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Genetic variability and fumonisin production by *Fusarium proliferatum* isolated from durum wheat grains in Argentina

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ABSTRACT

Fusarium proliferatum is a member of the *Fusarium fujikuroi* species complex (FFSC) involved in the maize ear rot together with *Fusarium verticillioides*, which is a very closely related species. Recently, different studies have detected natural fumonisin contamination in wheat kernels and most of them have shown that the main species isolated was *F. proliferatum*. *Fusarium* strains obtained from freshly harvested durum wheat samples (2008 to 2011 harvest seasons) from Argentina were characterized through a phylogenetic analysis based on translation elongation factor-1 alpha (EF-1α) and calmodulin (CaM) genes, determination of mating type alleles, and evaluation of fumonisin production capability. The strains were identified as *F. proliferatum* (72%), *F. verticillioides* (24%) and other *Fusarium* species. The ratio of mating type alleles (MAT-1 and MAT-2) obtained for both main populations suggests possible occurrence of sexual reproduction in the wheat fields, although this seems more frequent in *F. proliferatum*. Phylogenetic analysis revealed greater nucleotide variability in *F. proliferatum* strains than in *F. verticillioides*, however this was not related to origin, host or harvest year. The fumonisin-producing ability was detected in 92% of the strains isolated from durum wheat grains. These results indicate that *F. proliferatum and F. verticillioides*, among the fumonisin producing species, frequently contaminate durum wheat grains in Argentina, presenting a high risk for human and animal health.

1. Introduction

Fusarium proliferatum (Matsushima) Nirenberg is a member of the *Fusarium fujikuroi* species complex (FFSC), a group of over 40 closely related *Fusarium* species defined by morphological traits, sexual compatibility, and DNA-based phylogenetic analysis (Leslie and Summerell, 2006). *Fusarium proliferatum* is a polyphagous fungus, having a broad host range and is often isolated from several agriculturally important plants as the main pathogenic agent, as in crown and root rot of asparagus, bulb rot in garlic and onion, in citrus where it causes fruit rot and in palms where it is an agent of seedling blight, wilt and dieback at worldwide level (Abdalla et al., 2000; Armengol et al., 2005; Hyun et al., 2000; Stankovic et al., 2007; von Bargen et al., 2009). However, the highest concern regarding *F. proliferatum* is its ability to be a main pathogen of maize worldwide, where it is involved in the ear rot together with *Fusarium verticillioides* (Sacc.) Nirenberg (= *Fusarium moniliforme* Sheldon), which is a morphologically very closely related species (Ghiasian et al., 2006; Logrieco et al., 2002). Both species can produce several mycotoxins which accumulate on maize kernels (Desjardins, 2006). Among these mycotoxins, the fumonisins are the most dangerous and FB_1 has been evaluated as a possible carcinogen to humans (class 2B) by the International Agency for Research on Cancer (IARC, 2002). Fumonisins cause a number of severe mycotoxicoses in animals, such as equine leukoencephalomalacia in horses and porcine pulmonary edema in swine (Desjardins, 2006), and they have been associated with esophageal cancer and also with neural tube defects in humans (Marasas et al., 2004; Missner et al., 2006).

Generally, *F. proliferatum* is recovered at lower frequencies than *F. verticillioides*, when they co-occur on maize (Proctor et al., 2010). However, *F. proliferatum* has also been reported as the main contaminant of maize in some maize areas depending on specific environmental and geographical conditions (Chulze et al., 1996; Logrieco et al., 1995).

Wheat has also been reported to be contaminated by *F. proliferatum* (Conner et al., 1996; Moretti et al., 1999), but no fumonisins associated with the contaminated kernels were reported. However, wheat based foods have been reported to be contaminated by fumonisins (Cirillo et al., 2003). More recently, different studies have detected natural fumonisin contamination in wheat kernels and in most cases the main

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species isolated from the kernels was *F. proliferatum* (Busman et al., 2012; Cendoya et al., 2014; Palacios et al., 2011; Stankovic et al., 2012). In the literature, some studies regarding the toxicological and genetic variability of *F. proliferatum* exist, but only limited information on strains isolated from wheat is available (Jurado et al., 2010; Proctor et al., 2010). Since wheat is an important crop in the human diet, its quality and safety is of major concern. Therefore, investigating the genetic traits and the mycotoxin profile of this emerging *F. proliferatum* population from Argentinean wheat would provide useful information for greater efficacy in control of this pathogen in the field.

The aims of this study were to analyze: a) the genetic variability of *F. proliferatum* isolated from wheat through a phylogenetic analysis; and b) the fumonisin-producing capability of *F. proliferatum* strains isolated from durum wheat in Argentina.

2. Material and methods

2.1. Fusarium strains isolation and identification

The 130 *Fusarium* strains analyzed in this study were deposited in the fungal culture collection of Institute of Sciences of Food Production — CNR (ITEM collection: http://www.ispa.cnr.it/Collection/) (Table 1). The strains were isolated from freshly harvested durum wheat samples randomly collected during four consecutive harvest seasons (2008 to 2011) in different commercial fields from 8 different localities in the major durum wheat production area in Argentina (south of Buenos Aires province, Fig. 1). Nine *F. proliferatum* strains isolated from maize from the south of Buenos Aires province (Reynoso et al., 2004), and some species-reference strains provided by ITEM collection were included in the analysis.

From each sample, 100 wheat kernels were plated (10 grains per Petri dish) onto a modified pentachloronitrobenzene medium (PNBC). The PNBC plates were incubated at 25 °C for 7 days under 12/12 h photoperiod cold white and black fluorescent lamps. Fungal colonies were selected for subculture based on morphological traits typical of the two main fumonisin producing species *F. proliferatum* and *F. verticillioides*, i.e. the presence of microconidia formed in chains in aerial mycelium. Representative cultures of the species isolated were grown from single conidia for 10–14 days on Petri dishes of carnation leaf agar (CLA) and potato dextrose agar (PDA) slants, at 25 °C with a 12/12 h photoperiod under cold white and black fluorescent lamps. *Fusarium* species were identified according to the guidelines of Leslie and Summerell (2006).

2.2. DNA extraction

Fusarium strains were grown in Wickerham's medium (glucose, 40 g; peptone, 5 g; yeast extract, 3 g; malt extract, 3 g; and distilled water to 1 l) and incubated in an orbital shaker (150 rpm) for 48 h at 25 ± 1 °C. Following incubation, the mycelia were filtered and lyophilized for total DNA extraction. The fungal DNA was extracted starting from 10 mg of lyophilized mycelium, grinded with 5 mm iron bead in Mixer Mill MM 400 (Retsch), and processed with "Wizard® Magnetic DNA Purification System for Food" kit (Promega). The quality of genomic DNA was determined by electrophoresis and the quantification using a Spectrophotometer ND-1000 (Nano Drop).

2.3. PCR amplification and sequencing reaction

Fusarium calmodulin (CaM) and translation elongation factor-1 alpha (EF-1α) genes were amplified in all 130 strains (Table 1), using PCR conditions and primers described in literature: CL1 and CL2A primers from O'Donnell et al. (2000) and EF-1 and EF-2 primers from O'Donnell et al. (1998). PCR reactions were carried out on a thermal cycler 9700 (Applied Biosystems, Foster City, CA) and the reaction conditions were: denaturation at 94 °C for 5 min; 35 cycles of the denaturation at 94 °C for 50 s, annealing at 57 °C and 59 °C for 50 s, for caM and ef-1α respectively, extension at 72 °C for 1 min; final extension at 72 °C for 7 min, followed by cooling at 4 °C to develop the next step. After amplification, the products were purified with the enzymatic mixture EXO/SAP (Exonuclease I, *Escherichia coli*/Shrimp Alkaline Phosphatase). Sequence analysis using the BigDye Terminator Cycle Sequencing Ready reaction Kit for both strands was set up. The PCR program for the amplification was: one cycle of the denaturation 96 °C for 10 s; 35 cycles of annealing to 50 °C for 5 s, extension at 60 °C for 4 min and then one cycle of held at 4 °C. All the sequencing reactions were purified by gel filtration through Sephadex G-50 (Amersham Pharmacia Biotech) equilibrated in double-distilled water and analyzed on an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA).

The alignments of CaM and EF-1α sequences were performed using the software package BioNumerics (Applied Maths) and manual adjustments were made where necessary.

2.4. Mating type determination

The mating types (MAT-1 and MAT-2) of the strains were identified by PCR using the primers Gfmat1a/Gfmat1b and Gfmat1c/Gfmat1d as described by Steenkamp et al. (2000). The annealing temperature for MAT-2 primers hybridization was adjusted to 53 °C. The MAT-1 allele corresponds to a fragment of approximately 200 bp and the MAT-2 allele to a fragment of 800 bp.

2.5. Phylogenetic analysis

Calmodulin gene (CaM) and translation elongation factor alpha gene (EF-1α) of the 130 strains were subjected to Bayesian phylogenetic analysis using MrBayes 3.2 (Huelsenbeck and Ronquist, 2001). The Metropolis-coupled Markov chain Monte Carlo (MCMC) sampling approach was used to calculate posterior probabilities. The General Time Reversible model (GTR + I + G) was used. Four simultaneous chains were run 1×10^7 generations, with random starting trees, and sampled every 1000 generations. The burn-in period was set at 25%. Gaps were treated as "fifth state". Sequences of other species of the FFSC obtained from the *Fusarium*-ID database (*F. fujikuroi* NRRL 13566, *Fusarium subglutinans* NRRL 22016, *Fusarium circinatum* NRRL 25331, *Fusarium sacchari* NRRL 13999, *Fusarium thapsinum* NRRL 22045, *Fusarium andiyazi* F15910) and sequences of additional strains obtained from ITEM collection (*F. verticillioides* ITEM 7583, *F. verticillioides* ITEM 7581, *F. proliferatum* ITEM 7595 and *F. proliferatum* ITEM 7596), were analyzed in the same way. The trees were rooted by the outgroup method using sequences of *F. oxysporum* strain NRRL 22902.

2.6. Fumonisin production

The ability of the 121 strains isolated from durum wheat to produce fumonisins was assessed on autoclavated durum wheat grains. Erlenmeyer flasks containing 30 g of durum wheat grains and 13.5 ml of distilled water were autoclaved twice for 30 min at 121 °C. After cooling, durum wheat was inoculated with an aqueous suspension of conidia (1 ml) of 10^6 spores obtained from CLA culture of each strain and incubated in the dark at 25 °C for 28 days. To avoid clump formation, the cultures were hand-shaken during the first days of incubation and thereafter as necessary. Durum wheat cultures were then dried at 50 °C, finely ground with a laboratory mill, and stored at 4 °C until fumonisin analysis.

2.6.1. Fumonisin detection and quantification

Aliquots of the ground cultures (1 g) were shaken with 10 ml of methanol/water (3:1, v/v) (HPLC-grade, Mallinckrodt Baker, Milan, Italy. Ultrapure water was produced by a Millipore Milli-Q system Millipore, Bedford, MA, USA) for 60 min and filtered through Whatman