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ISSR markers detect high genetic variation among *Fusarium poae* isolates from Argentina and England

María I. Dinolfo · Sebastian A. Stenglein · María V. Moreno · Paul Nicholson · Philip Jennings · Graciela L. Salerno

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Abstract *Fusarium poae* is one of the *Fusarium* species isolated from cereal grains infected by Fusarium head blight (FHB), and in recent years it has been identified as a major FHB component. In this study, 97 *F. poae* isolates from Argentina (n=62) and England (n=35) were analysed by inter-simple sequence repeats (ISSR) to examine the genetic diversity and to determine whether intraspecific variation could be correlated with geographic and/or host origin. The molecular analysis showed high intraspecific variability within *F. poae* isolates, but did not reveal a clear relationship between variability and the host/geographic origin. *Fusarium poae* isolates from the same geographic region or host appeared in different subclusters. Conversely, isolates with the same haplotype were also collected from different geographic regions. However, we did observe subclusters consisting of isolates from Argentina only or from England only. Furthermore, a single seed sample was found to host different haplotypes. Analysis of molecular variance (AMOVA) indicated a high genetic variability in *F. poae*, with most of the genetic variability explained by differences within, rather than between Argentinean and English populations. This is the first report on genetic diversity of *F. poae* using ISSR markers. Moreover, ISSR fingerprinting generates highly polymorphic markers for *F. poae* and proved to be a useful and reliable assay for genetic variability studies.

Keywords *Fusarium poae* · Genetic variability · Inter-simple sequence repeats

M. I. Dinolfo · S. A. Stenglein (✉) · M. V. Moreno
Laboratorio de Biología Funcional y Biotecnología (BIOLAB)-CEBB, Facultad de Agronomía de Azul. UNCPBA,
Av. República de Italia # 780,
CP 7300 Azul, Buenos Aires, Argentina
e-mail: stenglein@faa.unicen.edu.ar

S. A. Stenglein · M. V. Moreno · G. L. Salerno
Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET),
Av. Rivadavia # 1917,
CP C1033AAJ Buenos Aires, Argentina

P. Nicholson
John Innes Centre, Norwich Research Park,
Colney Lane,
Norwich NR4 7UH, UK

P. Jennings
Central Science Laboratory,
Sand Hutton,
York YO41 1LZ, UK

G. L. Salerno
Fundación para Investigaciones Biológicas Aplicadas (FIBA)-CEBB,
Vieytes 3103,
CP 7600 Mar del Plata, Argentina

Introduction

Fusarium head blight (FHB) is a disease caused by a complex of fungal species and has a severe impact through reductions in yield and grain quality. The predominant *Fusarium* species worldwide is *F. graminearum* Schwabe. However, recent studies have cited *F. poae* (Peck) Wollenweber as one of the most frequently isolated FHB pathogens (e.g. Birzele et al. 2002; Xu et al. 2005; Bourdages et al. 2006; Stenglein et al. 2008). Among the mycotoxins produced by *Fusarium* species, *F. poae* produces both type A and B trichothecenes and other toxins such as beauvericin (BEA), enniatin (ENNs) and fusarin (Jestoi et al. 2004, 2008; Thrane et al. 2004; Desjardins 2006; Chelkowski et al. 2007; Vogelgsang et al. 2008a, b). In contrast to *F. graminearum* and *F. culmorum* (W.G. Smith) Saccardo, *F. poae* is a relatively weak pathogen of cereal plants and the lack of extensive visible symptoms on grains after infection constitutes a potential risk to humans and animals (Wong et al. 1992; Xu et al. 2007) since contaminated cereal lots could be consumed or fed.

Several molecular markers have been used to analyse the diversity of *Fusarium* plant pathogens at the genome level: amplified fragment length polymorphism (AFLP) in *F. graminearum* and *F. asiaticum* O'Donell, Aoki, Kistler & Geiser (Qu et al. 2008), random amplified polymorphic DNA (RAPD) in *F. graminearum* and *F. poae* (Ouellet and Seifert 1993; Kerényi et al. 1997), simple sequence repeats (SSR) in *F. graminearum* and *F. culmorum* (Vogelgsang et al. 2009), and inter-simple sequence repeats (ISSR) in *F. graminearum* and *F. culmorum* (Mishra et al. 2003, 2004). AFLP analysis has been used also for studying relationships at taxonomic levels within a world-wide collection of *F. sporotrichioides* Sherbakoff, *F. langsethiae* Torp & Nirenberg and *F. poae* isolates (Schmidt et al. 2004). In addition, internal transcribed region (ITS), intergenic spacer region (IGS), β-tubulin, mitochondrial small subunit rDNA (*mtSSU*), and translation elongation factor 1-alpha (*EF-1*α) sequences of *F. poae* isolates were studied (Yli-Mattila et al. 2004; Stenglein et al. 2010). ISSR is a PCR-based technique that consists of the amplification of DNA sequences between SSR by means of anchored or non-anchored SSR homologous primers (Zietkiewicz et al. 1994). Unlike SSR, ISSR does not require a previous knowledge of the sequence and generates specific and reproducible patterns due to the highly stringent conditions of the reaction (Bornet and Branchard 2001).

Based on the increasing awareness of *F. poae* in the FHB complex (Yli-Mattila et al. 2008; Kulik and Jestoi 2009; Stenglein 2009) and the lack of information relating to the population genetics and variability of this species, the aims of our study were (a) to analyze the genetic variability of *F. poae* isolates originating from two geographically distant countries, Argentina and England, recovered mostly from wheat and barley (plus five isolates obtained from oat and one obtained from tomato), by ISSR markers and (b) to examine whether intraspecific variation could be correlated with geographic and/or host origin.

Materials & methods

Wheat and barley seed samples (500 g) collected by farmers were obtained from different locations within the main production area in Argentina. Two-hundred seeds per sample (obtained using a seed divider) were surface-sterilised by dipping successively into 70% ethanol for 2 min, 5% sodium hypochlorite for 2 min and finally rinsed twice in fresh sterilised distilled water. The surface-sterilised seeds were placed onto potato dextrose agar (PDA) with 0.25 g chloramfenicol^{-1} and incubated for 4–7 days at 25±2°C under 12 h light/dark. Isolates were morphologically identified on PDA and on "Spezieller Nährstoffarmer Agar" (SNA) according to Leslie and Summerell (2006). One to six isolates were obtained from each seed sample, which allowed us to evaluate if one sample can host two or more haplotypes at a time. English isolates were obtained all from wheat seeds in a similar manner. MICA isolates obtained from wheat and oat seeds were provided by H. González and the tomato isolate was obtained from flower tissues (Stenglein et al. 2009). All isolates were collected between 1998–2008 and the monosporic isolates were stored under sterile mineral oil on SNA at 4°C.

Genomic DNA from a total of 97 *F. poae* and one *F. sporotrichioides* (used as an external group) monosporic isolates (Table 1) was extracted using the CTAB method described by Stenglein and Balatti (2006). The DNA concentration was estimated with a fluorometer (Qubit™-Invitrogen, Buenos Aires, Argentina). To confirm visual identifications, *F. poae*-specific PCR was performed for the 97 isolates

Table 1 Identification and geographic origin of *Fusarium poae* isolates used in this study

Argentinean isolates[a]	Gepgraphic origin Locality/Province	Argentinean isolates[a]	Geographic origin Locality/Province	English isolates[b]	Geographic origin
HSu1a	Cnel. Suarez/Buenos Aires	TSa1b	Saladillo/Buenos Aires	4/4343/1	Suffolk
HSu1b	Cnel. Suarez/Buenos Aires	TSm1a	San Manuel/Buenos Aires	1,021	Lancashire
HPu1a	Puán/Buenos Aires	TSm2a	San Manuel/Buenos Aires	1,020	Cheshire
HPu2a	Puán/Buenos Aires	TBig1a	Bigand/Santa Fé	1,019	W. Yorkshire
HPu3a	Puán/Buenos Aires	TBig1b	Bigand/Santa Fé	887	W. Yorkshire
HPu4a	Puán/Buenos Aires	TBig2a	Bigand/Santa Fé	751	Dorset
HPu5a	Puán/Buenos Aires	TMa1a	25 de Mayo/Buenos Aires	750	Dorset
HPu5b	Puán/Buenos Aires	TMa1b	25 de Mayo/Buenos Aires	747	Buckinghamshire
HPu5c	Puán/Buenos Aires	TJu1a	Junín/Buenos Aires	743	Hertfordshire
HPu5d	Puán/Buenos Aires	TJu1b	Junín/Buenos Aires	736	Norfolk
HPu5e	Puán/Buenos Aires	TPe1a	Pergamino/Buenos Aires	735	Bedfordshire
HPu5f	Puán/Buenos Aires	SP1a	San Pedro/Buenos Aires	731	Hereford & Worcester
HTA1a	Tres Arroyos/Buenos Aires	TAz1a	Azul/Buenos Aires	721	Humberside
HBe1a	Bellocq/Buenos Aires	T-MICA-01	Bragado/Buenos Aires	720	Merseyside
HBe1b	Bellocq/Buenos Aires	T-MICA-02	Bragado/Buenos Aires	718	Northumberland
HBe1c	Bellocq/Buenos Aires	T-MICA-03	Arrecifes/Buenos Aires	563	Oxfordshire
HBe1d	Bellocq/Buenos Aires	T-MICA-04	Arrecifes/Buenos Aires	552	Surrey
HBig1a	Bigand/Buenos Aires	T-MICA-05	San Antonio de Areco/Buenos Aires	527	County Durham
TCa1a	Castelar/Buenos Aires	T-MICA-06	San Antonio de Areco/Buenos Aires	522	Cambridgesgire
TSS1a	Sancti Spiritu/Santa Fé	T-MICA-07	Junín/Buenos Aires	507	Lincolnshire
TSS1b	Sancti Spiritu/Santa Fé	T-MICA-08	Junín/Buenos Aires	506	Somerset
TSS2a	Sancti Spiritu/Santa Fé	T-MICA-09	Pergamino/Buenos Aires	504	Suffolk
TSS2b	Sancti Spiritu/Santa Fé	A-MICA-01	Gualeguaychú (Entre Ríos)	444	Hereford
TSS2c	Sancti Spiritu/Santa Fé	A-MICA-02	Gualeguaychú (Entre Ríos)	303	Kent
THo1a	Los Hornos/Buenos Aires	A-MICA-03	Urdinarraín (Entre Ríos)	301	Lincolnshire
THo1b	Los Hornos/Buenos Aires	A-MICA-04	Urdinarraín (Entre Ríos)	300	Nottinghamshire
THo1c	Los Hornos/Buenos Aires	A-MICA-05	Basavilbaso (Entre Ríos)	295	Norfolk
THo1d	Los Hornos/Buenos Aires			173	Hampshire
THo2a	Los Hornos/Buenos Aires			141	Wiltshire
TPu1a	Puán/Buenos Aires			F18	Cambridge
TPu1b	Puán/Buenos Aires			F600	NN
TPu2a	Puán/Buenos Aires			4/3084/1	Surrey
TPu3a	Puán/Buenos Aires			4/3084/2	Surrey
TPu4a	Puán/Buenos Aires			4/3084/3	Surrey
TSa1a	Saladillo/Buenos Aires			4/3084/4	Surrey

NN, without data

[a] For Argentinean isolates, the first capital letter identifies the host (H: *Hordeum vulgare*; T: *Triticum aestivum*; S: *Solanum lycopersicum*; A: *Avena sativa*), the numbers identify different samples and the small letters identify different isolates of a sample. MICA isolates were provided by H. González, Facultad Ciencias Exactas y Naturales, UBA, Buenos Aires, Argentina

[b] All English isolates were obtained from wheat seeds and provided by P. Nicholson and P. Jennings

and for one *F. sporotrichioides* isolate used as negative control using primers Fp82F 5′-CAAGCAAACAGGCTCTTCACC-3′ and Fp82R 5′-TGTTCCACCTCAGTGACAGGTT-3′. All *F. poae* isolates produced a fragment of 220 bp identical to that observed by Parry and Nicholson (1996). Over a collection of 25 ISSR primers, six, $(GAG)_5$ CAG, $(AG)_8$, CTC $(GT)_8$, CT $(GA)_8$, $(GCC)_5$, and $(CAC)_5$, were used to assess diversity of *F. poae* isolates. These primers were selected based on their ability to consistently amplify the same fragment(s) from a given isolate. Furthermore, the fragments were polymorphic in that the same fragment(s) were not amplified from all isolates. ISSR amplifications were performed in a XP thermal cycler (Bioer Technology Co, Hangzhou, China) according to Stenglein and Balatti (2006). The annealing temperature was modified according to the sequence of the primer used: 48°C for $(AG)_8$ and CTC $(GT)_8$; 53°C for $(GAG)_5$ CAG, $(CAC)_5$ and CT $(GA)_8$; 66°C for $(GCC)_5$. A small number of samples (3) showed some bands with nearby migration. Each reaction was performed at least twice, running eight DNA samples plus the DNA ladder and the negative control in one gel, simultaneously. Products from ISSR reactions were examined by electrophoresis in 1.5% (*w*/*v*) agarose gels containing GelRed™ (Biotium, Hayward, USA) at 80 V in 1X Trisborate-EDTA buffer for 3–4 h at room temperature. Fragments were visualised under UV light. The size of the DNA fragments were estimated by comparing the DNA bands with a 1 kb and 100 bp DNA ladder (Genbiotech S.R.L., Buenos Aires, Argentina). Gel images were photographed with a digital DOC 6490 system (Biodynamics S.R.L., Buenos Aires, Argentina).

Clear, reproducibly amplified fragments were scored as present (1) or absent (0) and fragments of the same size were considered homologous. The data were assembled in a matrix and genetic similarities between all the pairs of isolates were computed using the Jaccard coefficient (Sneath and Sokal 1973). The data were used to construct a dendrogram using the unweighted pair group method with arithmetic average (UPGMA). The cophenetic correlation coefficient (CCC) was chosen to indicate the level of distortion between the similarity matrix and cluster analysis. NTSYSpc version 2.0 was used to perform these analyses (Rohlf 1998). Bootstrap values were calculated for 1,000 replicates using WINBOOT software (Yap and Nelson 1996).

Analysis of molecular variance (AMOVA, Excoffier et al. 1992) was used to calculate the variance among and within *F. poae* populations formed on the basis of the molecular markers. This procedure is based on an analysis of variance using distances between haplotypes. The distance chosen was an Euclidean metric equivalent to the number of differences between two individuals in their multilocus profile. Gene 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 the two populations (Argentinean and English isolates), with the assumption that the populations are in Hardy–Weinberg equlibrium. Arlequin 2000 was the software used to perform these analyses (Schneider et al. 2000).

Results & discussion

A total of 54 fragments were amplified and among them 48 (89%) were polymorphic, in that they were not amplified from all isolates. An example of amplification reactions with primer CT $(GA)_8$ and $(CAC)_5$ are presented in Fig. 1. Cluster analysis of ISSR data defined 90 *F. poae* haplotypes among the 97 isolates analysed, showing that 93% had unique banding patterns (Fig. 2). The *F. poae* isolates were resolved first in two clusters (I, II) with an average similarity between groups of 53%. Cluster I included eight English isolates. Cluster II included all the remaining isolates, which were further resolved into 12 subclusters that were designated subclusters IIa–VIIb. Subcluster IIa included a total of 11 Argentinean isolates. Subcluster IIb resolved into additional subclusters IIIa and IIIb (61% genetic similarity). Subcluster IIIa included five isolates from England. Subcluster IIIb resolved into subclusters IVa and IVb (65% genetic similarity), where IVa included four isolates from Argentina. Again, subcluster IVb resolved two subclusters, Va and Vb (68% genetic similarity). Subcluster Va included only two isolates from Argentina. Subcluster Vb resolved into subclusters VIa and VIb (70% genetic similarity). In this way five Argentinean plus two English isolates were included in VIa. Finally, subcluster VIb, resolved into subclusters VIIa and VIIb (72% genetic similarity). Subcluster VIIa included the remaining 20 English isolates, and subcluster VIIb the remaining 41 isolates from Argentina.

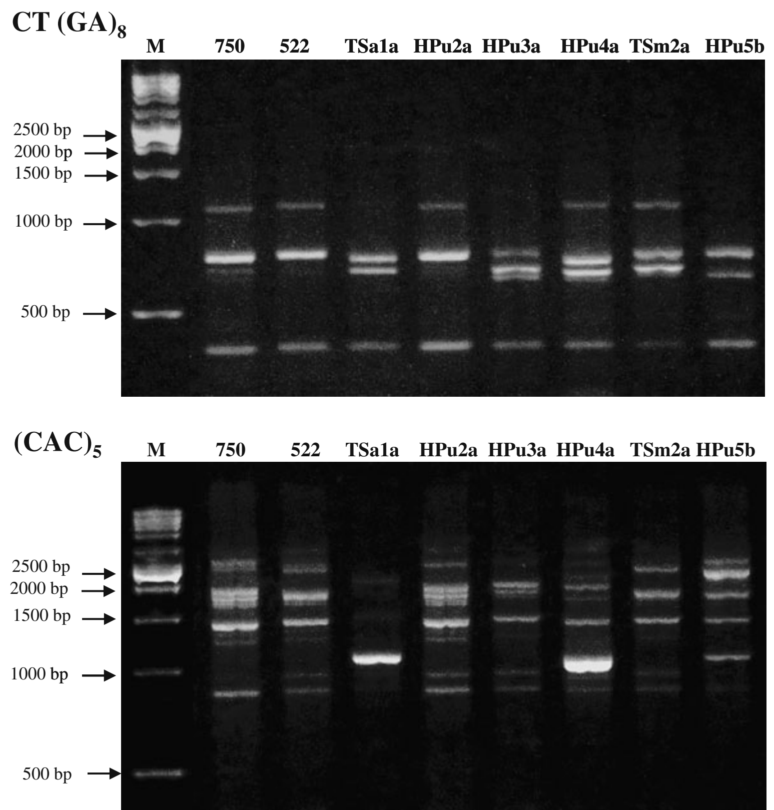


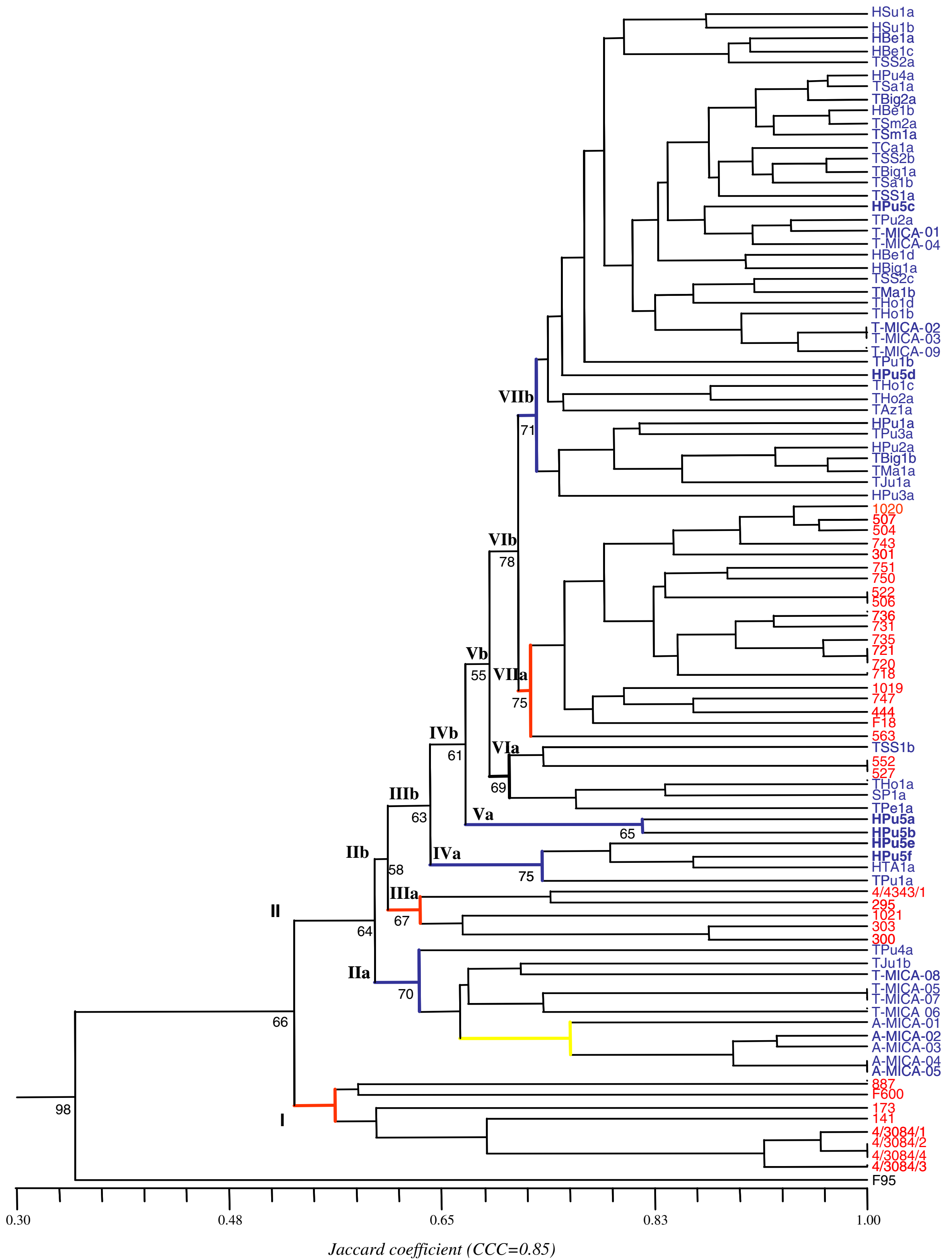
Fig. 1 Amplification patterns of eight *F. poae* isolates with primers $CT(GA)_8$ and $(CAC)_5$. M = molecular size marker

It's important to note that subcluster VIIa included 21% of all isolates analysed in the current study (57% of all English isolates) and that subcluster VIIb included almost the half of the isolates (42%), representing 66% of all *F. poae* isolates from Argentina.

Stenglein et al. (2010), analysing a great number of the *F. poae* isolates also used in this study, reported that there was an extensive and moderate conservation in the *mtSSU* and the *EF-1*α-1 sequences. The molecular analysis using ISSR markers showed high intraspecific variability within *F. poae* isolates, but it did not reveal a clear relationship between variability and geographical regions, in agreement with the results obtained with other studies on *Fusarium* species (e.g. Gargouri et al. 2003; Bayraktar et al. 2007). Moreover, the present cluster analysis did not reveal any correlation between host and ISSR profiles of *F. poae* isolates, except for isolates obtained from oat seeds (all from Entre Ríos, Argentina), which formed a small subcluster, but the number of isolates is too small (only five isolates) to suggest host or geographic specialisation (Fig. 2). Diversity studies using a greater number of isolates from oat and other cereal crops are needed to determine their host-dependent population structure.

Two populations of isolates with geographically distant origins (Argentinean and English) were not clearly separated by ISSR data, a result which parallels findings from *mtSSU* and *EF-1*α-1 sequence analyses, where isolates with distinct country origin were resolved in the same branches (Stenglein et al. 2010). However, we found a series of subclusters formed by isolates from Argentina only (subclusters IIa, IVa, Va, VIIb) or England (subclusters I, IIIa, VIIa), except for subcluster VIa that resolved a low number of isolates from both countries. *Fusarium poae* has been reported to produce type A and B trichothecenes, among other mycotoxins (Langseth et al. 1999; Jestoi et al. 2004; Thrane et al. 2004; Desjardins 2006; Jestoi et al. 2008; Vogelgsang et al. 2008a, b), and a possibility, that needs further studies, is that the isolates were resolved based on their abilities to synthesise particular toxins.

Based on the current ISSR data analysis, it was shown that *F. poae* isolates from the same geographic region or host sample (e.g. isolates TPu1a and TPu1b;


HSu1a
HSu1b
HBe1a
HBe1c
TSS2a
HPu4a
TSa1a
TBig2a
HBe1b
TSm2a
TSm1a
TCa1a
TSS2b
TBig1a
TSa1b
TSS1a
HPu5c
TPu2a
T-MICA-01
T-MICA-04
HBe1d
HBig1a
TSS2c
TMa1b
THo1d
THo1b
T-MICA-02
T-MICA-03
T-MICA-09
TPu1b
HPu5d
THo1c
THo2a
TAz1a
HPu1a
TPu3a
HPu2a
TBig1b
TMa1a
TJu1a
HPu3a
1020
507
504
743
301
751
750
522
506
736
731
735
721
720
718
1019
747
444
F18
563
TSS1b
552
527
THo1a
SP1a
TPe1a
HPu5a
HPu5b
HPu5e
HPu5f
HTA1a
TPu1a
4/4343/1
295
1021
303
300
TPu4a
TJu1b
T-MICA-08
T-MICA-05
T-MICA-07
T-MICA 06
A-MICA-01
A-MICA-02
A-MICA-03
A-MICA-04
A-MICA-05
887
F600
173
141
4/3084/1
4/3084/2
4/3084/4
4/3084/3
F95
VIIb
71
VIb
78
Vb
55
VIIa
75
IVb
61
VIa
69
IIIb
63
Va
65
IIb
58
IVa
75
IIIa
67
II
64
IIa
70
66
98
I
0.30
0.48
0.65
0.83
1.00
Jaccard coefficient (CCC=0.85)

◀ **Fig. 2** Cluster analysis dendrogram of the 97 *F. poae* isolates plus *F. sporotrichioides* isolate F95 using ISSR data. *Blue*: Argentinean isolates. *Red*: English isolates. *Bold black*: isolates from both Argentina and England. Isolates in *bold* are examples of different haplotypes obtained from the same seed sample. Branches in *yellow* resolved isolates obtained from oat seeds. Cluster I included eight English isolates. Cluster II was resolved into 12 subclusters: IIa, IVa, Va and VIIb included 58 Argentinean isolates; IIIa and VIIa included 25 English isolates; VIa included four Argentinean and two English isolates. For Argentinean isolates, the first capital letter identifies the host (H: *Hordeum vulgare*; T: *Triticum aestivum*; S: *Solanum lycopersicum*; A: *Avena sativa*). All English isolates were obtained from wheat seeds. *Numbers* under the nodes indicate bootstrap values (%)

HBe1a and HBe1b) appeared in different subclusters. Conversely, isolates with the same haplotype were collected from different geographic regions (e.g. isolates 552 and 527). Considering the diverse agro-environments where we obtained the isolates, results suggest that geographic isolation, ecological conditions and crop rotation systems did not have a significant effect on the haplotype distribution of *F. poae* isolates. Although the distribution of haplotypes in Argentina may have arisen through reproduction and dispersal, it is also possible that different haplotypes might have been dispersed and introduced in the fields by planting of asymptomatic seeds.

The AMOVA (fixation index of differentiation between population divisions $\Phi ST=0.174$; $P<0.001$) indicated that most of the variation resulted from genetic differences within (83%), rather than from differences between Argentinean and English populations (17%). The Argentinean population had 30% of polymorphic loci, whereas the English population had 32%. Diversity of the entire population was estimated to be 0.998 ($SD=0.018$) showing that the majority (99%) of the genotypes present within the *F. poae* population were different. Furthermore, expected heterozygosis of the Argentinean and English populations were 0.998 ($SD=0.003$) and 0.993 ($SD=0.008$), respectively. Previous DNA analyses using molecular markers suggested that there was a moderate level of genetic variability within *F. poae* (Kerényi et al. 1997), perhaps because of the low number of isolates (54) and/or the use of RAPD markers. However, the AFLP analyses for *Fusarium sporotrichioides*, *F. langsethiae* and *F. poae*, resolved several dendrogram branches among *F. poae* isolates (Schmidt et al. 2004). The amount of expected heterozygosis detected in our study was high, and although 62 isolates are from Argentina and 35 are from England, diversity based on ISSR markers was very similar, suggesting that these levels of diversity might be an intrinsic characteristic of the fungus. However, since isolates from only two geographic regions were analysed further research is needed to address this aspect. The high genetic variability found in our study and the partitioning of genetic variation in *F. poae* are in agreement with the estimates of expected heterozygosis reported for other *Fusarium* species (e.g. Miedaner et al. 2001; Mishra et al. 2004). Both the *MAT-1* and *MAT-2* mating types present in heterothallic *Fusarium* species occur and are transcribed in *F. poae* (Kerényi et al. 2004), and while the teleomorph is not known at the moment, sexual reproduction may provide the potential for recombination in this fungus. However, evidence that high levels of haplotypic diversity can be maintained in asexually reproducing fungi through parasexual reproduction has been obtained (Zeigler et al. 1997). Chromosomal translocations, deletions and segmental chromosome loss have the capability to increase diversity in fungi, contributing in this way to high haplotypic diversity (Kistler and Miao 1992).

Another important result was the demonstration that all Argentinean *F. poae* isolates obtained from a single seed sample can host different haplotypes (e.g. THo1a, b, c, d; HPu5a, b, c, d, e, f) (Fig. 2). This coexistence might have importance in the development of haplotype diversity, and might also have epidemiological implications, since the presence of more than one haplotype in the same field may permit parasexual recombination between isolates. Carefully planned surveys might help to elucidate the effect of infection and co-infection on *F. poae* diversity.

In conclusion, the ISSR fingerprinting described in our study generated highly polymorphic markers for *F. poae* and proved to provide useful and reliable molecular markers for further genetic diversity studies. The high genetic diversity could expedite the emergence of new genotypes of *F. poae* that may generate novel haplotypes with enhanced pathogenic properties, adaptation to different environments/hosts and/or differences in toxigenicity.

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