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Species composition and genetic structure of *Fusarium graminearum* species complex populations affecting the main barley growing regions of South America

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Members of the *Fusarium graminearum* species complex (FGSC), such as *F. graminearum* and *F. asiaticum*, are the main cause of fusarium head blight (FHB) of wheat and barley worldwide. In this study, 117 FGSC isolates obtained from commercial barley grain produced in Argentina (n = 43 isolates), Brazil (n = 35), and Uruguay (n = 39) were identified to species and trichothecene genotypes, and analysed using amplified fragment length polymorphism (AFLP) and sequence-related amplified polymorphism (SRAP) markers. In addition, reductase (*RED*) and trichothecene 3-O-acetyltransferase (*Tri101*) were sequenced for a subset of 24 isolates. The majority of the isolates (n = 103) were identified as *F. graminearum*, which was the only species found in Argentina. In Uruguay, only one *F. cortaderiae* isolate was found among *F. graminearum* isolates. In Brazil, *F. graminearum* also dominated the collection (22/35), followed by *F. meridionale* (8/35), *F. asiaticum* (2/35), *F. cortaderiae* (2/35) and *F. austroamericanum* (1/35). Species were structured by trichothecene genotype: all *F. graminearum* were of the 15-acetyldeoxynivalenol (ADON), *F. meridionale*, *F. asiaticum* and *F. cortaderiae* were of the nivalenol (NIV), and *F. austroamericanum* was of the 3-ADON genotype. Both AFLP and SRAP data showed high levels of genetic variability, which was higher within than among countries. Isolates were not structured by country of origin. SRAP analysis grouped *F. graminearum* in a separate cluster from the other species within the complex. However, AFLP analysis failed to resolve the species into distinct clades with partial clustering of *F. meridionale*, *F. austroamericanum*, *F. asiaticum* and *F. graminearum* isolates.

Keywords: AFLP, FGSC, *Hordeum vulgare*, SRAP, trichothecene genotype

Introduction

Fusarium head blight (FHB) is amongst the most damaging fungal diseases of wheat and barley worldwide. *Fusarium graminearum* species complex (FGSC) comprises 16 phylogenetic species but FHB is caused mainly by *F. graminearum sensu stricto* (hereafter *F. graminearum*) or *F. asiaticum*, depending on the region (Aoki *et al.*, 2012). In barley, FHB significantly reduces grain yield and affects grain quality due to contamination with mycotoxins and association with quality defects (gushing) of beer (Minervini *et al.*, 2004; Desjardins, 2006). Among the various mycotoxins produced by FGSC members, special attention has been given to deoxynivalenol (DON), a type B trichothecene, for which maximum tolerated limits have been established in several countries, including Brazil and Uruguay. Species of this complex also produce zearalenone (ZEA), a non-steroidal oestrogenic mycotoxin associated with numerous mycotoxicoses in swine (Desjardins, 2006).

Fusarium graminearum is the dominant FGSC member causing FHB of wheat in the Americas. However, surveys on wheat and barley crops grown in South America identified five other species affecting these crops: *F. asiaticum*, *F. austroamericanum*, *F. brasilicum*, *F. cortaderiae* and *F. meridionale*. While in Argentina FHB in wheat seems to be caused exclusively by *F. graminearum* (Alvarez *et al.*, 2009; Reynoso *et al.*, 2011), other species of the complex occur in Brazil and Uruguay in a relatively small proportion (Scoz *et al.*, 2009; Astolfi *et al.*, 2011, 2012; Pan *et al.*, 2013; Umpiérrez-Failache *et al.*, 2013; Del Ponte *et al.*, 2015).

FGSC isolates are known to possess three specific profiles of trichothecene production (chemotypes) including: nivalenol (NIV) and its acetyl derivatives, DON and primarily 3-acetyldeoxynivalenol (ADON), and DON

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and primarily 15-ADON (Desjardins, 2008). PCR-based assays targeting portions of genes predictive of trichothecene synthesis have been used to determine the trichothecene genotype as predictive of the chemotype (Desjardins, 2008). While a few FGSC members appear to comprise only one trichothecene genotype (e.g. *F. meridionale*) others may possess two or three different genotypes, depending on the geographic origin (e.g. *F. graminearum* and *F. asiaticum*) (Aoki *et al.*, 2012).

An earlier review on the population structure of FGSC showed that: (i) a larger amount of variation (haplotype diversity ranging from 60 to 100%) is present within population than among the populations, (ii) population subdivision within a population is negligible, and (iii) gene flow is high (Miedaner *et al.*, 2008). In more recent studies conducted in South America, these results were confirmed with *F. graminearum* populations from Brazilian wheat (Astolfi *et al.*, 2012) and maize (Sampietro *et al.*, 2011). As reviewed recently, the several studies conducted thus far have employed a large set of different molecular markers, including random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), sequence-related amplified polymorphism (SRAP), variable number of tandem repeats (VNTR) and single nucleotide polymorphism (SNP) (Wang *et al.*, 2011). The marker choice seems to be a matter of availability, knowledge or preference by the researcher. AFLP is the most commonly used marker in genetic diversity and population structure studies in FGSC (Akinsanmi *et al.*, 2006; Ramirez *et al.*, 2007; Qu *et al.*, 2008; Lee *et al.*, 2009; Alvarez *et al.*, 2011; Astolfi *et al.*, 2012). Although there are some advantages such as that multiple bands can be derived from across the entire genome, AFLP is relatively costly and time-consuming compared to other marker systems, and is not highly reproducible (Wang *et al.*, 2011). Although less used to study variability for *F. graminearum* (Fernando *et al.*, 2006; Guo *et al.*, 2008) and *F. poae* (Dinolfo *et al.*, 2015), SRAP is a simple, reliable and cost-effective PCR-based assay. SRAP was developed to target open reading frames (ORFs) that encode proteins of interest and, as AFLP, requires no knowledge of nucleotide sequence (Li & Quiros, 2001). Studies comparing results of these different markers for the analysis of the FGSC populations have not been reported to date.

Studies on the species diversity of FGSC affecting barley in the main producing regions of South America, such as Argentina, Brazil and Uruguay, are scarce. In Brazil, 92 isolates associated with infected barley grain were identified to trichothecene genotypes using PCR assays, but only nine isolates representative of distinct tricothecene genotypes were identified to species by sequencing informative genes (Astolfi *et al.*, 2011). In Argentina, *F. graminearum* isolates were also identified to trichothecene genotypes and assessed for their *in vitro* mycotoxin production (Castañares *et al.*, 2014). Thus far, the genetic structure of FGSC populations in South America has been assessed only for isolates from wheat using AFLP (Ramirez *et al.*, 2007; Astolfi *et al.*, 2012) and from maize using VNTR markers (Sampietro *et al.*, 2011). Based on previous knowledge of the populations from wheat, it can be hypothesized that populations from barley in South America are structured by species, which can be revealed in a consistent manner by two molecular marker systems. The aim of this study was to test this hypothesis using a collection of over 100 isolates obtained from barley grains produced in the main barley growing regions of South America. In addition, the isolates were assessed for their potential to produce trichothecenes and ZEA using a range of PCR assays. Finally, a sample of isolates representative of different trichothecene genotypes were sequenced for reductase (*RED*) and trichothecene 3-*O*-acetyltransferase (*Tri101*) genes to confirm and compare their species assignment by the molecular marker data of reference isolates.

Materials and methods

Study region, isolation and PCR-based clade identification

A collection of 117 monosporic FGSC isolates was obtained from barley fields located in the main barley growing areas of Argentina (A, $n = 43$ isolates), Brazil (B, $n = 35$) and Uruguay (U, $n = 39$). The Argentinian isolates were obtained from six locations during two seasons (2010 and 2011); the Brazilian isolates were obtained from 19 locations during three seasons (2007, 2008 and 2009); and the Uruguayan isolates were obtained from one location during 2011 (Table S1). Argentinian and Brazilian isolates have been identified previously to the complex level (FGSC) and had their trichothecene genotype determined based on *Tri3*, *Tri7* and *Tri12* gene portions (Astolfi *et al.*, 2011; Castañares *et al.*, 2014). In addition, six previously identified *F. graminearum* isolates from wheat in Argentina were included in this study for comparison.

All isolates were stored on synthetic nutrient-poor agar (SNA) (Leslie & Summerell, 2006) and further grown on potato dextrose agar (PDA) media for mycelial production. Genomic DNA of 7-day-old cultures was extracted with a CTAB (cetyltrimethylammonium bromide) method as described elsewhere (Stenglein & Balatti, 2006). All isolates were identified using the clade-specific (*F. graminearum sensu lato*) Fg16N and polymorphic Fg16 primer set (Table 1; Nicholson *et al.*, 1998; Waalwijk *et al.*, 2003; Castañares *et al.*, 2014). Reference isolates for six species of the complex (*F. graminearum*, *F. meridionale*, *F. asiaticum*, *F. austroamericanum* and *F. cortaderiae*) were included as positive controls (Table S1). One *F. pseudograminearum* and one *F. cerealis* isolate from Argentinian barley were used as PCR negative controls.

Potential for DON, NIV and ZEA production

The potential to produce the main type B trichothecenes was determined for all isolates based on two PCR assays. First, NIV and DON genotypes were differentiated based on a PCR assay targeting portions of *Tri13* genes (primers Tri13NIVF, Tri13NIVR, Tri13DONF, Tri13DONR; Table 1; Chandler *et al.*, 2003). Secondly, the genotypes NIV, DON and the two DON acetylates, 3-ADON and 15-ADON, were differentiated based on a multiplex PCR assay targeting portions of the *Tri7* and

Table 1 Primer sequences used in the present study

Primer name	Primer sequence (5′–3′)	Reference
Fg16NF	ACAGATGACAAGATTCAGGCACA	Nicholson
Fg16NR	TTCTTTGACATCTGTTCAACCCA	*et al.* (1998)
Fg16F	CTCCGGATATGTTGCGTCAA	
Fg16R	GGTAGGTATCCGACATGGCAA	
Tri13NIVF	CCAAATCCGAAAACCGCAG	Chandler
Tri13NIVR	TTGAAAGCTCCAATGTCGTG	*et al.* (2003)
Tri13DONF	CATCATGAGACTTGTKCRAGTTTGGG	
Tri13DONR	GCTAGATCGATTGTTGCATTGAG	
Tri7F340	ATCGTGTACAAGGTTTACG	Quarta
Tri7R965	TTCAAGTAACGTTCGACAAT	*et al.* (2006)
3551H	ACTTTCCCACCGAGTATTTT	
4056H	CAAAAACTGTTGTTCCACTGCC	
Tri3F971	CATCATACTCGCTCTGCTG	
Tri3R1679	TTRTAGTTTGCATCATTRTAG	
Tri3F325	GCATTGGCTAACACATGA	
Tri3R1679	TTRTAGTTTGCATCATTRTAG	
PKS4F	CGTCTTCGAGAAGATGACAT	Meng *et al.*
PKS4R	TGTTCTGCAAGCACTCCG A	(2010)
REDF	AGACTCATTCCAGCCAAG	O'Donnell
REDR	TCGTGTTGAAGAGTTTGG	*et al.* (2000)
Tri101F	CAAGATACAGCTCGACACC	
Tri101R	CTGGGTAGTTGTTCGAGA	
ME1	TGAGTCCAAACCGGATA	This study
ME6	TGAGTCCAAACCGGTCA	
EM17	GACTGCGTACGAATTCCA	
ME5	TGAGTCCAAACCGGAAG	
EM13	GACTGCGTACGAATTGGT	

Tri3 genes (primers Tri7F340, Tri7R965, 3551H, 4056H, Tri3F971, Tri3R1679, Tri3F325, Tri3R1679; Table 1; Quarta *et al.*, 2006). One NIV *F. meridionale* isolate (NRRL 28436), one 3-ADON *F. austroamericanum* isolate (NRRL 2903) and one 15-ADON *F. graminearum* isolate (B26.1) were used as positive controls (Table S1). In addition, the potential of the isolates to produce ZEA was determined using the PKS4 primer set (PKS4F, PKS4R; Table 1) according to Meng *et al.* (2010). DNA of a ZEA-producer *F. cerealis* isolate and a ZEA-non-producer *F. poae* isolate were used as controls.

Sequence-based identification

Twenty-four isolates, selected from the overall collection based on their trichothecene genotype, were assigned to species based on their sequences of the *RED* and *Tri101* genes using primers REDF, REDR, Tri101F and Tri101R (Table 1; O'Donnell *et al.*, 2000). Among them, there were 12 NIV (B = 11 and U = 1), one 3-ADON (B = 1) and 11 15-ADON isolates selected at random (A = 6, B = 2, U = 3). PCR products were purified using the Purelink Quick Gel Extraction & PCR Purification Combo kit (Invitrogen) and sequenced in a BigDye Terminator v. 3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems) in a 3130 Hitachi Genetic Analyzer Sequencer (ABI). The sequences were compared and deposited in the GenBank database (accession numbers KR456289–KR456336; Table S2).

AFLP and SRAP analyses

Genomic DNA was completely digested with two restriction enzymes, *Eco*RI and *Mse*I, and ligated to AFLP adapters at 16°C overnight. After a preselective amplification, the selective amplification was carried out with two combinations of primers: *Eco*RI + TG/*Mse*I + TT and *Eco*RI + AC/*Mse*I + CA. The AFLP-PCR was performed according to Leslie & Summerell (2006). Amplified fragments were separated by vertical electrophoresis in polyacrylamide gels and then revealed by silver staining according to Bassam *et al.* (1991). AFLP fragments ranging from 150 to 450 bp were analysed.

Five of nine combinations of SRAP primers (Wang *et al.*, 2010) were selected based on efficiency to produce polymorphic fragments: ME1, ME5, ME6, EM13 and EM17 (Table 1). PCR conditions were as described elsewhere (Li & Quiros, 2001). Amplified fragments were separated and revealed as described for AFLP markers and all generated fragments were analysed. Some fragments were randomly selected to evaluate the primers' capacity to amplify ORFs and determine whether these fragments encoded part of any *Fusarium* protein. For this, two polymorphic and three monomorphic fragments were cut from a polyacrylamide gel and each transferred to a 1·5 μL Eppendorf. Then, 50 μL distilled water was added and the tubes were placed in a dry heat block at 95°C for 5 min. Finally, a new PCR-SRAP was performed using 5 μL of this final solution as DNA template. PCR fragments were purified and sequenced as described earlier. The sequences were compared with the FUSARIUM ID (Geiser *et al.*, 2004) database and were deposited in GenBank (Table 2).

All AFLP and SRAP reactions were repeated independently at least twice.

Analysis of genetic marker data

Amplified fragments of each of the markers were scored manually in a binary matrix (1 = presence, 0 = absence of fragment). All fragments were scored, including those that were monomorphic. Fragments migrating at the same position were assumed to be homologous. Unresolved data and missing data were treated as missing data. The genetic distance between individuals was estimated based on the simple matching similarity coefficient. Dendrograms were constructed using the UPGMA algorithm in statistical software NTSYSpc v. 2.0 (Rohlf, 1998). An analysis of molecular variance (AMOVA) was conducted to examine hierarchical population structure using distances among haplotypes. The distance chosen was a Euclidean metric equivalent to the number of differences between two individuals in their multilocus profile. Genotypic diversity (expected heterozygosis, *H*; Nei, 1987), or the average probability that two randomly chosen alleles at a locus are different, was estimated for the entire population, as well as for populations of each country and considering all FGSC isolates or only *F. graminearum* isolates, with the assumption that the populations are in Hardy–Weinberg equilibrium. These analyses were performed in Arlequin 2000 (Schneider *et al.*, 2000). The Mantel test (MXCOMP program of the NTSYSpc v. 2.0) was used to calculate the similarity between the matrices generated by both markers.

Results

PCR-based identification

All isolates amplified a single fragment of *c.* 280 bp with Fg16N, thus confirming that they belong to *F. graminearum sensu lato*. The differences in fragments sizes (from *c.* 380 to 580 bp) using the Fg16 primer set among the isolates suggest they belong to different spe-

Table 2 SRAP sequences and BLAST results

Selected fragment code	Primer combination	Type of fragment	GenBank accession number	BLASTX score (bits)	FUSARIUM ID
HM	SRAPME1/ EM13	Monomorphic	AR154224	49·2914	*F. oxysporum* PHW808: FOPG_02257.1: hypothetical protein
				49·2914	*F. oxysporum melonis*: FOMG_05224.1: hypothetical protein
				49·2914	*F. verticillioides* 7600 (FV3): FVEG_08236.5: hypothetical protein
IM	SRAPME1/ EM17	Monomorphic	AR154225	55·4546	*F. graminearum* PH-1 (FG3): FGSG_02271.3: hypothetical protein
				55·0694	*F. oxysporum* PHW808: FOPG_13898.1: primary-amine oxidase
				55·0694	*F. oxysporum melonis*: FOMG_03336.1: primary-amine oxidase
I5M	SRAPME1/ EM17	Monomorphic	AR154226	58·151	*F. graminearum* PH-1 (FG3): FGSG_02271.3: hypothetical protein
				58·151	*F. oxysporum* PHW808: FOPG_13898.1: primary-amine oxidase
				58·151	*F. oxysporum melonis*: FOMG_03336.1: primary-amine oxidase
JP	SRAPME6/ EM13	Polymorphic	AR154227	29·261	*F. verticillioides* 7600 (FV3): FVEG_10221.5: hypothetical protein
				28·4906	*F. oxysporum* II5: FOIG_07990.1: hypothetical protein
				27·7202	*F. graminearum* PH-1 (FG3): FGSG_07469.3: myosin-2B
2F	SRAPME6/ EM17	Polymorphic	AR154228	26·5646	*F. oxysporum* PHW808: FOPG_00483.1: hypothetical protein
				26·5646	*F. graminearum* PH-1 (FG3): FGSG_03906.3: hypothetical protein
				25·409	*F. graminearum* PH-1 (FG3): FGSG_09278.3: hypothetical protein

cies within FGSC. The two controls, *F. pseudograminearum* and *F. cerealis* showed no amplification.

Toxigenic potential

Using the singleplex PCR assay targeting *Tri13* gene portions, 13 isolates amplified a fragment of 312 bp with Tri13NIV primers, and did not amplify with the Tri13DON primers. The remaining 104 isolates amplified a fragment of 282 bp with primers Tri13DON and did not amplify with the Tri13NIV primer set. All controls amplified the expected fragments for the DON and NIV genotypes. Using the multiplex PCR assay targeting *Tri7* and *Tri3*, the same 13 isolates amplified a fragment of 625 bp, predictive of the NIV genotype, and the remaining isolates amplified a fragment of 525 bp, predictive of the DON genotype. For the DON genotypes, only one isolate amplified the 354 bp predictive of the 3-ADON genotype and the remainder amplified the fragment of 708 bp predictive of the 15-ADON genotype (Table S1). All surveyed isolates and the positive control amplified the fragment of 280 bp predictive of their potential to produce ZEA, while no amplification was observed for the *F. poae* isolate used as negative control.

RED and *Tri101*-based identification

The subsample of 24 sequenced isolates showed the highest percentage of similarity with five members of the FGSC from the GenBank database. Among them, all ($n = 11$) 15-ADON genotypes were assigned to *F. graminearum* (A = 6, B = 2, U = 3) and the only 3-ADON genotype (B = 1) was assigned to *F. austroamericanum*. Among the 12 NIV isolates, three species were identified: three *F. cortaderiae* (B = 2, U = 1), seven *F. meridionale* (B = 7) and two *F. asiaticum* (B = 2) (Tables S1 & S2).

AFLP and SRAP clustering

The out-groups *F. pseudograminearum* and *F. cerealis* were separated from FGSC isolates by AFLP markers (Fig. 1). Four main clusters were formed. Six *F. meridionale* isolates grouped with the reference *F. meridionale* isolate (cluster I), two *F. asiaticum* clustered together but not with the reference *F. asiaticum* isolate (cluster II), two *F. cortaderiae* clustered with the reference isolate (cluster III), and one isolate plus the reference formed the *F. austroamericanum* cluster (cluster IV). The remaining two *F. meridionale* and *F. cortaderiae* isolates, which were identified through sequencing, were distributed along the last portion of the dendrogram. The *F. graminearum* isolates were not clearly clustered (Fig. 1).

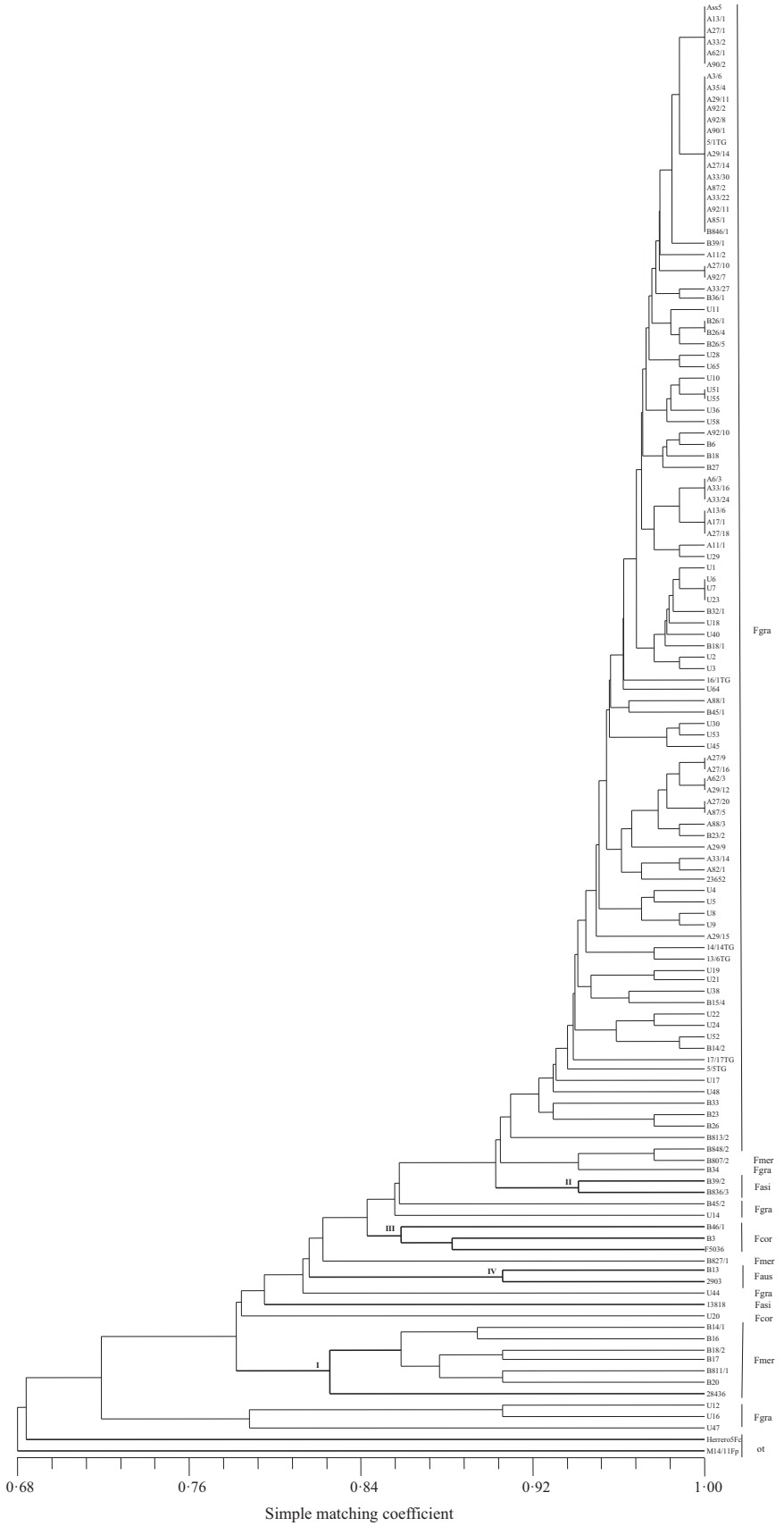
Using SRAP markers, the out-groups were not totally separated from FGSC isolates (Fig. 2). Two reference isolates (*F. cortaderiae* and *F. asiaticum*) were grouped together (cluster I). Only clusters II and IV comprised isolates from a single species, grouping two *F. asiaticum* and all *F. graminearum* isolates, respectively. In cluster III, there were three *F. cortaderiae*, eight *F. meridionale* and one *F. austroamericanum*, all identified by sequencing (Fig. 2).

The FGSC isolates were not structured by country of origin (Figs 1 & 2).

Genotypic diversity

The number of polymorphic loci was always higher for isolates from Brazil and Uruguay than those from Argentina for both molecular markers.

The estimated genotypic diversity based on AFLP markers was lower in Argentina (Nei's $H = 0{\cdot}88$) and higher in Uruguay and Brazil (Nei's $H > 0{\cdot}98$) whether considering all isolates or only *F. graminearum* isolates. The percentage of haplotypes was >90% in Brazil and Uruguay and around 40% in Argentina (Table 3).


Ass5
A13/1
A27/1
A33/2
A62/1
A90/2
A3/6
A35/4
A29/11
A92/2
A92/8
A90/1
5/1TG
A29/14
A27/14
A33/30
A87/2
A33/22
A92/11
A85/1
B846/1
B39/1
A11/2
A27/10
A92/7
A33/27
B36/1
U11
B26/1
B26/4
B26/5
U28
U65
U10
U51
U55
U36
U58
A92/10
B6
B18
B27
A6/3
A33/16
A33/24
A13/6
A17/1
A27/18
A11/1
U29
U1
U6
U7
U23
B32/1
U18
U40
B18/1
U2
U3
16/1TG
U64
A88/1
B45/1
U30
U53
U45
A27/9
A27/16
A62/3
A29/12
A27/20
A87/5
A88/3
B23/2
A29/9
A33/14
A82/1
23652
U4
U5
U8
U9
A29/15
14/14TG
13/6TG
U19
U21
U38
B15/4
U22
U24
U52
B14/2
17/17TG
5/5TG
U17
U48
B33
B23
B26
B813/2
B848/2
B807/2
B34
B39/2
B836/3
B45/2
U14
B46/1
B3
F5036
B827/1
B13
2903
U44
13818
U20
B14/1
B16
B18/2
B17
B811/1
B20
28436
U12
U16
U47
Herrero5Fc
M14/11Fp
Fgra
Fmer
Fgra
Fasi
Fgra
Fcor
Fmer
Faus
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Fmer
Fgra
ot
I
II
III
IV
0·68
0·76
0·84
0·92
1·00
Simple matching coefficient

Figure 1 Dendrogram obtained from AFLP marker data, showing clustering of *Fusarium graminearum* species complex (FGSC) isolates from barley grown in Argentina (A), Brazil (B) and Uruguay (U). The genetic distance between individuals was estimated based on the simple matching similarity coefficient. Fgra, *F. graminearum*; Fcor, *F. cortaderiae*; Faus, *F. austroamericanum*; Fmer, *F. meridionale*; Fasi, *F. asiaticum*; ot, out-group.

Conversely, the SRAP markers indicated that the genotypic diversity in Argentina, Uruguay and Brazil was similar (Nei's $H \geq 0{\cdot}99$). The percentage of haplotypes was ≥97% for all countries (Table 3).

The AMOVA results for both types of marker showed higher variability for populations within countries than among countries, whether using all isolates or only *F. graminearum* isolates. For AFLP, the estimated variability ranged from 7·9 to 10·3% for populations among countries and from 89·6 to 92·1% for populations within countries. For SRAP the estimated variability ranged from 7·4 to 12·6% for populations among countries and 87·3 to 92·5% for populations within countries.

Mantel test and SRAP sequences

The correlation coefficient obtained by the Mantel correspondence test to compare similarity matrices for different marker types showed a moderate correspondence ($r = 0{\cdot}75$), suggesting that the analysis should be made separately. All sequenced SRAP fragments were homologous with hypothetical proteins of *F. graminearum* or other *Fusarium* species, confirming the efficacy of this marker to amplify ORFs (Table 2).

Discussion

In this study, knowledge of the species composition of the FGSC pathogens infecting barley grains in the southern cone of South America has been expanded.

The results confirm the hypothesis from previous work, based on Fg16 amplifications, that *F. graminearum* is the only FGSC member causing FHB in barley in Argentina (Castañares *et al.*, 2014). In addition, this is the first detailed information on the FGSC members associated with barley in Uruguay, which showed that all but one (*F. cortaderiae*) isolate belonged to *F. graminearum*. This result contrasts with the higher diversity of FGSC members found in Uruguayan wheat, based on a multilocus genotyping (MLGT) assay (Umpiérrez-Failache *et al.*, 2013). In the Brazilian collection, two additional species, *F. cortaderiae* and *F. asiaticum*, are reported; in a previous study based on the polymorphism in Fg16 amplification, these same isolates could not be differentiated from *F. meridionale* (Astolfi *et al.*, 2011). These two species have been recently reported on wheat in southern Brazil during a large molecular survey based on a MLGT assay where *F. cortaderiae* was found to be much more prevalent than *F. asiaticum* (Del Ponte *et al.*, 2015).

The reasons for the higher diversity of FGSC found in Brazil compared, for example, to Argentina, where FHB is caused mainly by *F. graminearum*, is not well known. It is hypothesized that the subtropical environment may favour epidemics almost every year and the high number of hosts, including summer crops, may allow the survival of multiple species throughout the year. In the higher latitudes of Argentina and southern Uruguay, where the climate is mostly temperate, FHB epidemics are caused mainly by *F. graminearum*. For example, all isolates from wheat samples from Buenos Aires province, Argentina, were identified as *F. graminearum* (Ramirez *et al.*, 2007; Alvarez *et al.*, 2011). However, in the northern and central regions of Uruguay, where the climate is more similar to southern Brazil, other FGSC species have been described for wheat, including *F. asiaticum* in regions where rice is the dominant crop (Umpiérrez-Failache *et al.*, 2013). In fact, the widespread occurrence and dominance of NIV-producing *F. asiaticum* was also reported in a sampling of FGSC isolates from Brazilian rice, where *F. asiaticum* inoculum that could also infect wheat may survive and build up (Gomes *et al.*, 2015).

In the present study, none of the barley isolates segregated for trichothecene genotypes. *Fusarium graminearum* isolates were solely of the 15-ADON genotype, *F. austroamericanum* of the 3-ADON and *F. asiaticum*, *F. cortaderiae*, *F. meridionale* of the NIV genotype. Thus far, evidence indicates that FGSC members in South America are structured by trichothecene genotype and that higher diversity is found in Brazil (due to prevalence of multiple species) than in Uruguay and Argentina, where *F. graminearum* dominates. The dominance of 15-ADON isolates has been reported in previous studies with populations from wheat in Argentina and Uruguay. For example, Reynoso *et al.* (2011) found that most *F. graminearum* isolates in Argentina were of the 15-ADON genotype, although some amplified for both DON/NIV genotypes. Two NIV isolates were also detected by Pan *et al.* (2013) in Uruguay, but these isolates were identified as *F. graminearum* by AFLP analysis. Isolates with the 3-ADON genotype have also been identified in a small proportion of FGSC isolates from wheat produced in Argentina (Alvarez *et al.*, 2009) and Uruguay (Umpiérrez-Failache *et al.*, 2013).

In the present study, *F. asiaticum*, *F. cortaderiae* and *F. meridionale* showed potential to produce NIV and *F. austroamericanum* to produce 3-ADON, agreeing with previous studies of wheat and barley in the region (Astolfi *et al.*, 2011, 2012; Umpiérrez-Failache *et al.*, 2013; Del Ponte *et al.*, 2015). The current investigation also showed, for the first time in the region, that all isolates, irrespectively of the FGSC assignment, had the genetic potential to produce ZEA, which agrees with previous results of chemical analysis conducted with FGSC isolates (O'Donnell *et al.*, 2000).


Ass5
U5
U7
U10
U23
U8
B23/2
U30
A3/6
A92/7
A87/5
A33/22
A27/14
U52
U9
B36/1
A27/16
A27/20
A92/8
A87/2
A88/3
B39/1
A82/1
13/6TG
5/1TG
A33/2
A62/3
A33/14
A62/1
U55
U58
U1
U18
U51
A90/1
A90/2
U21
U24
U28
U40
A11/2
A17/1
A29/15
U4
B32/1
B846/1
U29
B26/1
B26/4
U3
U12
B14/2
B26/5
A11/1
A29/11
A63
A92/2
B18/1
U53
U36
U45
A13/1
A13/6
A92/10
A27/18
A33/30
B813/2
A27/1
U22
A33/24
A33/27
U64
A33/16
U6
A92/11
A85/1
14/14TG
A27/10
A29/9
A29/14
16/1TG
5/5TG
17/17TG
U19
A27/9
A35/4
A29/12
B45/1
B45/2
B15/4
U38
U48
B23
U44
U65
U2
U14
B26
B34
U17
A88/1
B33
U11
23652
U16
B848/2
B27
B18
B6
U47
U20
B14/1
B18/2
B46/1
2903
B3
B16
B17
B20
28436
B13
B811/1
B807/2
B827/1
B39/2
B836/3
Herrero5Fc
13818
F5036
M14/11Fp
IV
III
II
I
Fgra
Fcor
Fmer
Fcor
Faus
Fcor
Fmer
Faus
Fmer
Fasi
ot
Fasi
Fcor
ot
0·63
0·72
0·81
0·91
1·00
Simple matching coefficient

Figure 2 Dendrogram obtained from SRAP marker data, showing clustering of *Fusarium graminearum* species complex (FGSC) isolates from barley grown in Argentina (A), Brazil (B) and Uruguay (U). The genetic distance between individuals was estimated based on the simple matching similarity coefficient. Fgra, *F. graminearum*; Fcor, *F. cortaderiae*; Faus, *F. austroamericanum*; Fmer, *F. meridionale*; Fasi, *F. asiaticum*; ot, out-group.

Table 3 Comparison of estimates of genotypic diversity based on AFLP and SRAP loci for three populations comprising isolates of all five species within *Fusarium graminearum* species complex or only for *F. graminearum* isolates (within parentheses) obtained from barley grains produced in Argentina, Brazil and Uruguay

		AFLP			SRAP		
Population	No. isolates	No. polymorphic loci	% haplotypes	Nei's *H*	No. polymorphic loci	% haplotypes	Nei's *H*
Argentina	43 (43)	14 (14)	41·8 (41·8)	0·88 (0·88)	30 (30)	97·7 (97·7)	0·99 (0·99)
Brazil	35 (22)	52 (31)	97·1 (95·4)	0·99 (0·99)	82 (58)	100 (100)	1 (1)
Uruguay	39 (38)	55 (50)	92·3 (92·1)	0·99 (0·99)	72 (60)	97·4 (97·4)	0·99 (0·99)
Overall	117 (103)	69 (56)	74·3 (70·8)	0·98 (0·97)	94 (77)	98·3 (98·0)	0·99 (0·99)

In most AFLP studies on FGSC, isolates have been successfully clustered with reference isolates for the different species (Qu *et al.*, 2008; Alvarez *et al.*, 2011; Astolfi *et al.*, 2012). However, in one study, isolates representative of different FGSC species grouped in the same AFLP cluster, which discouraged the use of this marker for species identification (Akinsanmi *et al.*, 2006). In the present study, the species to which sequenced isolates were assigned was the same regardless of which of the two informative genes were used (O'Donnell *et al.*, 2000). The results were partially, but not totally, congruent with AFLP and SRAP clustering, as some species of the complex were not grouped together with their representative reference isolate. For instance, SRAP markers were able to cluster all *F. graminearum* isolates together.

The lack of clustering by geographic region suggests a possible genetic exchange among these regions, with the isolate being part of a metapopulation in which the fungus is capable of dispersing over long distances (Schmale *et al.*, 2006). Transport of infected seed may contribute to dispersal of toxigenic genotypes.

Analysis of the genetic diversity using SRAP markers showed a higher number of haplotypes (115) than using AFLP (87). Among the countries, the Argentinian populations had a lower number of unique haplotypes than Brazil or Uruguay using AFLP, but a similar haplotype number was found between them using SRAP. In contrast, a higher proportion of haplotypes in relation to total isolates was reported in previous studies using AFLP in Argentina and Brazil: 96/103 (Astolfi *et al.*, 2012), 104/113 (Ramirez *et al.* (2007) and 183/183 (Alvarez *et al.*, 2011). The high number of haplotypes suggests that sexual recombination is prevalent within *Fusarium* populations, explaining the high level of genetic diversity found in the present study with both molecular markers. The difference between results from the present and previous studies for AFLP analysis may be related to the different number and/or type of primers used in each study or to a lower genetic polymorphism for *F. graminearum* isolates obtained from barley grains.

Differences in dendrograms and AMOVA analyses between AFLP and SRAP markers may be related to the number of primer combinations used in each technique. In addition, each technique has different aims; while SRAP is targeted to amplify ORFs, AFLP is based on the amplification of restriction fragments. The number of amplicons per AFLP assay is a function of the number of selective nucleotides in the AFLP primer combination, the selective nucleotide motif, GC content, and physical genome size and complexity (Agarwal *et al.*, 2008). Moreover, the differences among techniques may also explain the moderate, rather than high, correlation among matrices obtained with Mantel test, although both methods showed a high level of genetic diversity, as previously reported for *F. graminearum* (Akinsanmi *et al.*, 2006; Ramirez *et al.*, 2007; Guo *et al.*, 2008; Astolfi *et al.*, 2012).

Compared to the expensive and time-consuming AFLP technique, SRAP combines simplicity, reliability, moderate throughput ratio, sequencing of selected fragments and the possibility of targeting ORFs. The SRAP technique has been used to evaluate genetic variability in *F. graminearum* in previous studies (Fernando *et al.*, 2006; Guo *et al.*, 2008), although the fragments were not sequenced to identify ORFs. Dinolfo *et al.* (2015) used SRAP markers to study genetic variability of *F. poae* and, as in the present investigation, sequenced many fragments of interest generated by this marker, corroborating the efficacy of this technique to amplify ORFs.

In summary, the results of this investigation suggest that FHB management in barley, including regulatory measures, should consider regional variations in the species and the toxigenic ability of the populations, especially their potential to produce NIV, which is much more toxic than DON (Minervini *et al.*, 2004). The ability of the pathogen to disperse long distance by wind (Schmale *et al.*, 2006) and seeds may contribute to dissemination of toxigenic genotypes across the continent, especially considering future variations or changes that may lead to a warmer climate. This, therefore, poses a challenge to researchers and growers to reduce the impact of *Fusarium* on the food and beer industry.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. Country of origin, locality, trichothecene genotype, species and host of the *Fusarium graminearum* species complex isolates used in this study.

Table S2. GenBank accession numbers of 24 *Fusarium graminearum* species complex isolates.