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SHORT COMMUNICATION

SRAP as an Informative Molecular Marker to Study the *Fusarium poae* Genetic Variability

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Abstract

Fusarium poae is one of the *Fusarium* species isolated from grains associated with Fusarium head blight (FHB), whose occurrence has increased in the last years. In this study, a total of 105 *F. poae* isolates from Argentina, Belgium, Canada, England, Finland, France, Germany, Hungary, Italy, Luxembourg, Poland, Switzerland and Uruguay were evaluated using sequence-related amplified polymorphism (SRAP) to analyse the capacity of this molecular marker to evaluate the *F. poae* genetic variability. The molecular analysis showed high intraspecific variability within *F. poae* isolates, and a partial relationship was revealed between variability and the host/geographic origin. Analysis of molecular variance (AMOVA) indicated a high genetic variability in the *F. poae* collection, with most of the genetic variability resulting from differences within, rather than between American and European populations. The analysis of sequenced SRAP fragments targets into hypothetical proteins from different *Fusarium* species showing that the SRAP technique not only allows studying *F. poae* genetic variability, but also targets coding regions into the *F. poae* genome. To our knowledge, this is the first report on genetic variability of *F. poae* using SRAP technique and also demonstrates the efficacy of this molecular marker to amplify open reading frames in fungus.

Introduction

Fusarium head blight (FHB) is one of the most economically important diseases affecting the production of wheat, barley and other small grains worldwide, which causes yield losses and reduces seed quality not only by the fungus presence, but also by mycotoxin production (Parry et al. 1995; McMullen et al. 1997; Windels 2000; Bottalico and Perrone 2002). Some *Fusarium* species such as *Fusarium graminearum, F. avenaceum, F. culmorum* and *F. poae* have been isolated from grains with FHB symptoms (Xu et al. 2008; Kulik et al. 2011).

Although *Fusarium graminearum* is the most frequently isolated species, in the last few years, a gradual increase in incidence of *F. poae* has been reported (Pereyra and Dill-Macky 2010; Lenc 2011; Infantino et al. 2012; Stenglein et al. 2012). *Fusarium poae* is a cosmopolitan pathogen able to synthesize mycotoxins including enniatins, beauvericin and type A (e.g. diacetoxyscirpenol) and B (e.g. nivalenol) trichothecenes with recognized cytotoxic, neurotoxic and carcinogenic activities (Gutleb et al. 2002; Thrane et al. 2004; Meca et al. 2010). Although *F. poae* is not an important T-2 and HT-2 producer, several isolates from Norwegian cereals, which resembled *F. poae*, produced these mycotoxins and the isolates were named 'powdery *F. poae*' (Torp and Langseth 1999). However, based on morphological observations, sequences analyses of the internal transcribed spacer region (ITS), intergenic spacer region (IGS), β-tubulin and translation elongation factor 1α (EF-1α), the

'powdery *F. poae*' isolates were designed as a new species named *F. langsethiae* (Knutsen et al. 2004; Torp and Nirenberg 2004; Yli-Mattila et al. 2004a). Moreover, important T-2-producing isolates morphologically similar to *F. langsethiae* and *F. poae* were isolated from northern Asia. Using multilocus phylogenetic and phenotypic analyses, Yli-Mattila et al. (2011) described these Asian isolates as a new species named *F. sibiricum,* which is more related to *F. sporotrichioides* than to *F. langsethiae* and *F. poae.*

Several studies have been developed to analyse intraspecific variability within *F. poae* using different available tools such as vegetative compatibility tests (VCG), random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), amplified fragment length polymorphisms (AFLP) and microsatellites (SSR) (Kerényi et al. 1997; Schmidt et al. 2004; Dinolfo et al. 2010; Somma et al. 2010; Vogelgsang et al. 2010). Moreover, DNA sequences derived from the ITS, IGS, β-tubulin, EF-1α regions and mitochondrial small subunit rDNA (mtSSU) have been used to study *F. poae* genetic variability and phylogenetic analyses (Knutsen et al. 2004; Konstantinova and Yli-Mattila 2004; Mach et al. 2004; Yli-Mattila et al. 2004a,b; Stenglein et al. 2010; Alberti et al. 2011).

A molecular marker called sequence-related amplified polymorphism (SRAP) was developed not only to analyse genetic variability, but also to amplify open reading frames (ORFs). Firstly, SRAP has been developed to fingerprint and target coding sequences in the *Brassica oleracea* genome (Li and Quiros 2001). Later, some researchers have adopted this molecular marker to analyse genetic variability in some fungus species such as *Puccinia striiformis*, *Ganoderma* sp. and *F. culmorum.* In these studies, the authors concluded that SRAP is an efficient technique to identify polymorphisms useful to evaluate variability among individuals (Sun et al. 2006; Pasquali et al. 2010; Irzykowska et al. 2013).

Based on that the exon region is conserved among individuals, polymorphism levels are so low, so two primers based in exon regions do not show high polymorphism levels. However, this region could amplify GC-rich sequences. Therefore, the technique uses two primers: one to target an exon region and another to target an intron region rich in AT regions that is more variable than exon regions among individuals, so this region could be the responsible of the high polymorphism level found in this technique (Li and Quiros 2001). Briefly, this molecular marker uses two primers with 17 or 18 nucleotides long, where the first 10 are nonspecific, followed by the sequence CCGG plus three nonspecific nucleotides in the forward primer, with the aim to target exon regions, and AATT plus three nonspecific nucleotides in the reverse primer to target promoters and/or intron regions. The forward primer is based on the fact that exons are GC-rich regions (43–44%) (Li and Quiros 2001). Sequence-related amplified polymorphism is a technique that allows random amplification of coding regions in genome with the possibility of isolating and sequencing fragments to target ORFs. The aims of this work were to test the SRAP molecular marker for *F. poae* genetic variability studies and to assess the efficacy of this technique to amplify ORFs by sequencing some fragments selected randomly.

Materials and Methods

A total of 105 monosporic *F. poae* isolates from different countries and hosts and a *F. sporotrichioides* isolate (F95) used as out-group were evaluated (Table S1). All *F. poae* isolates were previously identified by a species-specific PCR according to Parry and Nicholson (1996). The isolates were conserved in Spezieller Nährstoffarmer Agar (SNA) according to Leslie and Summerell (2006). The genomic DNA from all *Fusarium* isolates was extracted using a cetyltrimethylammonium bromide (CTAB) method according to Stenglein and Balatti (2006). The quality of fungus DNA was examined by electrophoresis in 0.8% (w/v) agarose gels containing GelRed™ (Biotium, Hayward, USA) at 80 V in 1× Trisborate-EDTA buffer for 3 h at room temperature. The DNA was visualized under UV light. The DNA concentration was estimated with a fluorometer (Qubit™-Invitrogen, Buenos Aires, Argentina).

Sequence-related amplified polymorphism amplifications were performed with eight randomly selected primer combinations: SRAP1F/SRAP1R, SRAP1F/SRAP3R, SRAP2F/SRAP1R, SRAP2F/SRAP2R, SRAP2F/SRAP3R, SRAP3F/2R, SRAP3F/SRAP1R and SRAP3F/SRAP3R that were selected based on the results of an initial screening of nine primer combinations against 32 representative *F. poae* isolates (Table 1).

Polymerase chain reactions (PCRs) were carried out using 10–25 ng of DNA in a total volume of 25 μl containing 10× reaction buffer, 0.5 μM of each primer, 200 μM of each dNTP (Inbio-Highway, Tandil, Argentina), 2.5 mM $MgCl_2$ and 1.25 U of Taq DNA polymerase (Inbio-Highway). DNA amplifications were performed in a XP Thermal cycler (Bioer Technology Co., Hi-tech, Hangzhou, China) with the following

Table 1 Primer sequences used for SRAP markers

Primers	Sequence (5′–3′)
SRAP1F	TGA GTC CAA ACC GGA TA
SRAP1R	GAC TGC GTA CGA ATT GTC
SRAP2F	TGA GTC CAA ACC GGT CA
SRAP2R	GAC TGC GTA CGA ATT CCA
SRAP3F	TGA GTC CAA ACC GGA AG
SRAP3R	GAC TGC GTA CGA ATT GGT

programme: an initial denaturing at 94°C for 5 min followed by five cycles at 94°C for 2 min (denaturing), 35°C for 1 min (annealing) and 72°C for 1 min (extension). Then, the annealing temperature was raised to 50°C for other 35 cycles with a final extension at 72°C for 10 min. Each reaction was performed at least twice. The amplification products were separated by vertical gel electrophoresis using 4% polyacrylamide DNA sequence gel with 5 M urea. Gel fixing, staining and developing were followed to visualize the DNA fragments using the silver-staining protocol according to Bassam et al. (1991). Presence or absent of SRAP fragments was scored manually and recorded in a binary, present/absent format considering fragments with the same size as homologous. Cluster analysis based on simple matching coefficient and an analysis of molecular variance (AMOVA) in American and European population and among them (American and European) were performed as described Dinolfo et al. (2010).

A total of eight SRAP fragments, four of them polymorphic and four monomorphic, were cut from the gels and introduced individually into 1.5-ml sterile tubes. Fifty microlitres of distilled water was added into the tubes, and the fragments were broken with the help of tips. The final solution was heated at 95°C for 5 min using a dry heating block (Major Science Co., Ltd., Pan-Chiao City, Taipei Hsien, Taiwan). Finally, 5 μl of this final solution was taken to carry out a new PCR-SRAP reaction with the conditions described above. The SRAP fragments were purified using the *PureLink*TM Quick Gel Extraction & PCR Purification Combo Kit (Invitrogen, Löhne, Germany). DNA sequencing, from both the sense and antisense ends of the fragments, was carried out using BigDye Terminator version 3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) in an Applied Biosystems Sequencer (ABI/Hitachi Genetic Analyzer 3130). All sequences obtained were deposited in the NCBI/GenBank database under the accession numbers: KJ805783–KJ805790 (Table 2) and were compared with the FUSARIUM ID database (Geiser et al. 2004).

Results and Discussion

Fusarium poae is an important pathogen whose occurrence has increased in the last years (Pettersson 1991; Torp and Langseth 1999; Torp and Nirenberg 2004; Pereyra and Dill-Macky 2010; Yli-Mattila 2010; Lenc 2011; Infantino et al. 2012; Stenglein et al. 2012). Previous studies on the genetic variability of *F. poae* have been developed (Liu and Sundheim 1996; Kerényi et al. 1997; Konstantinova and Yli-Mattila 2004; Mach et al. 2004; Yli-Mattila et al. 2004a,b; Dinolfo et al. 2010). In our study, the SRAP technique generated a total of 117 fragments among 105 isolates evaluated, of which 96 (82.05%) were polymorphic among all the *F. poae* isolates, showing that SRAP markers are a useful tool to analyse the *F. poae* genome.

Cluster analysis of SRAP data defined 104 haplotypes among the 105 *F. poae* isolates analysed which were partially grouped according to their country of origin (Fig. 1). Cluster analysis resolved subcluster I that include only *F. sporotrichioides* (F95) and subcluster II that include all the *F. poae* isolates evaluated (Fig. 1). Subcluster II was resolved into two subclusters designed as subcluster IIb, which included only a *F. poae* isolate from England, and subcluster IIa, which included the remaining *F. poae* isolates. Subcluster IIa was resolved into two: subcluster IIa2, which included *F. poae* from Germany, France, Finland, Belgium and Poland, and subcluster IIa1, which included the 92.38% of the total of *F. poae* analysed. Subcluster IIa1 was resolved into two subclusters IIa1a and IIa1b, which included isolates from Poland, Belgium, Germany, Luxembourg and France. Subcluster IIa1a included isolates from Argentina, Uruguay, Switzerland, Hungary and Italy, and some isolates from Finland, England, Poland and Germany. In general, European isolates were distributed throughout the dendrogram unlike American ones. This result is in agreement with the result of Kerényi et al. (1997) who reported partial clustering between the VCG and RAPD and the host/geographic origins of *F. poae* isolates (Kerényi et al. 1997). Vogelgsang et al. (2010) analysed *F. poae* from Switzerland, Canada, Germany, Denmark, Finland, Hungary, Norway, Poland, Russia and the United States of America using microsatellite markers and not grouping related with their origin was reported. Moreover, Dinolfo et al. (2010) evaluated *F. poae* isolates from Argentina and England using ISSR and observed partial relationship

Table 2 Analysis of different SRAP fragments

Selected fragment code	Primers	Type of fragment	GenBank accession number	BlastX score(bits)	FUSARIUM ID
S6107	SRAP3F/3R	Polymorphic	KJ805783	147.1	*F. oxysporum* PHW808: FOPG_17988.1
				147.1	*F. oxysporum* Fo5176:FOXB_20935.1
				147.1	*F. oxysporum* PHW815:FOQG_17741.1
				146.7	*F. oxysporum* PHW815:FOQG_17269.1
				146.7	*F. oxysporum* PHW815:FOQG_17321.1
				115.9	*F. verticillioides* 7600:FVEG_06582.5
				71.2	*F. graminearum* PH-1:FGSG_07754.3
				68.5	*F. verticillioides*7600:FVEG_13223.5
S3107T	SRAP3F/3R	Monomorphic	KJ805790	98.2	*F. verticillioides* 7600:FVEG_10146.5
				96.2	*F. oxysporum* PHW808:FOPG_13871.1
				87.8	*F. graminearum* PH-1:FGSG_03986.3
S45	SRAP2F/2R	Polymorphic	KJ805787	43.1	*F. graminearum* PH-1:FGSG_08699.3
S48T	SRAP2F/2R	Monomorphic	KJ805786	43.1	*F. graminearum* PH-1:GFSG_08699.3
S59	SRAP2F/3R	Polymorphic	KJ805784	48.1	*F. oxysporum melonis*:FOMG_13602.1
				40.0	*F. verticillioides* 7600:FVEG_16745.5
S59T	SRAP2F/3R	Monomorphic	KJ805789	55.0	*F. oxysporum* PHW808:FOPG_08978.1
				55.0	*F. verticillioides* 7600:FVEG_09114.5
				41.5	*F. graminearum* PH-1:FGSG_02583.3
S110	SRAP3F/1R	Polymorphic	KJ805788	98.5	*F. graminearum* PH-1: FGSG_06426.3
				73.5	*F. oxysporum* PHW808:FOPG_01692.1
				72.7	*F. verticillioides* 7600:FVEG_06490.5
S110T	SRAP 3F/1R	Monomorphic	KJ805785	83.5	*F. graminearum* PH-1:FGSG_06426.3
				55.8	*F. verticillioides* 7600:FVEG_06490.5
				51.6	*F. oxysporum melonis*:FOMG_12090.1

between clusters and the country of origin/host. In the same year, Somma et al. (2010) evaluated 81 *F. poae* isolates from Italy using AFLP and not relationship between variability and other well-characterized traits such as mycotoxins profile was found (Somma et al. 2010).

Diversity of the entire population was estimated to be 0.998 ($SD = 0.018$). The American population diversity was estimated to be 1.00 ($SD = 0.063$) while the European population was estimated to be 0.9985 ($SD = 0.0029$). According to this, Dinolfo et al. (2010) and Somma et al. (2010) observed a high level of genetic diversity in the *F. poae* isolates analysed using ISSR (99%) and AFLP (61%), respectively.

The AMOVA ($\Phi ST = 0.174$; $P < 0.001$) indicated that most of the variation resulted from genetic differences within (90.83%), rather than between American and European populations (9.17%). Dinolfo et al. (2010) indicated that the differences within groups were 83%, while that the differences between Argentinian and English isolates were 17%. However, similar values in differences between (70%) and within groups (70%) were observed in Italian *F. poae* isolates (Somma et al. 2010). These differences could be attributed not only in the technique used in each study, but also that in the first one, the analysis was realized between Argentinian and England groups, and in the last, only Italian isolates were evaluated. Our results indicated that the SRAP technique allows detecting a high polymorphism level, similar to other molecular markers.

The GC content of the total SRAP-sequenced fragments was over 44.71% (data not shown), which indicates a high possibility to fall into GC-rich regions such as ORFs. The BLASTx analysis found that the total of the sequences analysed were homologous with hypothetical proteins of different *Fusarium* species, showing that SRAP markers are able to amplify coding regions in the *F. poae* genome (Table 2). Therefore, SRAP technique is an anonymous molecular marker that allows amplifying coding regions in unknown genomes such as *F. poae*. Moreover, hypothetical proteins from polymorphic fragments could demonstrate a differential protein expression among individuals.

Although there are some reports that study fungus genetic variability using the SRAP markers (Sun et al. 2006; Pasquali et al. 2010; Irzykowska et al. 2013), to

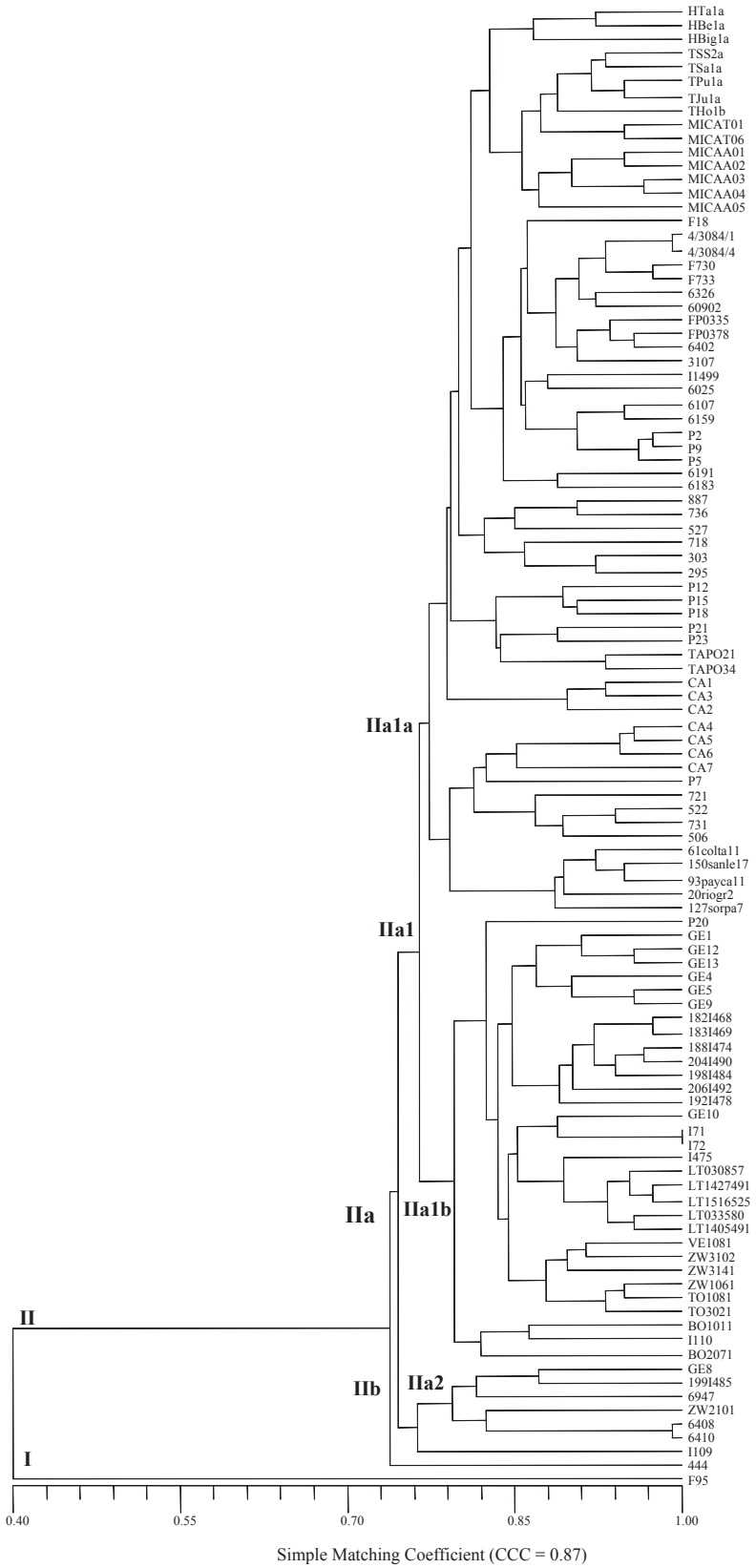

Fig. 1 Cluster analysis dendrogram of the 105 *Fusarium poae* isolates obtained by sequence-related amplified polymorphism.

our knowledge, this is the first work that sequences SRAP fragments to corroborate the efficacy to this molecular marker to amplify ORF.

In summary, SRAP allows analysing the genetic variability and amplifying coding regions in *F. poae*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. *Fusarium* isolates used in this study.