal control. This size corresponds to the estimated size of the amplified IGS fragment of *F. oxysporum* [26] (Fig. 2c).

**RFLP Analysis of the IGS-PCR Products:** The 4-base cutting enzyme *AluI* digested the IGS-PCR products into six to ten fragments, some very faint (Fig. 3a). The 6-base cutting enzyme *HindIII* digested the PCR products into one to three distinct fragments and some small, faint ones (Fig. 3b).

The RFLP patterns of IGS-PCR products digested with the 6-base cutting enzyme *HindIII* and 4-base cutting enzyme *AluI* were polymorphic not only among isolates with different IGS-PCR products length but also among isolates with the same IGS-PCR product length.

Despite Pecchia et al. [9] results, in our study the isolates with the same IGS length showed different RFLP patterns with *HindIII* enzyme. So, it demonstrates that Iranian isolates can be discriminated using *HindIII* enzyme. Moreover, this results indicates that Iranian isolates are genetically different from Pecchia’s ones.

**DISCUSSION**

The IGS region is a part of the rDNA repeat unit which occurs in a tandem array on one or more chromosomes [33]. Through concerted evolution, a mutation within one copy of IGS may spread to neighboring spacer regions. As a result, the multiple copies of IGS are homogenized within each individual and fixation of this region occurs within populations of sexually reproducing species [22].

The rDNA IGS region has already been used successfully to study taxonomic relationships in other fungi at the intraspecific level, including *Pseudomonas oxysporum* [34, 27], *Metarhizium anisopliae* [35], *Microdochium rivasii* [36], *Habekula cynodontisporum* [37], *Cryptococcus neoformans* [38], *Verticillium albo-atrum* [39] and *Diaporthe helianthi* [40]. The IGS region is one of the most rapidly evolving sequences and provides large data sets that are considered to be phylogenetically useful for delineating relationships within species [22].

Under stringent conditions with a two-temperature PCR protocol [41] and an annealing/extension time of 60 s per kilobase of target DNA [42], a single amplification fragment of the whole IGS region of *F. graminea* was
obtained for all isolates tested. Length polymorphism of amplification products among *P. gramineae* isolates indicates that considerable genetic variation occurs in the IGS region of this species and accordingly could be used for primary discrimination of the isolates. The occurrence of insertion and/deletion in such a large nonconserved region is not unexpected and could account for the length polymorphisms observed in the amplification products. Length variation has been reported in many fungi including *Cochliobolus heterostrophus* [43], *Coprinus cinereus* [44], *Cronartium ribicola* [45], *Laccaria proxima* [23], *Pythium ultimum* [20] and *Pleurotus cornucopiae* [46]. Additionally, the IGS is commonly considered the most variable part of the rDNA unit in restriction site, too.

In this study, IGS-RFLP analysis provided quantitatively different information on the genetic variability of the *P. gramineae* pathogen, depending on the enzymes used. Based on the observations, it was clear that digestion of the amplified IGS region with restriction endonucleases is a good indicator of genetic polymorphism in *P. gramineae* and the digestion patterns revealed that this could be used as a sound fingerprinting technique for this pathogen. Variability of the IGS region could also be useful for diagnostic purpose, allowing routine detection of *P. gramineae* in barley seeds.

PCR-RFLP analysis of the IGS is a rapid and reproducible technique to resolve genetic variation among the isolates. The main advantage of this technique is that the IGS region is amplified using PCR and digested to produce RFLPs, avoiding the need to time-consuming probe digests of whole cell DNA. The choice of enzyme allows the screening ability of the method to be fine-tuned. There are several reports indicating that this method allows categorization of groups of closely related strains of different fungi at an intraspecific level with simple banding patterns [24, 26, 27, 47].

The studied isolates, demonstrated a wide range of variation in the population of this pathogen. In this study, despite the isolates were obtained from close geographical regions, there was a high level of genetic diversity among them.

Our data revealed that while there was significant variation in the pathogenicity of *P. gramineae*, there was no clear pattern of distribution linked to the pathogenicity of the isolates. The pathogenic or weakly pathogenic isolates were not concentrated in any one particular region. This can be due to genotype-isolate interactions where different virulence genes are operating in the pathosystem [48].

The phylogenetic structures, however, did not correlate with pathogenicity to barley. The lack of correlation between pathogenicity and the phylogenetic structure suggests that *P. gramineae* isolates causing leaf stripe either have evolved recently from non-pathogenic isolates, or that some of the field isolates have lost their ability to cause leaf stripe. Moreover, the isolates may have been derived from the same source population and disseminated from one area to another in association with their hosts. In addition, the lack of high correlation between pathogenicity and IGS variation may be attributed to the fact that the IGS variation was not influenced by the process in which pathogenic genes have been obtained or lost from the isolates. Therefore, virulence has been developed independent of IGS taxonomic evolution. As external factors such as cultural practices, soil structure and humidity conditions influence pathogenicity of fungal populations [49], this could help explain the lack of correlation between pathotype and phylogenetic structure observed in the present study.

Our results represent the first genetic study of *P. gramineae* in Iran. The high degree of molecular variation found among isolates was unexpected. Additional data, especially from the sequencing of the IGS region, could provide useful information on the variation and dispersal of *P. gramineae*.

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**REFERENCES**


