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IGS region polymorphisms are responsible for failure of commonly used species-specific primers in *Fusarium proliferatum* isolates from diseased garlic

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Fusarium proliferatum is a globally distributed fungal pathogen that affects a range of crop hosts and is one of the main producers of mycotoxins, such as fumonisins, in foods. Specific PCR primers are commonly used for detection and identification of this pathogen. The aim of this study was to validate previously published *F. proliferatum*-specific primers targeting the intergenic spacer (IGS) region and characterize intraspecific variation and homologous recombination events for isolates obtained from diseased garlic bulbs in Spain. Sixty-nine isolates were morphologically identified as *F. proliferatum*, and their identity was confirmed by sequencing of the translation elongation factor; however, specific IGS primers did not

result in an amplification product for nine of these isolates. Further analysis showed that this was due to polymorphism in the IGS region and six isolates were classified as IGS type I, while the remaining isolates were type II. Sequencing of the complete IGS region revealed numerous sequence polymorphisms amongst *F. proliferatum* isolates, and regions of recombination. Duplication and deletion events may have occurred via unequal crossing over during mitotic or meiotic recombination. These results suggest that the IGS region may be too variable as a reliable target for *F. proliferatum*-specific identification.

Keywords

genetic variability, IGS region, mating type, recombinant regions

1. Introduction

Garlic (*Allium sativum*) is a horticultural crop cultivated worldwide with a total production of around 28.16 Mt. Spain is the largest producer of garlic within the European Union, with 274,712 t produced in 2017 (FAOSTAT, 2019); exports accounted for 151,731 t.

Garlic is affected by several postharvest fungal diseases, with the most important being dry bulb rot primarily caused by *Fusarium proliferatum*. The pathogen was first reported in Germany (Seefelder *et al*., 2002) and later described in other production areas. In Spain, *F. proliferatum* is the pathogenic fungus most frequently associated with garlic rot during storage. Moreover, *F. proliferatum* is an important mycotoxigenic species, producing a broad range of toxins, which may pose a risk for food safety. Disease mainly occurs after the drying process and can cause losses of up to 30% of bulbs (Tonti *et al*., 2012). Fusarium bulb rot symptoms initially consist of superficial dry brown necrotic spots that progress

toward the clove and, in severe cases, the presence of white mycelium and a water-soaked rot can be observed. *Fusarium proliferatum* belongs to the *Liseola* section within the *Fusarium* genus. Its teleomorph, *Gibberella intermedia*, belongs to the *Fusarium fujikuroi* species complex (FSSC), which comprises of at least 12 reproductively different biological species (mating populations). *F. proliferatum* is distinguished from the other species within the FFSC complex by the morphology of its microconidia, macroconidia, and absence of chlamydospores.

Biologically, *F. proliferatum* is a heterothallic species with two different mating types identified through *MAT-1* and *MAT-2* idiomorphs. Mating type used to be determined by sexual crossing with tester strains, and the subsequent observation of the formation of the fruiting bodies with ascospores. However, the cloning and sequencing of different mating type genes for several ascomycetes has led to PCR assays which allow the identification of MAT gene idiomorphs in different fungi including *F. proliferatum* (Kerényi *et al*., 2002).

The importance of the damage caused by *F. proliferatum*, its wide distribution, and the high number of crops affected has led to the development of specific primers for detection and identification of the pathogen in diverse materials including food, plant material, and soil. Variation within the internal transcribed spacer (ITS) and the intergenic spacer (IGS) regions of the ribosomal RNA gene (rDNA) have been used to develop species-specific primers for a range of fungal plant pathogens (Bridge *et al*., 2003) and these regions are present in multiple copies per genome and organized as tandem arrays. The IGS region is considered to be the most rapidly evolving area within the rDNA array and displays the highest variability (Mirete *et al*., 2013; Gil-Serna *et al*., 2016), and has therefore been used to identify and distinguish closely related *Fusarium* species (Sampietro *et al*., 2010).

Specific primer pairs for PCR assays have been developed for the rapid identification of *F. proliferatum* based on calmodulin gene sequences for isolates from asparagus (Mulè *et al*., 2004a; 2004b) and on the IGS region of the rDNA gene for isolates from infected cereal crops (Jurado *et al*., 2006). This IGS primer pair has been used previously to identify *F. proliferatum* in a range of different crops and situations (Visentin *et al*., 2009; Sampietro *et al*., 2010; Scarpino *et al*., 2015).

This study initially aimed to validate published species-specific IGS primers (Jurado *et al*., 2006) for their ability to identify *F. proliferatum* isolates from garlic in Spain. Following unexpected non-amplification of some isolates, the intraspecific variation and recombination within the IGS region in *F. proliferatum* garlic isolates was subsequently examined and compared with closely related *Fusarium* species.

2. Materials and methods

2.1 *F. proliferatum* isolates and DNA extraction

Sixty-nine putative *F. proliferatum* isolates were collected from stored garlic bulbs with fusarium bulb rot symptoms, originating from different cooperatives in Spain from different years (Table 1). Cloves with symptoms were cut, surface-disinfected for 3 min in a 2% sodium hypochlorite solution, rinsed twice with sterile distilled water and cultured for 5 days at 25 °C on potato dextrose agar (PDA, Conda). After 5 days, isolations were made by transferring emerging fungal mycelia to a Petri plate containing PDA (Difco), and monosporic cultures were subsequently obtained. Isolates were maintained on PDA at 25 °C and morphological identification was performed following the taxonomic criteria of Leslie and Summerell (2006). Genomic DNA was extracted from all isolates using a

hexadecyltrimethylammonium bromide (CTAB) method according to Stępień *et al*. (2003) and DNA concentrations were adjusted to 10 ng/ml following quantification using a NanoDrop spectrophotometer.

2.2 PCR assays and sequencing

Identification of isolates as *F. proliferatum* based on morphology was confirmed by PCR amplification and sequencing of part of the elongation factor 1-α (*EF-1α*) gene using EF1-T/EF2-T primers (O'Donnell *et al*., 1998). Next, the *F. proliferatum* species-specific primers Fp3-F/Pr4-R as described by Jurado *et al*. (2006) were tested for their ability to amplify DNA from all 69 *F. proliferatum* isolates. Where isolates did not produce the expected 230 bp amplicon, the PCR test was repeated and a further PCR amplification of the *EF-1α* gene was carried out. This control assay demonstrated that DNA from each isolate was readily amplifiable, thus confirming that the lack of amplification with Fp3-F/Pr4-R primers was most probably a result of primer mismatch and not due to other possible causes, such as DNA degradation or the presence of PCR inhibitors. To identify potential mismatch of Fp3-F/Pr4-R primers, new primers were designed (IGSseqF/IGSseqR; Table 2) to examine a 700 bp region of the IGS covering the Fp3-F/Pr4-R binding sites for 20 of the 69 *F. proliferatum* isolates, including the 9 where no PCR products were obtained. Furthermore, two specific PCR assays using primer pairs IGSTIF/R and IGSTIIF/R (Table 2) were also carried out to identify two non-orthologous IGS types (types I and type II) as identified in *F. proliferatum* and *F. globosum* by Jurado *et al*. (2012). Finally, PCR amplification and sequencing of the complete IGS region was carried out for three *F. proliferatum* isolates displaying different IGS sequence types using universal primers iNL11 and CNS1 (Table 2) and nuclear ribosomal operons were sequenced with primers iNL11, iCNS1, NLa (5′-TCT AGGGTAGGCKRGTTTGTC-3′) and CNSa (5′-TCTCATRTACCCTCCGAGACC-3′). The presence of mating type genes in *F. proliferatum* isolates (MAT-1 and MAT-2) were also

identified by PCR using the primers GfMAT1-F/GfMAT1-R for MAT-1 allele, and GfMAT2-F/GfMAT2-R for MAT-2 allele as described by Kerenyi *et al*. (2002). All PCR amplifications were carried out in volumes of 25 µl containing 0.8 µM of each primer (Sigma-Aldrich), 0.2 mM of dNTP, 1× NH_4 reaction buffer, 2 mM $MgCl_2$, 0.75 U *Taq* DNA polymerase (BIOTAQ, Bioline), and 20 ng genomic DNA.

The new IGS primers IGSseqF/IGSseqR were designed using the Primer3 program (http://primer3.ut.ee/) following alignment (ClustalW method) and analysis of IGS sequences from different *F. proliferatum* isolates (AJ879946, HQ165887, GU737458, AY249383, DQ831905) and related species downloaded from the NCBI database, including *F. sacchari* (AJ8796944, KC869398), *F. fujikuroi* (AJ879945, HQ165889, AY249382), *F. globosum* (AY249384), and *F. mangiferae* (GU737449). PCR amplification conditions were as follows: 5 min at 94 °C; 40 cycles of 30 s at 95 °C, 20 s at 58 °C, 35 s at 72 °C; and 3 min at 72 °C.

Primer sequences and references of the PCR conditions used in this study are as shown in Table 2. The PCR amplifications were performed with a TC-PRO thermal cycler (BOECO). Amplicons for all PCRs were visualized following gel electrophoresis with the presence of ethidium bromide under UV light. PCR amplicons were purified with the UltraClean 15 DNA Purification Kit (MOBIO Labs) and sequencing was conducted using the primers on an ABI 3730xl genetic analyser by Stab Vida Ltd (Portugal). Sequences were processed and edited using the 4peaks v. 1.8 program and compared in the GenBank database and Fusarium-ID.

2.3 Data analysis

Sequences were aligned and analysed using Geneious v. 4.8.3 software (Biomatters,). The SVARAP program was used to visualize conserved and more variable regions of the IGS sequence alignment.

Complete IGS region aligned sequences were then subjected to recombination analysis using the Recombination Detection Program (RDP4 Beta 4.95, Simmonics). Several methods were implemented to identify recombination breakpoints in the alignment of the *F. proliferatum* FPG24, FPG64, and FPG82 isolates and in seven closely related *Fusarium* species: *F. proliferatum* (AJ879946), *F. fujikuroi* (AJ879945), *F. sacchari* (AJ879944), *F. mangiferae* (GU737449), *F. circinatum* (AY249403), *F. fractiflexum* (AY249386), and *F. globosum* (AY249384). The specific algorithms used were GENECOV, Bootscan/Rescan, Chimaera, MaxChi, SiScan, 3Seq, and RDP. Because different algorithms do not always identify the same recombination events, only events detected by at least three out of four algorithms were counted as valid ($p \leq .05$). The minor parent was defined as the one contributing the smaller fraction of the recombinant, while the major parent was the one contributing the larger fraction of the recombinant.

3. Results

3.1 Validation of *F. proliferatum*-specific PCR primers

Sixty-nine *Fusarium* isolates from garlic were morphologically identified as *F. proliferatum* based on the presence of club-shaped catenate microconidia with a flattened base, aseptate, produced on mono- and polyphialides. Curved macroconidia usually had 3 to 5 septa. Sequences of the *EF-1α* (c.730 bp) for all 69 isolates showed 99% identity and 100% query coverage with *F. proliferatum* NRRL 31071 and NRRL 25082 on the NCBI database. However, the *F. proliferatum*-specific primers Fp3-F/Pr4-R (Jurado *et al*., 2006) did not amplify the expected PCR product (c.230 bp) for nine isolates (FPG05, FPG06, FPG07, FPG54, FPG56, FPG63, FPG73, FPG74, FPG82; Table 1). This was explained by the results of the analysis of the partial IGS region sequences (IGSseqF/IGSseqR) where a

polymorphism in the Fp3F primer annealing sequence and a deletion of 67 nucleotides bases within the primer annealing region of Fp4R were detected (Figure 1).

3.2 Identification of IGS type

Two non-orthologous IGS types, named type I and II, were identified within *F. proliferatum* garlic isolates using the two primer pairs IGSTI-F/R and IGSTII-F/R (Jurado *et al*., 2012). Six isolates were classified as type I (FPG4, FPG 41, FPG59, FPG64, FPG65, FPG75), while the remaining 63 isolates were classified as type II (Table 1). When comparing IGS type with the results using the *F. proliferatum*-specific primers Fp3-F/Pr4-R, the nine isolates which did not result in amplification were all type II isolates (Table 1; Figure 2).

There was a strong correlation between mating type (MAT) and successful amplification using the specific primers Fp3-F/Pr4-R (r = .71) and moderate correlation between mating type and IGS type (r = .44) was detected ($p \leq .001$). Five of the six IGS type I isolates and all nine isolates that did not amplify using the *F. proliferatum*-specific primers Fp3-F/Pr4-R showed the *MAT-1* idiomorph. All but two of the 54 IGS type II strains that amplified using Fp3-F/Pr4-R primers showed the *MAT-2* idiomorph (Table 1; Figure 2).

3.3 Intraspecific comparison of the IGS organization

To assess the extent of IGS variation, partial IGS sequences of 20 *F. proliferatum* isolates obtained using IGSseqF/IGSseqR primers were initially examined. This revealed that there were variable regions corresponding to a 67 bp deletion at positions 550–600, and an insertion of 11 bp at position 450 (Figure 3). This was confirmed following sequencing of the complete IGS region (iNL11, iCNS1, NLa, and CNSa primers) for *F. proliferatum* isolates FPG24, FPG64 and FP82, which corresponded to different IGS groups based on amplification with Fp3F/Fp4R primers, IGS type and mating type (Figure 2). These complete

IGS sequences also showed that a region spanning position 859 to 1800 bp was characterized by an abundance of transition and transversion events, whereas the rest of the sequence (1800–2352 bp) was generally conserved with only one SNP detected (Figure 3).

Pairwise identity values were calculated for each alignment of the three isolates FPG24, FPG64, and FP82. The levels of identity between the FPG24 and FPG64, FPG24 and FPG82, and FPG24 and FPG64 strains were 96%, 93.4% and 92.8%, respectively (Table 3). FPG24-FPG64, FPG24-FPG82, and FPG64-FPG82 strains differed by single nucleotide polymorphisms (SNP) at 85, 69, and 90 sites, respectively. Sequence comparison of the three strains revealed the presence of an 11 bp insertion in the FPG24 sequence, as well as three deletions, 1, 67, and 11 bp in length (positions 368, 571–637, and 1121–1131, respectively) in the FPG82 isolate.

The short sequence AGGGTAGGTA was found repeated five times in the isolate FPG24 while it was repeated twice in FPG64 and FPG82. The similar motif AGGTAGG was repeated 19 times in isolate FPG24 while it was repeated 16 times in FPG64 and FPG82. In contrast, the motif GGCTGTGTG was repeated twice in both FPG24 and FPG64 while in isolate FPG82 it was detected only once.

The consensus GRTVYAGGGTAG sequence motif, as reported by Mbofung *et al*. (2007) and O'Donnell *et al*. (2009) in *F. oxysporum*, was also present 17 times in FPG24 and FPG64 isolates and 15 times in FPG82 isolate and dispersed over approximately 1.5 kb in the central portion of the IGS rDNA. A 106 bp consensus sequence (with 87.9% pairwise identity level) was repeated in the IGS region at the 742–848 and 1796–1902 positions.

3.4 Recombination analysis

The RDP4 software was used to detect homologous recombination events. The alignment of the IGS region of the three *F. proliferatum* isolates FPG24, FPG64, and FP82 suggested two significant recombination regions (Table 4). Recombination breakpoints in FPG82 for all five recombination methods (RDP, Geneconv, Bootscan, MaxChi, and 3Seq) were detected. FPG64 and FPG24 were identified as the major and minor parents, respectively. Moreover, four of the methods identified isolate FPG24 as a recombinant, and isolates FPG82 and FPG64 as the major and minor parents, respectively.

To detect recombination events in closely related *Fusarium* species, an analysis of an alignment within seven species (*F. proliferatum*, *F. fujikuroi*, *F. sacchari*, *F. mangiferae*, *F. circinatum*, *F. fractiflexum*, and *F. globosum*) was performed. The program detected two recombination events; again, isolates FPG82 and FPG24 were identified as recombinants, but from different parents. In the case of the FPG82 recombinant, five methods implemented in RPD4 software identified the major and minor parents as *F. globosum* and FPG24, respectively. In the case of the FPG24 recombinant, four methods identified *F. fujikuroi* and another isolate of *F. proliferatum* as the major and minor parents, respectively.

4. Discussion

All *Fusarium* isolates from garlic were morphologically identified as *F. proliferatum* and their identification was confirmed through *EF-1α* sequencing. The failure of the species-specific PCR using Fp3-F/Fp4-R primers (Jurado *et al*., 2006) for nine out of the 69 studied isolates was unexpected and found to be caused by primer mismatch due to polymorphism in the target IGS region. These results are consistent with a report by Visentin *et al*. (2009), who observed a similar lack of amplification for three of six *F. proliferatum* isolates from maize originating in north-western Italy.

Dissanayake *et al*. (2009) reported that PCR analysis using specific primers for *F. verticillioides* (VERT-1/VERT-2) and *F. proliferatum* (PRO1/PRO2) were inconsistent with morphological and sequence analysis for Japanese isolates from Welsh onion, demonstrating that other species-specific primers can lead to the misidentification of *F. verticillioides* and *F. proliferatum* in onion. Both *F. verticillioides* and *F. proliferatum* isolates from maize in Hungary, identified by morphological characters, yielded species-specific PCR fragments, in contrast to four strains identified as *F. subglutinans*, of which only two gave specific fragments when PCR amplified using the SUB-1 and SUB-2 primers (Szécsi *et al*., 2011).

Our initial investigation of intraspecific variability within the *F. proliferatum* isolates from garlic identified two non-orthologous IGS types (type I and type II), as also reported by Jurado *et al*. (2012), for a range of isolates from different hosts and locations. However, in contrast to the findings of Jurado *et al*. where most of the isolates were IGS type I (70.5%), the majority of the Spanish garlic isolates in the present study were type II (91.3%).

In another previous study, the relationship between the presence of the two different *MAT* alleles and the *EF-1α* and *FUM1* gene phylogenies was studied for *F. proliferatum* isolates (Gálvez *et al*., 2017). Clusters observed on both phylogenies were correlated with groups based on MAT alleles (r = .788 for *EF-1α* and r = .745 for *FUM1*). In the main group for both phylogenetic analyses, the majority of isolates displayed the *MAT-2* allele while *MAT-1* was the prevailing allele for the remaining groups. In the present study, there was a strong correlation between mating type and amplification with the *F. proliferatum* species-specific primers while there was a moderate correlation between mating type allele and IGS type.

Further analysis of the IGS region for *F. proliferatum* isolates from garlic revealed that the most variable regions corresponded to a 67 bp deletion and an 11 bp insertion, with

the former corresponding with the hybridization site for the species-specific reverse primer Fp4-R. Pairwise identity values for alignments of three *F. proliferatum* isolates representing different IGS groups indicated that FPG24 and FPG64 were more similar (96% nucleotide identity) than FPG64 and FPG82 (92.8% nucleotide identity). These differences were based on 69–90 SNPs and 4 indels within the IGS rDNA region, which ranged from 1 to 67 bp in length. Similarly, Mbofung *et al*. (2007) reported numerous sequence polymorphisms consisting of insertions, deletions, and single nucleotide transitions and substitutions within the IGS region among formae speciales of *F. oxysporum*. This high intraspecific variability for IGS sequences has been detected as repeated sequence motifs in many fungi (Pramateftaki *et al*., 2000; Mishra *et al*., 2002). In the present study, several repeat elements were distributed across much of the IGS region of *F. proliferatum*. Three short repeats (7, 9, and 10 bp) were observed in different numbers within the isolates from garlic. This is in contrast to the results of Mirete *et al*. (2013), who studied the partial IGS sequences of 22 strains of *F. proliferatum* isolated mainly from cereals in Spain and revealed that the number of short sequences was conserved. The consensus sequence motif GRTVYAGGGTAG was identified in the majority of IGS sequences in the *F. proliferatum* garlic strains in the present study and this sequence has also been duplicated across the IGS region within *F. oxysporum*, as reported by Mbofung *et al*. (2007) and O'Donnell *et al*. (2009). The high intraspecific variability detected in *Fusarium* spp. due to variable numbers of subrepeats within the IGS rDNA may lead to discordant phylogenies (Mbofung *et al*., 2007; Fourie *et al*., 2009; O'Donnell *et al*., 2015).

These examples of structural variants of the IGS region might have arisen as a result of unequal crossing over and thus might be considered intermediates in the process of concerted evolution (Dover, 1986; Hillis and Davis, 1988; Pramateftaki *et al*., 2000). The alignment of the entire IGS region of the three *F. proliferatum* strains in the present study

suggested different intraspecific and interspecific recombinants. Meiotic recombination may be associated with the situation where FPG82 was identified as a recombinant, and isolates FPG24 and FPG64 were identified as the parents; this is not only because sexual reproduction is reported to be relatively common in *F. proliferatum* (Leslie and Klein, 1996), but also because both *MAT-1* (FPG64) and *MAT-2* (FPG24) alleles and different IGS types are represented within *F. proliferatum* isolates from garlic and share the same geographic region. In the situation where FPG24 was identified a recombinant, and isolates FPG82 and FPG64 were identified as the parents, both contained the *MAT-1* allele; hence, this could have occurred by unequal mitotic crossing over rather than meiotic recombination, which has been suggested as one of the major driving forces in the evolution of rDNA units (Eickbush and Eickbush, 2007). Mitotic crossing over can have an important impact on concerted evolution and can occur between sister chromatids and between repeats (intrachromatid recombination) (Ganley and Scott, 1998; James *et al*., 2001). The simple sequence motifs within the repeat arrays, in addition to the long repeat elements, may act as recognition sites for the initiation of recombination. Thus, we hypothesize that these duplication and deletion events may have occurred via unequal crossing over via mitotic recombination.

Moreover, the possibility of producing recombination events in this region with closely related *Fusarium* species was detected: FPG82 was a recombinant of *F. globosum* and FPG24 while FPG24 was a recombinant of *F. fujikuroi* and another isolate of *F. proliferatum* (GenBank database). In this case, *F. proliferatum* strains are interfertile with *F. fujikuroi* (Desjardins *et al*., 1997; Leslie *et al*., 2007) although naturally occurring hybrids between *F. proliferatum* and *F. fujikuroi* have been recovered from native prairie grass in Kansas (Leslie *et al*., 2004). The existence of *F. proliferatum* hybrids potentially limits the use of a single region to identify an unknown isolate of *F. proliferatum*. Although, initially, the use of specific PCR assays for *F. proliferatum* was reported to be effective, it is not clear

how reliable the assays are over the entire range of genetic diversity of *F. proliferatum* (Proctor *et al*., 2010). This work shows that the design of the more widely used *F. proliferatum*-specific primers based on the IGS region is not adequate due to the occurrence of a significant number of false negatives at least for strains from garlic. If identification of *F. proliferatum* through sequencing of a single gene or region can sometimes be insufficient to distinguish it from other species or hybrids, then it is likely that specific PCR assays based on a single primer pair from one gene will not always suffice. PCR assays that employ multiple primer pairs and amplify fragments from multiple loci should overcome limitations of assays based on a single primer pair (Proctor *et al*., 2010). O'Donnell *et al*. (2007) have developed rapid, multiple primer extension assays to identify *F. proliferatum* and other *Fusarium* species among pathogenic strains from humans. The assay exploited SNPs among *Fusarium* species in the RNA polymerase II gene and combined the SNPs with microsphere and laser technology. This provides an additional contribution to the arsenal of tools for identifying and detecting *F. proliferatum* as well as other *Fusarium* species.

In summary, commonly used species-specific primers failed to detect all *F. proliferatum* isolates collected from garlic in Spain due to extensive variability In the IGS region. However, it is not known if there are populations with this same IGS variation among *F. proliferatum* populations affecting other crops (e.g., maize or asparagus). A multiple primer assay may therefore be required for the accurate identification of *F. proliferatum*.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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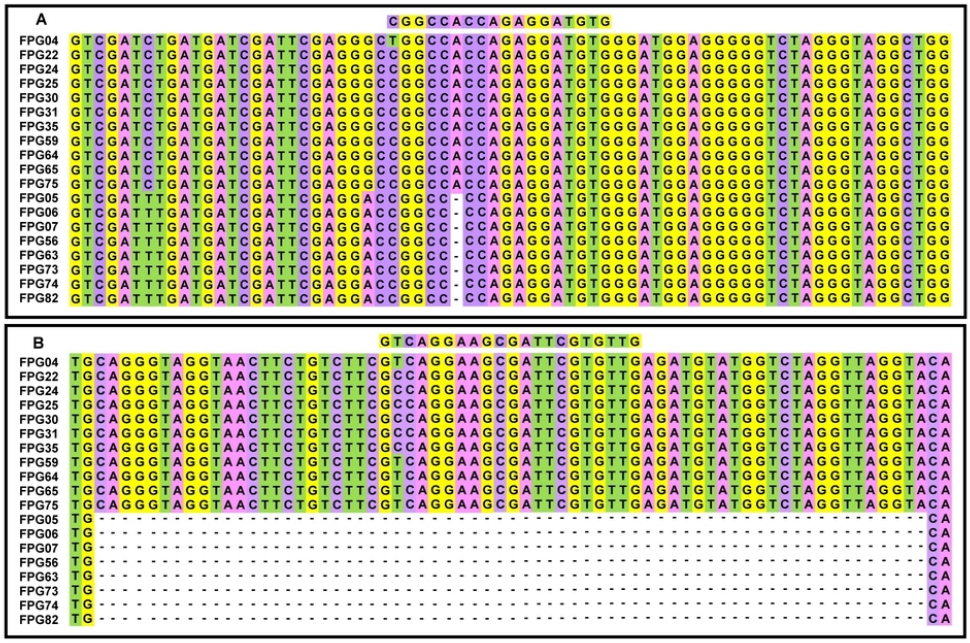
Figure legends

Figure 1 Nucleotide polymorphisms detected in the intergenic spacer region of *Fusarium proliferatum* isolates, corresponding with the positions of primers Fp3F (a) and Fp4R (b).

Figure 2 Dendrogram of *Fusarium proliferatum* isolates based on the presence or absence (asterisk) of specific amplification with Fp3F / Fp4R primers, on the intergenic spacer (IGS) type (● Type I and ○ Type II), and on the mating type (*MAT-1* and ☐ *MAT-2*).

Figure 3 Percentage mean variability of different positions along the intergenic spacer (IGS) region of 20 *Fusarium proliferatum* isolates, with the most variable regions indicated; a schematic representation of the sequence alignment showing the nucleotide polymorphisms and gaps (upper); and a comparison of the IGS sequence organization among three *F. proliferatum* isolates (lower).



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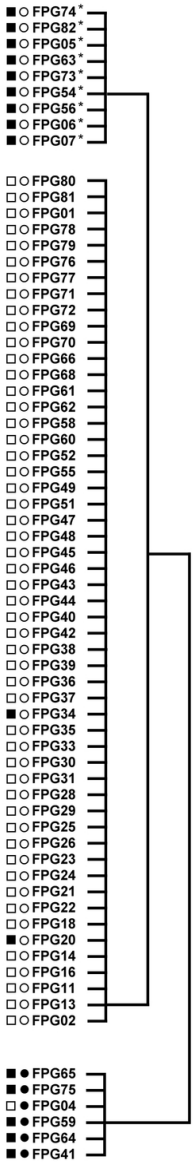
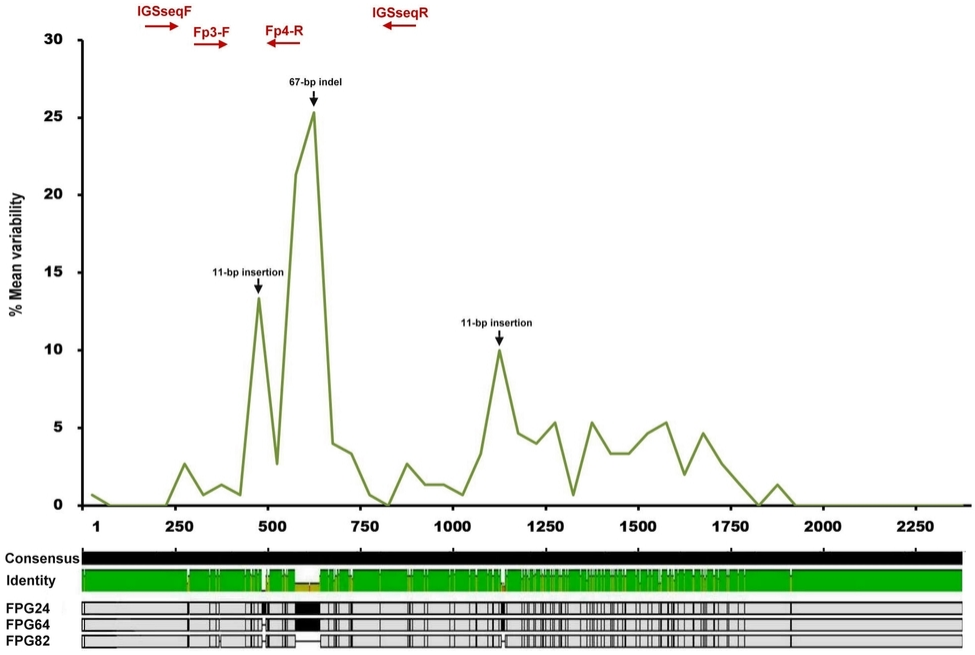


50x254mm (150 x 150 DPI)



169x115mm (150 x 150 DPI)

Table 1 Description of the *Fusarium proliferatum* isolates from infected garlic indicating their year of isolation, presence or absence of PCR amplification with the specific primers Fp3-F/F4e-R, intergenic spacer (IGS) type and *MAT* idiomorph

Isolate	Isolation year	Specific primers (Fp3-F/Pr4-R)	IGS type	*MAT*
FPG01	2009	+	II	2
FPG02	2009	+	II	2
FPG04	2011	+	I	2
FPG05	2011	−	II	1
FPG06	2011	−	II	1
FPG07	2011	−	II	1
FPG11	2012	+	II	2
FPG13	2012	+	II	2
FPG14	2012	+	II	2
FPG16	2013	+	II	2
FPG18	2013	+	II	2
FPG20	2013	+	II	1
FPG21	2013	+	II	2
FPG22	2013	+	II	2
FPG23	2013	+	II	2
FPG24	2013	+	II	2
FPG25	2013	+	II	2
FPG26	2013	+	II	2
FPG28	2013	+	II	2
FPG29	2013	+	II	2

FPG30	2013	+	II	2
FPG31	2013	+	II	2
FPG32	2013	+	II	2
FPG33	2013	+	II	2
FPG34	2013	+	II	1
FPG35	2014	+	II	2
FPG36	2014	+	II	2
FPG37	2014	+	II	2
FPG38	2014	+	II	2
FPG39	2014	+	II	2
FPG40	2014	+	II	2
FPG41	2014	+	I	1
FPG42	2014	+	II	2
FPG43	2014	+	II	2
FPG44	2014	+	II	2
FPG45	2014	+	II	2
FPG46	2014	+	II	2
FPG47	2014	+	II	2
FPG48	2014	+	II	2
FPG49	2014	+	II	2
FPG51	2014	+	II	2
FPG52	2014	+	II	2
FPG54	2014	−	II	1
FPG55	2014	+	II	2

FPG56	2014	−	II	1
FPG58	2014	+	II	2
FPG59	2014	+	I	1
FPG60	2014	+	II	2
FPG61	2014	+	II	2
FPG62	2014	+	II	2
FPG63	2014	−	II	1
FPG64	2014	+	I	1
FPG65	2014	+	I	1
FPG66	2014	+	II	2
FPG68	2014	+	II	2
FPG69	2014	+	II	2
FPG70	2014	+	II	2
FPG71	2014	+	II	2
FPG72	2014	+	II	2
FPG73	2014	−	II	1
FPG74	2014	−	II	1
FPG75	2014	+	I	1
FPG76	2014	+	II	2
FPG77	2014	+	II	2
FPG78	2014	+	II	2
FPG79	2014	+	II	2
FPG80	2014	+	II	2
FPG81	2014	+	II	2

FPG82	2014	–	II	1

Table 2 Primer sequences, amplicon size and primer target site used for the specific identification of *Fusarium proliferatum* isolates and analysis of the intergenic spacer (IGS) region

Primer names	Sequence (5′–3′)	Amplicon size (bp)	Target site	Reference
Fp3-F/Pr4-R	CGGCCACCAGAGGATGTG CGGCCACCAGAGGATGTG	230	Specific *for F. proliferatum*	Jurado *et al.* (2006)
EF1T/EF2T	ATGGGTAAGGAGGACAAGAC GGAAGTACCAGTGATCATGTT	750	Elongation factor 1-α	O'Donnell *et al.* (1998)
IGSseqF/R	GCCGTCCTTCGACTCGATT GGACGAACGCCAGACCGGACT	700	Partial IGS region	This study
IGSTIF/R	GGATAGCTCTAGGGTAGTT CTAGACRGACACRCAGGAR	250	IGS type I	Jurado *et al.* (2012)
IGSTIIF/R	CTGGTCGGGATGAGGG CTGGACGGACACRCAG	300	IGS type II	Jurado *et al.* (2012)
iNL11/ CNS1	AGGCTTCGGCTTAGCGTCTTAG TTTCGCAGTGAGGTCGGCAG	2220	Complete IGS region	O'Donnell *et al.* (2009)
GfMAT1-F/R	GACCAACTCAAACCTCGTGGCG TCATCAAAGGGCAAGCGATACCC	320	*MAT-1* allele	Kerényi *et al.* (2002)
GfMAT2-F/R	ACCGTAAGGAGCGTCACCATT GGGGTACTGTCGGCGATGTT	212	*MAT-2* allele	Kerényi *et al.* (2002)

Table 3 Statistics from the alignments of complete sequences of the intergenic spacer (IGS) region of isolates of *Fusarium proliferatum*, showing the pairwise identity, number of single nucleotide polymorphisms (SNPs) and size of indels in the nucletotide sequences

Isolates	Length (bp)	Identical sites	Pairwise identity (%)	SNPs	Indel sizes (bp)
FPG24–FPG64	2352	2259	96.0	85	11
FPG24–FPG82	2352	2197	93.4	69	1, 11, 67, 11
FPG64–FPG82	2341	2172	92.8	90	1, 67, 11

Table 4 Recombination breakpoints and origin of major and minor related sequences in the alignment of the complete sequences of the intergenic spacer (IGS) regions of *Fusarium proliferatum* isolates

Recombinant isolate		Recombinant region		Parental isolate[a]		Detection method
		Begin	End	Major	Minor	
Intraspecific	FPG82	1071	1534	FPG64	FPG24	RDP, Geneconv, BootScan, MaxChi, 3Seq
	FPG24	2062	834	FPG82	FPG64	Geneconv, BootScan, MaxChi, 3Seq
Interspecific	FPG82	891	1425	*F. globosum*	FPG24	RDP, Geneconv, BootScan, MaxChi, 3Seq
	FPG24	503	1372	*F. fujikuroi*	*F. proliferatum* NRRL 22944	Geneconv, BootScan, MaxChi, 3Seq

[a]Major sequence, sequence most closely related to the sequence surrounding the smaller, transferred fragment. Minor Sequence, sequence closely related to the transferred fragment in the recombinant.