Restriction analysis of PCR amplified nrDNA regions revealed intraspecific variation within populations of *Fusarium culmorum*

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Abstract

Seventy-five isolates of *Fusarium culmorum* with diverse geographical origin and host were analyzed using restriction digestion of polymerase chain reaction amplified nuclear ribosomal DNA intergenic spacer (IGS) and 28S gene regions. The 28S gene was conserved and has produced identical restriction patterns, however, the IGS region was substantially variable. The isolates were divided into 29 unique IGS haplotypes. There was limited resolution between clustering of isolates and their origin and/or host. The variability was distributed largely equally at both macro- and micro-geographical scale. The phylogeographic distribution pattern suggests a seed-borne dispersal of *F. culmorum*.

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1. Introduction

*Fusarium culmorum* (W.G. Smith) Sacc, classified in *Fusarium* section *Discolor* [1], is known to cause diseases of economic importance in many field crops resulting in significant losses to agriculture [2]. It can cause additional loss to agriculture due to its potential for producing detrimental mycotoxins, such as zearalenone and trichothecene deoxynivalenol [3]. These mycotoxins have been associated with livestock toxicoses or feed refusal [4] apart from posing considerable health hazards to humans [5]. Despite its substantial importance both in plant pathology and in mycotoxicology, there is a lack of appropriate and adequate information regarding population structure and genetic diversity in this fungus at a molecular level.

A range of various methods of molecular biology has been introduced in the field of fungal research. The polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) analysis has been used to address the research problems of fungal population biology such as the differentiation of species, species forms and isolates [6,7]. This method has often utilized the analysis of nuclear ribosomal DNA (nrDNA) sequences that are found in all eukaryotic cells and contain both variable and conserved regions. The analysis of these regions showed substantial resolution at different taxonomic units in a majority of fungi including *Fusarium* species [8-12].

Non-coding nrDNA regions such as the intergenic spacer (IGS) appear to be the most rapidly evolving spacer regions, whereas, among the coding regions, the 28S gene was found to be more variable due to divergent domains D1 and D2 [13,14]. These regions have been extensively utilized in inter- as well as intraspecific comparisons in many fungi [15,16]. Because of the potential for intraspecific variation within the IGS and the 28S gene, we examined these regions of nrDNA in *F. culmorum*. The purpose of our investigation was to assess the extent of variability in these regions and to determine whether or not the observed variability served to clarify intraspecific relationships in *F. culmorum*. Of particular interest were the geographical distribution of variability and its relationships with the origins and/or hosts of *F. culmorum* isolates.
2. Materials and methods

2.1. Fungal isolates and DNA extraction

Seventy-five isolates of *F. culmorum* of different geographic origins and hosts were examined. Each single spore strain was grown on potato-dextrose agar (PDA) medium for 1 week at 25±1°C and stored at 4°C for further study. Fungal tissue for DNA extraction was harvested from the isolates grown on filter paper placed over PDA plates and ground in liquid nitrogen. The total genomic DNA was extracted using a CTAB method [17], resuspended in 1× Tris-EDTA and stored at −20°C.

2.2. PCR Amplification of the IGS and 28S gene regions

The PCR primers CNL12 and CNS1 [18] were used to amplify the IGS region, whereas primers CTB6 and TW14 [19] were used for the amplification of the 28S gene. Amplification reactions contained 1× PCR buffer (100 mM Tris–Cl, pH 8.0; 500 mM KCl; and 0.8% Nonidet P40, Helena Biosciences, UK), 1 U Taq polymerase (Bioline, UK), 0.2 mM each dNTP, 2 mM MgCl2, 0.35 μM of each primer and 1 ng of genomic DNA per μl of reaction mixture. PCR was performed in a Thermocycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA, USA). Initial denaturation of 94°C for 1 min was followed by 35 cycles of 94°C for 1 min, 52°C for 30 s (primer annealing), 72°C for 1 min (primer extension) with a final extension of 7 min at 72°C.

2.3. Restriction digestion, gel electrophoresis and data analysis

In separate reactions, 8.0 μl of the PCR reaction was digested for 3 h with five different endonucleases following manufacturer’s recommendations (MBI Fermentas, UK). Restriction endonucleases *Rsa*I, *Alu*I, *Mbo*I, *Eco*RI and *Hae*III were used for the digestion of 28S gene, whereas, *Rsa*I, *Hin*II, *Mbo*I, *Eco*RI and *Hae*III were used for the restriction of the IGS region.

Digests were size-fractioned by electrophoresis through 2% agarose gels that were photographed under UV light after staining with ethidium bromide, and presence or absence of bands was recorded. The data were converted to a distance matrix using Nei and Li’s coefficient [20], which was used to construct a dendrogram by the UPGMA using the software MVSP v3.1 (Kovach Co., UK).

3. Results

3.1. PCR amplification and RFLPs of the IGS region

PCR amplification with primers CNS1 and CNL12 yielded a single DNA fragment ranging from about 2.3 to 2.6 kb in size from the isolates of *F. culmorum*, representing the IGS region of nrDNA. For any given isolate, the size of IGS, estimated by summing the sizes of constituent restriction fragments, varied with the endonucleases used to generate the fragments. Averaged across the isolates, size estimates for IGS varied from 1.375 kb, based on a *Hae*III digest, to 2.6 kb, based on *Eco*RI digests (Fig. 1). Differences in expected size are likely to reflect a failure to detect very small fragments and/or comigrating fragments.

Enzymatic digests of the IGS with five restriction endonucleases revealed RFLPs reflecting intraspecific variation (47.69%) in this nrDNA region of *F. culmorum*. Each unique fingerprint produced by an endonuclease was designated by a letter (A–N). For the enzyme *Eco*RI, five distinct patterns were recognized, for *Hin*II and *Mbo*I there were four, for *Rsa*I there were seven, and for *Hae*III there were 14 restriction patterns (Figs. 1 and 2). Isolates were scored for the patterns revealed by these five restriction enzymes, and each unique five-letter code was regarded as a different IGS haplotype. A total of 29 IGS haplotypes were identified amongst 75 isolates examined (Fig. 4).

3.2. PCR amplification and RFLPs of 28S gene

Primers CTB6 and TW14 successfully amplified a single DNA fragment of approximately 900 bp in size representing the 28S gene. Enzymatic digestion of the PCR ampli-con using five restriction endonucleases did not reflect any length polymorphism. All isolates produced identical restriction patterns (Fig. 3a,b).

3.3. Cluster analysis and phylogeographical distribution

A dendrogram constructed using the IGS RFLP data (Fig. 4) was consistent with the proposal that the isolates be divided into 29 haplotypes. There was substantial resolution regarding the clustering of the isolates and their geographical origins. Although isolates belonging to one country formed multiple clades, most of the isolates grouped together according to their country of origin. However, there were certain isolates that did not follow any such patterns and were clustered randomly (Fig. 4).

4. Discussion

This study presents the results of PCR-based RFLP analysis of nrDNA IGS and 28S gene regions, conducted with major emphasis to explain the extent of intraspecific variability within global populations of *F. culmorum*. Among the nrDNA loci analyzed, the IGS region showed substantial resolution amongst isolates; however, the 28S gene was conserved. The failure of the 28S gene to resolve the relationships within *F. culmorum* may have been due to
various reasons, for example absence of divergent sequence domains, absence of polymorphic restriction sites within the sequence, homogenization of this gene through concerted evolution and/or the functional considerations associated with this gene. The 28S gene is known as a housekeeping gene, which is under stringent functional constraint, thus it may be less prone to the changes.

There was a pronounced length variation in the IGS region of \textit{F. culmorum} that may perhaps be due to insertion/deletion (indel) events. Although the lengths of the IGS region are homogenous in some organisms, pronounced length heterogeneity has been reported in other fungi including \textit{Fusarium oxysporum} that are attributed to indel events [21]. The fingerprints generated from the restriction digestion of \textit{nrDNA} IGS region demonstrated high levels of intraspecific variability within the \textit{F. culmorum} population that is highlighted from the fact that there were 29 different haplotypes amongst 75 isolates. Such high levels of diversity may have arisen through point mutations, gene flow or recombination. Point mutations, especially at endonuclease target sites, play a crucial role in changing the restriction pattern; however, point mutation alone cannot explain the IGS diversity especially the length heterogeneity observed in this region. Gene flow may perhaps play a role in generation of genetic diversity at a small spatial scale, however, considering the limited dispersal of \textit{F. culmorum} spores [22], it is a matter of debate whether gene flow may have any major role in the diversity found in \textit{F. culmorum} populations, which were equally distributed both at the macro- and micro-

![Fig. 1. Schematic representations of RFLP fingerprints of \textit{F. culmorum} isolates generated from the restriction digestion of the IGS region of \textit{nrDNA}.](https://academic.oup.com/femsle/article-abstract/215/2/291/491867)
geographical scale. However, it could be easily explained by invoking a sexual recombination. The IGS region is a part of nrDNA repeat unit, which occurs in a tandem array on one or more chromosomes [23]. Through concerted evolution by means of unequal chromatid exchange and biased gene conversion, the multiple copies of IGS are homogenized within an individual and fixation of this region occurs within populations of sexually reproducing species [24,25]. The intraspecific variation observed in the IGS region may reflect the slow rate of concerted evolution particularly at a population level, perhaps due to low levels of sexual recombination. As a consequence, many structural variants of the IGS still exist in the population. This may perhaps be the reason for presence of substantial length variants (2.3–2.6 kb) in the IGS region of \textit{F. culmorum} that may have arisen through unequal crossing over, and thus might be considered as intermediates in the process of concerted evolution, as reported for other fungi [26]. \textit{F. culmorum} is considered as a mitosporic fungus and a sexual stage has not been observed yet. However, recent observations using molecular data have suggested substantial recombination in \textit{F. culmorum} populations and functional mating type genes have been identified in this fungus [27]. Thus it seems most reasonable that the diversity found in the IGS region in \textit{F. culmorum} may have been generated by unequal crossing over perhaps during a sexual process. Similar observations have been made for the asexual fungus \textit{Candida albicans}, where segregation patterns of alleles at certain loci suggested sporadic recombination [28] and recently, a sexual cycle has been discovered [29,30].

Variation within the IGS region was used to infer the global phylogeographic pattern of present day populations of \textit{F. culmorum}. While placed over multiple clades on a dendrogram (Fig. 4), the isolates were largely clustered according to their country of origin. However, there were certain genotypes that were shared by the isolates belonging to certain countries and/or some genotypes dispersed randomly across the continents. There could be various reasons for such a phylogeographic pattern; however, due to limited data only general comments can be made. The most plausible explanation for such a pattern seems to be the man-mediated introductions of \textit{F. culmorum} across the continents, perhaps through agricultural trade. \textit{F. culmorum} has frequently been reported to be seed borne [31] on a wide range of host plants [32]. I t seems most likely, then, that once the respective genotypes of \textit{F. culmorum} have emerged, they would have become established in seeds and/or host plants, and subsequently they became widespread through trans-global transportation of agricultural and horticultural plants, leading to the fixation of pioneer populations in different parts of the world. In addition, intercontinental transportation of infected seeds by migratory birds has also been reported for scab causing Fusaria [33] that may have additionally contributed towards the random dispersal of \textit{F. culmorum} giving rise to a global, mosaic population genetic structure.

In summary, the nrDNA RFLP is useful and a versatile technique for intraspecific comparisons in \textit{F. culmorum}. The IGS region is substantially divergent and can be
used for intraspecific analysis while the 28S gene is conserved. The phylogeographic distribution of IGS diversity suggests a man-mediated dispersal of *F. culmorum* genotypes across the continents. However, more data especially from the coding regions such as beta-tubulin or histone gene regions would be useful for further in-depth study of *F. culmorum* diversity and dispersal.

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