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Aggressiveness and genetic variability of *Fusarium graminearum* populations from the main wheat production area of Argentina

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ABSTRACT

Objectives: Differences in aggressiveness and mycotoxin production were previously found among isolates of *Fusarium graminearum*, the main incitant of Fusarium head blight (FHB) of wheat, from Argentina. This study aims at evaluating the genetic diversity present in these isolates and its relationship with their aggressiveness.
Methods: Inter-simple sequence repeats polymerase chain reaction (ISSR-PCR) was used to asses the genetic variability present in 112 *F. graminearum* isolates from 28 localities of Buenos Aires Province, Argentina. The analysis of molecular variance (AMOVA) was performed to examine the population structure.
Results: F. graminearum populations from Argentina showed a large genotypic variability. Seventy seven percent of the isolates included in the analysis were identified as a unique haplotype. The largest part of this variation resulted from genetic differences within (89%) rather than between populations (11%). The constructed dendrogram showed no genotype clustering according to geographic origin or aggressiveness of the isolates.
Conclusions: A high genetic heterogeneity was found in the *F. graminearum* populations from Argentina. This diversity can possibly reflect the occurrence of high frequencies of sexual outcrosses in the field and gene flow.

1. Introduction

Fusarium head blight (FHB), caused by *Fusarium graminearum* (Schwabe) is one of the most important diseases of wheat (*Triticum aestivum* L.) in Argentina (De Galich, 1997). The disease decreases yield and reduces the test weight and the protein content of the grain, affecting the value of the flour and its subproducts (Goswami and Kistler, 2004). Furthermore, the frequent contamination of FHB-affected grain with mycotoxins synthesized by the pathogen, mainly the trichothecenes nivanelol (NIV) and deoxynivalenol (DON), compromises its use as food or feed (Lori et al., 2003).

Fusarium graminearum has been ubiquitously isolated from wheat crops and the structure of the populations of this fungus has been thoroughly studied. Differences in aggressiveness have been reported for *F. graminearum* isolates collected from different world regions (Bai and Shaner, 1996; Miedaner et al., 2001), countries or states (Carter et al., 2002; Dusabenyagasani et al., 1999; Malbrán et al., 2012; Walker et al., 2001) and even individual fields (Miedaner and Schilling, 1996).

In a previous work, we studied the differences in aggressiveness of 112 *F. graminearum* isolates collected from the main wheat production area of Argentina (Malbrán et al., 2012). Differences in the percentage of symptomatic spikes and the lesion size induced by point inoculation of wheat spikes grown in the field were found among isolates. According to the severity of FHB symptoms the isolates were arranged as low, medium and highly aggressive. Furthermore, we concluded that the variation found was not geographically structured (Malbrán et al., 2012).

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High genotypic variability of *F. graminearum* populations has been found in Europe (Carter et al., 2000), USA (Schmale III et al., 2006; Walker et al., 2001; Zeller et al., 2004, 2003), Brazil (Astolfi et al., 2012) and Uruguay (Pan et al., 2016). In Canada, both low (Dusabenyagasani et al., 1999) and high (Mishra et al., 2004) levels of variability have been reported.

Even though some information regarding the genetic diversity of Argentinian *F. graminearum* populations is available, it circumscribes to a limited area of wheat production (Alvarez et al., 2011; Consolo et al., 2015; Ramírez et al., 2007, 2006) or to populations of the pathogen isolated from durum wheat (*Triticum turgidum* L. var. *durum*) (Palacios et al., 2017). A high genetic diversity was reported for *F. graminearum* populations from the north central wheat cropping area of Argentina when vegetative compatibility groups (VCG) and amplified fragment length polymorphism (AFLP) were used (Ramírez et al., 2007, 2006). Similar results were obtained by Alvarez et al. (2011, 2010) with the same molecular markers. The variability of *F. graminearum* isolates from 4 localities of 3 Argentinian provinces (Consolo et al., 2015) and 5 localities from the durum wheat production area of the country (Palacios et al., 2017) were studied using Inter-simple sequence repeats (ISSR) polymerase chain reactions (PCR).

The ecological variability comprised in the large extension of land cultivated with this cereal in Argentina justifies the need for improving the knowledge of the genetic variability present in the populations of *F. graminearum* in the country. Furthermore, to delve into the relationship between this variability and fenotipic traits such as aggressiveness towards wheat could help in the development of durable resistance to FHB and to the improvement of the methods aimed at its management (Bowden and Leslie, 1997; Miedaner et al., 2008; Schmale III et al., 2006).

The objectives of this work were: (i) to analyse the genetic diversity present in *F. graminearum* populations from the main wheat production area of Argentina and (ii) to study if such variation is related to the geographical origin and/or aggressiveness of the isolates.

2. Materials and methods

One hundred and twelve *F. graminearum* isolates were collected from grain samples of common wheat (*Triticum aestivum* L.) obtained from 28 localities of Buenos Aires Province, Argentina. All isolates were taxonomically and molecularly identified as *F. graminearum* and belonged to the 15-ADON trichothecene genotype as was previously reported (Malbrán et al., 2014, 2012). The aggressiveness of these isolates was evaluated by assessing the ability to produce symptoms (infection efficiency) (Pariaud et al., 2009) on point-inoculated field-grown wheat spikes (Malbrán et al., 2014, 2012). Furthermore, according to the percentage of the spike affected by the fungus (disease severity), the thousand kernel weight, and the area under the disease progress curve obtained for each isolate, we organized them in three groups: high, medium, and low aggressiveness (Table 1). All isolates were deposited in the fungal collection of the Centro de Investigaciones de Fitopatología (CIDEFI), Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata.

Genetic variability of the isolates was studied by means of ISSR-PCR. First, the ability of a set of 26 ISSR primers to produce polymorphic banding patterns was studied in 25 μL reaction volumes containing 12–15 ng of genomic DNA, 1.25 U of T-plus DNA polymerase (Highway Molecular Biology – InBio – UNICEN, Argentina), 0.75 μM of primer (FAGOS/Ruralex, Argentina), 200 μM of each deoxynucleoside triphosphate and 2.5 mM $MgCl_2$ in reaction buffer (500 mM KCl, 100 mM Tris-Cl pH 9.0, 1% Triton X-100 without Mg^{++}). A PTC-150 MiniCycler™ thermocycler (M.J. Research, INC., USA) was programed as follows: a single step of 7 min at 94 °C followed by 33 cycles of 1 min at 94 °C, 75 s at the annealing temperature (48 or 52 °C depending on the primer), and 4 min at 72 °C. A final step of 7 min at 72 °C was programmed. The products of amplifications were resolved on 1.5% (w/v) agarose gels, stained with ethidium bromide (0.5 μg/mL) and visualized under UV light using a GeneGenius (Syngene, USA) image analyzer. The size of the DNA fragments amplified was estimated by comparison with a 1 Kb DNA ladder (Highway Molecular Biology – InBio – UNICEN, Argentina). In each amplification reaction a negative control, containing all reagents but no DNA, was included.

Amplifications with all primers were repeated at least twice in separate experiments. To construct a binary matrix, fragments that were present or absent in all the repetitions of every primer for each isolate were scored as 1 and 0, respectively. This matrix was analyzed by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Sokal and Michener, 1958) using the DICE similarity coefficient (Dice, 1945). The PAST 3.19 package (Hammer et al., 2001) was used to build a dendrogram and its reliability was assessed by the cophenetic correlation coefficient (CCC). The statistical support of the resolved branches was evaluated by bootstrapping with 100 replicates. *Fusarium cerealis* strain RBG999 (kindly provided by Dr. Brett Summerell) was used as the outgroup.

An analysis of molecular variance (AMOVA) was performed to evaluate the genetic diversity among and between populations (Excoffier et al., 1992). Population structure was inferred using the distances between the DNA haplotypes and computed in the Arlequin 3.5 package (Excoffier and Lischer, 2010).

3. Results and discussion

Four polymorphic ISSR primers: BA3 (5-ACA CAC ACA CAC ACA CCT–3′), FA5 (5′-TAC GAG AGA GAG AGA GAG A-3′), KA5 (5′-CTA CAC ACA CAC ACA CAC-3′), and LA5 (5′-CAG AAC AAC AAC AAC AAC-3′) were selected from the larger set of 26 primers. All 4 primers were anchored, 2 at 5′ end (FA5 and LA5) and 2 at 3′ end (BA3 and KA5) and, except for primer LA5, consisted of 2 nucleotide motifs. Primers BA3 and KA5 only differed in their anchoring sequence. These primers amplified the DNA of the 112 *F. graminearum* isolates, except for primers KA5, that did not work with isolates R2 and BA8, and LA5 that did not work with isolate BA10. As a result, these isolates were not included in the analyses.

A total of 80 DNA fragments that ranged from 255 to 2315 bp were obtained for the remaining 109 isolates with the 4 ISSR primers. Among them, 57 (71%) were polymorphic. Even though the number of primers used in our work was small, it was enough to discern the variability present in *F. graminearum* populations of Argentina. Eighty four out of the 109 isolates included in the analysis (77%) were identified as unique haplotypes with a level of similarity that ranged between 97% and 80% for the most related and distant haplotypes, respectively (Fig. 1). This level of variability is consistent with the results obtained by Miedaner et al. (2008) who found haplotype diversity ranges between 60 and 100% in populations of *F. graminearum* from different countries and continents. It is also similar to those observed in populations of the fungus from USA (Schmale III et al., 2006; Zeller et al., 2004, 2003), Canada (Mishra et al., 2004), Brazil (Astolfi et al., 2012), Uruguay (Pan et al., 2016) and Argentina (Alvarez et al., 2011; Palacios et al., 2017; Ramírez et al., 2007). However, it differed from the small proportion of isolates with unique banding patterns (36%) found by Consolo et al. (2015) in Argentina.

The cluster analysis of the ISSR data obtained resulted in the grouping of 92 isolates in a major cluster, with a similarity coefficient of ~0.86, and several minor clusters (Fig. 1). The dendogram

Table 1
Aggressiveness group and locality of isolation of the 112 isolates of *Fusarium graminearum* used in this study.

Group*	Locality	Coordinates	Isolates
Low	Alberti	35° 1′ 53″ S 60° 16′ 48″ W	A4
	Bolivar	36° 14′ 20″ S 61° 7′ 32″ W	BV1
	Gral. Alvarado	38° 9′ 30″ S 58° 5′ 53″ W	GA1
	Gral. Belgrano	35° 45′ 56″ S 58° 29′ 49″ W	GB2, GB4
	Las Flores	36° 0′ 50″ S 59° 5′ 57″ W	LF3
	Mar del Plata	38° 0′ 19″ S 57° 32′ 33″ W	MP2
	Miramar	38° 16′ 13″ S 57° 50′ 22″ W	MR18, MR31, MR4
	Rojas	34° 11′ 51″ S 60° 44′ 1″ W	RJ5
	Roque Pérez	35° 24′ 2″ S 59° 20′ 5″W	RP2
	San Pedro	33° 40′ 32″ S 59° 39′ 46″ W	SP2
	Tandil	37° 19′ 43″ S 59° 8′ 12″ W	TD1
Medium	25 de Mayo	35° 25′ 56″ S 60° 10′ 17″ W	25M1
	30 de Agosto	36° 16′ 43″ S 62° 32′ 42″ W	30A3
	9 de Julio	35° 26′ 38″ S 60° 53′ 4″ W	9J1
	Alberti	35° 1′ 53″ S 60° 16′ 48″ W	A2
	Arrecifes	34° 3′ 46″ S 60° 6′ 9″ W	AR1
	Azul	36° 46′ 29″ S 59° 51′ 14″ W	AZ1
	Balcarce	37° 50′ 47″ S 58° 15′ 19″ W	BA1, BA2, BA10, BA12, BA13, BA8
	Bragado	35° 6′ 55″ S 60° 29′ 23″ W	BR1
	Carhué	37° 10′ 46″ S 62° 45′ 36″ W	CR2
	Chacabuco	34° 38′ 31″ S 60° 28′ 17″ W	CH1, CH2
	Colonia Rivadavia	35° 39′ 03″ S 63° 15′ 16″ W	CRV1
	Gral. Alvarado	38° 9′ 30″ S 58° 5′ 53″ W	GA2
	Gral. Belgrano	35° 45′ 56″ S 58° 29′ 49″ W	GB1, GB3
	Las Flores	36° 0′ 50″ S 59° 5′ 57″ W	LF1
	Los Hornos	34° 57′ 14″ S 57° 58′ 29″ W	IV II 3, LH1, LH6, LH9, LH12
	Mar del Plata	38° 0′ 19″ S 57° 32′ 33″ W	MP1
	Miramar	38° 16′ 13″ S 57° 50′ 22″ W	MR1, MR2, MR3, MR7, MR9, MR12, MR14, MR15, MR16, MR19, MR21, MR22, MR23, MR24, MR25, MR28, MR29, MR30, MR32, MR33, MR35, MR36, MR39, MR40, MR43, MR48
	Necochea	38° 33′ 16″ S 58° 44′ 22″ W	N1, N2, N3
	Pila	35° 59′ 59″ S 58° 8′ 44″ W	PL1, PL2, PL4
	Roque Pérez	35° 24′ 2″ 59° 20′ 5″ W	RP1
	San Pedro	33° 40′ 32″ S 59° 39′ 46″ W	SP3
	Trenque Lauquen	35° 58′ 23″ S 62° 43′ 57″ W	TL2, TL4
	Tres Arroyos	38° 22′ 39″ S 60° 16′ 30″ W	TA1
High	Alberti	35° 1′ 53″ S 60° 16′ 48″ W	A5, A6
	Balcarce	37° 50′ 47″ S 58° 15′ 19″ W	BA3, BA4, BA5, BA6, BA9, BA11, BA14, BA15
	Las Flores	36° 0′ 50″ S 59° 5′ 57″ W	LF2
	Los Hornos	34° 57′ 14″ S 57° 58′ 29″ W	LH2, LH4, LH5, LH7, LH8, LH11
	Miramar	38° 16′ 13″ S 57° 50′ 22″ W	MR5, MR13, MR26, MR37, MR38, MR41, MR42, MR45, MR46
	Pehuajó	35° 48′ 38″ S 61° 53′ 55″ W	PH1
	Rauch	36° 46′ 29″ S 59° 5′ 23″ W	R1, R2, R3
	Roque Pérez	35° 24′ 2″ S 59° 20′ 5″ W	RP4
	San Pedro	33° 40′ 32″ S 59° 39′ 46″ W	SP1
	Trenque Lauquen	35° 58′ 23″ S 62° 43′ 57″ W	TL3

* Aggressiveness group according to Malbrán et al. (2012).

obtained is reliable according to the CCC ($r = 0.986$) obtained and the AMOVA ($\varphi = 0.10$, $P < 0.001$) showed that most of the variation resulted from genetic differences within (89%) rather than between populations (11%). These results agree with previous reports of genotypic diversity of populations of the fungus from the world (Dusabenyagasani et al., 1999; Gale et al., 2002; Miedaner et al., 2001; Mishra et al., 2004; Pan et al., 2016) and from the durum wheat production area of Argentina (Palacios et al., 2017).

The geographic origin of the isolates was not correlated with their clustering, as illustrated by the inclusion of representatives from 21 of the 28 localities sampled in the largest cluster resolved (Fig. 1). These results are in agreement with those reported by Alvarez et al. (2011) but contrast with the relationship between haplotype and geographic origin found by Consolo et al. (2015).

Isolate grouping was not correlated with aggressiveness, either, as isolates belonging to the high aggressiveness group were clustered together with others belonging to the middle and low aggressiveness groups (Fig. 1). In our previous work, we organized the same isolates according to their infection efficiency and disease severity in three groups: high, medium, and low aggressiveness. These groups included approximately 31%, 57% and 12% of all the isolates, respectively (Malbrán et al., 2012). A similar proportion of each aggressiveness group was found in the major cluster of the UPGMA dendogram, which included 26 (28%), 55 (60%) and 11 (12%) isolates belonging to the high, medium and low aggressiveness groups, respectively.

The homothallic nature of *F. graminearum* allows the fungus to produce both homozygous and heterozygous perithecia as a result of sexual reproduction (Miedaner et al., 2001). Under laboratory conditions, Bowden and Leslie (1999) reported a high rate of sexual outcrosses between *F. graminearum* isolates and suggested that the production of heterozygous perithecia might also occur in the field. Schmale III et al. (2006) found evidence of significant genetic exchange among populations of the fungus and proposed that the high genotypic diversity of *F. graminearum* found worldwide may result from the combined effect of recombination and long distant transportation of spores. Therefore, a possible explanation for the high genotypic variability found in populations from Argentina could be the occurrence of frequent sexual outcrosses in the field and gene flow due to the long-distance dispersal of spores. Furthermore, the large variability found in the aggressiveness of *F. graminearum* (Bai and Shaner, 1996; Carter et al., 2002;

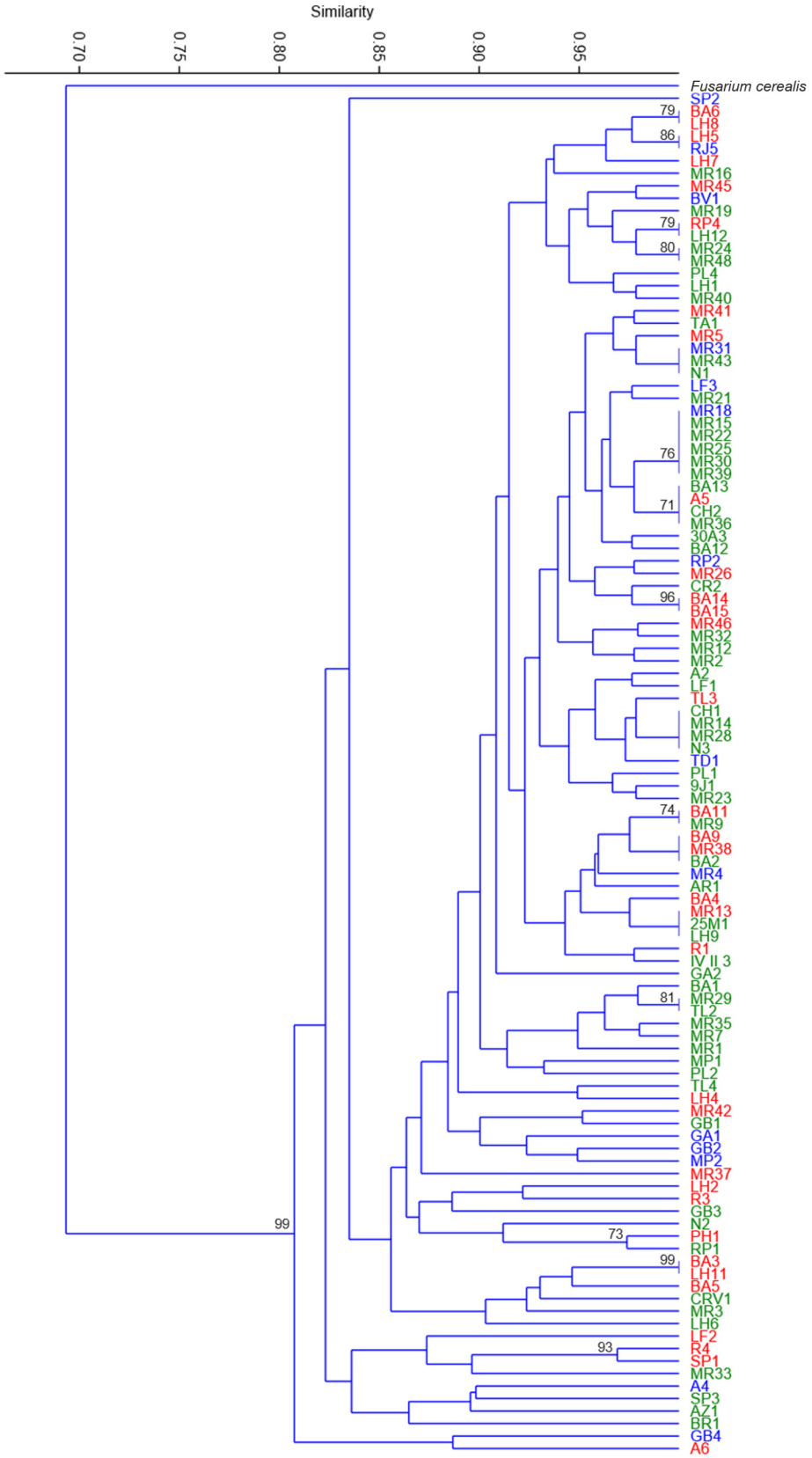

Fig. 1. Dendrogram obtained with the band fingerprints generated by 112 *Fusarium graminearum* isolates with 4 inter-simple sequence repeats (ISSR) polymorphic primers by means of polymerase chain reaction (PCR). Bootstrap values of 70 or greater are indicated above the branches. *Fusarium cerealis* strain RBG999 was used as the outgroup. Colors indicate the inclusion of the isolates in one of the groups described: red, green and blue for the high, medium and low aggressiveness, respectively.

Dusabenyagasani et al., 1999; Malbrán et al., 2012; Miedaner et al., 2001; Miedaner and Schilling, 1996; Walker et al., 2001) could be explained by these recombination events, while the migration of spores might be responsible for the lack of correlation between genotype and location.

4. Conclusions

The presence of a high heterogeneity in the *F. graminearum* populations from the main wheat production area of Argentina was demonstrated by ISSR-PCR, showing also the efficience of even small numbers of these molecular markers in resolving genetic variability in fungal populations. The clustering of isolates according to their genotype was not correlated with their geographic origin or with their aggressiveness, possibly because of gene flow due to long-distance dispersal of spores and a high frequency of sexual outcrosses in the field.

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Conflict of interest

None.

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