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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

Table 1
Species, origin, harvest year, mating type allele and fumonisin production by *Fusarium* strains isolated from durum wheat in Argentina.

Strain	Species	Origin	Year	MAT	Fumonisin production (μg/g)		
					FB_1	FB_2	FB_3
ITEM 15654	*F. proliferatum*	Bahia Blanca	2008	2	64	23	8
ITEM 15656	*F. proliferatum*	Bahia Blanca	2008	2	3	0.7	1
ITEM 15657	*F. proliferatum*	Bahia Blanca	2008	2	nd	nd	nd
ITEM 15658	*F. proliferatum*	Bahia Blanca	2008	2	0.9	0.5	nd
ITEM 15659	*F. proliferatum*	Bahia Blanca	2008	1	7	0.7	0.9
ITEM 15660	*F. proliferatum*	Bahia Blanca	2008	2	nd	nd	nd
ITEM 15661	*F. proliferatum*	Bahia Blanca	2008	1	7	45	0.6
ITEM 15662	*F. proliferatum*	Bahia Blanca	2008	2	139	89	24
ITEM 15663	*F. proliferatum*	Bahia Blanca	2008	2	0.6	0.5	nd
ITEM 15664	*F. proliferatum*	Bahia Blanca	2008	1	11	83	nd
ITEM 15665	*F. proliferatum*	Bahia Blanca	2008	1	25	14	12
ITEM 15666	*F. proliferatum*	La Dulce	2009	2	6	1	2
ITEM 15667	*F. proliferatum*	La Dulce	2009	2	168	105	40
ITEM 15668	*F. proliferatum*	La Dulce	2009	1	744	199	152
ITEM 15669	*F. proliferatum*	La Dulce	2009	1	42	9	7
ITEM 15670	*F. proliferatum*	La Dulce	2009	1	1616	382	74
ITEM 15671	*F. proliferatum*	La Dulce	2009	2	58	14	1
ITEM 15672	*F. proliferatum*	La Dulce	2009	2	10	3	0.7
ITEM 15673	*F. proliferatum*	La Dulce	2009	1	51	34	8
ITEM 15674	*F. proliferatum*	La Dulce	2009	2	2	16	nd
ITEM 15675	*F. proliferatum*	Pieres	2010	2	85	31	29
ITEM 15676	*F. proliferatum*	Pieres	2010	1	10	76	0.9
ITEM 15677	*F. proliferatum*	Pieres	2010	1	107	29	20
ITEM 15678	*F. proliferatum*	Pieres	2010	2	13	2	3
ITEM 15679	*F. proliferatum*	Pieres	2010	2	103	17	25
ITEM 15680	*F. proliferatum*	Pieres	2010	1	39	18	16
ITEM 15681	*F. proliferatum*	Barrow	2010	2	21	10	5
ITEM 15682	*F. proliferatum*	Miramar	2010	2	78	56	39
ITEM 15683	*F. proliferatum*	Miramar	2010	2	195	103	22
ITEM 15685	*F. proliferatum*	La Dulce	2010	1	25	6	8
ITEM 15689	*F. proliferatum*	Pieres	2011	1	69	30	21
ITEM 15691	*F. proliferatum*	Pieres	2011	1	21	8	3
ITEM 15692	*F. proliferatum*	Pieres	2011	2	88	51	29
ITEM 15693	*F. proliferatum*	Pieres	2011	1	56	32	8
ITEM 15694	*F. proliferatum*	Pieres	2011	2	137	42	13
ITEM 15695	*F. proliferatum*	Pieres	2011	1	179	79	16
ITEM 15696	*F. proliferatum*	Pieres	2011	1	5	41	nd
ITEM 15697	*F. proliferatum*	Pieres	2011	2	20	151	nd
ITEM 15698	*F. proliferatum*	Pieres	2011	1	153	43	21
ITEM 15699	*F. proliferatum*	Pieres	2011	2	152	83	30
ITEM 15700	*F. proliferatum*	Barrow	2011	2	7	3	2
ITEM 15701	*F. proliferatum*	Cabildo	2011	2	161	66	20
ITEM 15704	*F. proliferatum*	Barrow	2011	1	93	22	41
ITEM 15705	*F. proliferatum*	Bordenave	2011	2	25	11	3
ITEM 15706	*F. proliferatum*	La Dulce	2011	2	165	50	27
ITEM 15707	*F. proliferatum*	Bordenave	2011	2	21	10	9
ITEM 15708	*F. proliferatum*	Barrow	2011	2	52	28	9
ITEM 15709	*F. proliferatum*	Cabildo	2011	1	75	57	14
ITEM 15710	*F. proliferatum*	Barrow	2011	2	44	20	25
ITEM 15711	*F. proliferatum*	La Dulce	2011	2	111	52	23
ITEM 15712	*F. proliferatum*	Balcarce	2011	1	2	0.8	1
ITEM 15713	*F. proliferatum*	Barrow	2011	2	54	18	10
ITEM 15714	*F. proliferatum*	Cabildo	2011	1	2	0.5	0.5
ITEM 15715	*F. proliferatum*	La Dulce	2011	1	35	161	1
ITEM 16368	*F. proliferatum*	La Dulce	2009	1	0.1	3	nd
ITEM 16369	*F. proliferatum*	La Dulce	2009	2	68	106	3
ITEM 16370	*F. proliferatum*	La Dulce	2009	1	23	nd	nd
ITEM 16371	*F. proliferatum*	La Dulce	2009	1	0.05	5	0.7
ITEM 16372	*F. proliferatum*	La Dulce	2009	1	nd	nd	nd
ITEM 16374	*F. proliferatum*	La Dulce	2009	1	41	11	7
ITEM 16375	*F. proliferatum*	La Dulce	2009	2	19	nd	nd
ITEM 16376	*F. proliferatum*	La Dulce	2009	1	98	117	8
ITEM 16377	*F. proliferatum*	La Dulce	2009	1	104	29	23
ITEM 16378	*F. proliferatum*	Pieres	2010	1	5	8	3
ITEM 16379	*F. proliferatum*	Pieres	2010	2	1	2	0.2
ITEM 16380	*F. proliferatum*	Pieres	2010	1	5	nd	nd
ITEM 16382	*F. proliferatum*	Barrow	2010	2	7	761	2
ITEM 16384	*F. proliferatum*	Bordenave	2011	1	6	nd	8
ITEM 16386	*F. proliferatum*	La Dulce	2011	1	44	10	8
ITEM 16387	*F. proliferatum*	Bordenave	2011	2	6	6	4
ITEM 16388	*F. proliferatum*	Barrow	2011	1	14	8	1
ITEM 16389	*F. proliferatum*	Barrow	2011	1	12	7	6
ITEM 16391	*F. proliferatum*	Barrow	2011	2	5	nd	8
ITEM 16392	*F. proliferatum*	La Dulce	2011	2	0.2	0.3	0.3
ITEM 16393	*F. proliferatum*	La Dulce	2011	1	6	10	9
ITEM 16394	*F. proliferatum*	Pieres	2011	2	160	117	26
ITEM 16395	*F. proliferatum*	Pieres	2011	2	1	3	1
ITEM 16396	*F. proliferatum*	Pieres	2011	1	40	nd	nd
ITEM 16397	*F. proliferatum*	Pieres	2011	1	5	3	1
ITEM 16398	*F. proliferatum*	Pieres	2011	1	25	34	5
ITEM 16399	*F. proliferatum*	Pieres	2011	1	19	6	6
ITEM 16400	*F. proliferatum*	Pieres	2011	1	1,053	nd	nd
ITEM 16407	*F. proliferatum*	La Dulce	2009	1	3	0.5	2
ITEM 16408	*F. proliferatum*	La Dulce	2009	2	67	12	16
ITEM 16413	*F. proliferatum*	La Dulce	2009	1	16	4	2
ITEM 16425	*F. proliferatum*	Pieres	2010	2	1	0.3	0.3
ITEM 16439	*F. proliferatum*	Pieres	2011	1	41	12	7
ITEM 15655	*F. verticillioides*	Bahia Blanca	2008	2	98	43	160
ITEM 15684	*F. verticillioides*	Barrow	2010	2	3	1	0.2
ITEM 15686	*F. verticillioides*	Barrow	2010	1	28	9	12
ITEM 15687	*F. verticillioides*	La Dulce	2010	1	19	3	8
ITEM 15688	*F. verticillioides*	Miramar	2010	1	43	19	35
ITEM 15690	*F. verticillioides*	Pieres	2011	1	31	28	64
ITEM 15703	*F. verticillioides*	Bordenave	2011	1	26	15	31
ITEM 16403	*F. verticillioides*	La Dulce	2009	1	nd	0.05	nd
ITEM 16405	*F. verticillioides*	La Dulce	2009	1	16	3	8
ITEM 16406	*F. verticillioides*	La Dulce	2009	1	4	7	2
ITEM 16409	*F. verticillioides*	La Dulce	2009	2	0.05	0.1	nd
ITEM 16410	*F. verticillioides*	La Dulce	2009	2	0.6	0.8	1
ITEM 16415	*F. verticillioides*	La Dulce	2009	1	4	3	2
ITEM 16416	*F. verticillioides*	La Dulce	2009	1	nd	nd	Nd
ITEM 16419	*F. verticillioides*	Pieres	2010	1	1	2	4
ITEM 16421	*F. verticillioides*	Pieres	2010	2	175	62	80
ITEM 16422	*F. verticillioides*	Pieres	2010	1	53	30	63
ITEM 16423	*F. verticillioides*	Pieres	2010	1	0.3	0.1	nd
ITEM 16424	*F. verticillioides*	Pieres	2010	1	0.07	nd	nd
ITEM 16426	*F. verticillioides*	Barrow	2010	1	51	11	28
ITEM 16427	*F. verticillioides*	Miramar	2010	1	1	1	0.2
ITEM 16428	*F. verticillioides*	Barrow	2010	2	0.05	0.7	0.2
ITEM 16429	*F. verticillioides*	Barrow	2010	2	11	21	39
ITEM 16430	*F. verticillioides*	Miramar	2010	2	19	5	38
ITEM 16431	*F. verticillioides*	Barrow	2011	2	10	11	16
ITEM 16433	*F. verticillioides*	Bordenave	2011	1	2	1	7
ITEM 16434	*F. verticillioides*	Bordenave	2011	1	0.1	nd	0.1
ITEM 16436	*F. verticillioides*	Balcarce	2011	2	4	4	3
ITEM 16440	*F. verticillioides*	Pieres	2011	1	nd	nd	nd
ITEM 16373	*F. andiyazi*	La Dulce	2009	1	nd	nd	nd
ITEM 16411	*F. andiyazi*	La Dulce	2009	1	nd	nd	nd
ITEM 16418	*F. thapsinum*	Pieres	2010	1	nd	nd	nd
ITEM 16420	*F. thapsinum*	Pieres	2011	2	nd	nd	nd
ITEM 16435	*F. thapsinum*	Cabildo	2011	2	nd	nd	nd
ITEM 15716[a]	*F. proliferatum*	Bs As	1997	1	nd	nd	nd
ITEM 15717[a]	*F. proliferatum*	Bs As	1997	2	1344	280	23
ITEM 15718[a]	*F. proliferatum*	Bs As	1997	2	481	115	14
ITEM 15719[a]	*F. proliferatum*	Bs As	1997	2	2054	818	47
ITEM 15720[a]	*F. proliferatum*	Bs As	1997	2	1681	1132	47
ITEM 15721[a]	*F. proliferatum*	Bs As	1997	2	2	3	2
ITEM 15722[a]	*F. proliferatum*	Bs As	1997	2	177	3	2
ITEM 15723[a]	*F. proliferatum*	Bs As	1997	1	1495	1426	57
ITEM 15724[a]	*F. proliferatum*	Bs As	1997	2	nd	nd	nd

nd: not detected <0.05 μg/g.
[a] Strains isolated from maize reporting data on fumonisin production on autoclavated maize kernels from Reynoso et al. (2004).

No. 4 filter paper. Two milliliters of the filtered extract was applied to a Bond-Elut strong anion-exchange (SAX) cartridge (Varian, Harbor City, CA), previously conditioned by the successive passage of methanol (5 ml) and methanol/water (3:1, v/v, 5 ml). The cartridge was then washed with methanol/water (3:1, v/v, 5 ml) followed by methanol (3 ml), and fumonisins were eluted with 1% acetic acid in methanol (10 ml). The eluate was dried (50 °C, air stream) and reconstituted with 1000 μl of acetonitrile/water (30:70, v/v). Then, 50 μl of the sample

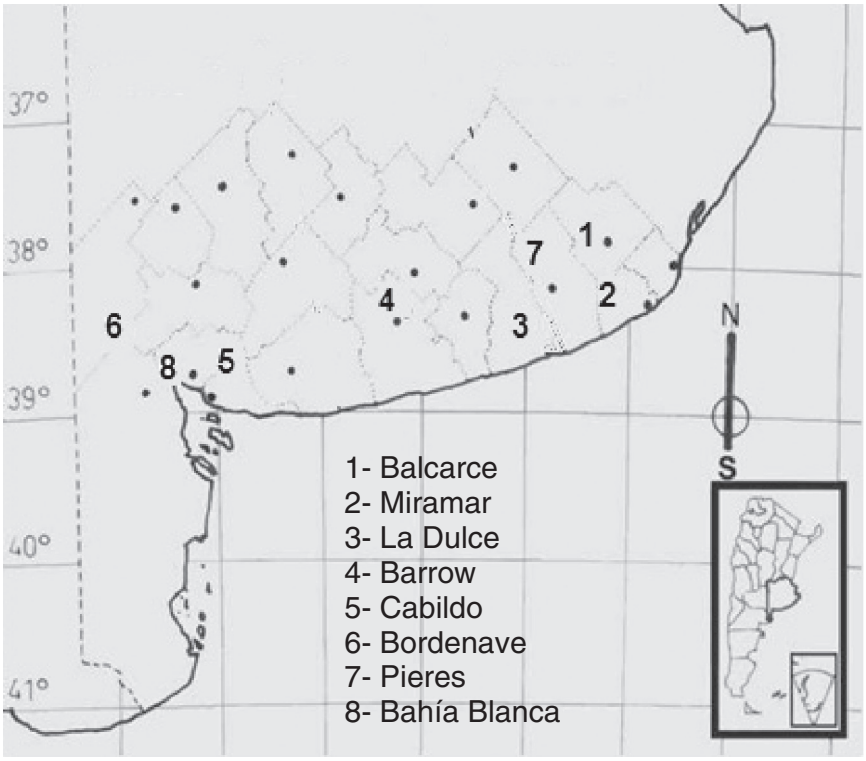


Fig. 1. Buenos Aires province map indicating the localities where durum wheat samples were obtained.

extract or standard was derivatized with 50 μl of o-phthaldialdehyde (OPA) (Sigma-Aldrich, Milan, Italy) reagent solution, mixed for 50 s, incubated for 2 min and then 100 μl the derivatized mixture was injected. The fumonisin OPA derivates were analyzed by using reversed-phase HPLC/fluorescence detection system. The HPLC instrument was the Agilent 1100 (Agilent, Waldbronn, Germany) equipped with a binary pump, column thermostat set at 30 °C. The analytical column was a Symmetry Shield RP18 15 cm × 4.6 mm, 5 μm (Waters, Milford, MA, USA) with a guard column inlet filter (0.5 μm × 3 mm diameter, Rheodyne Inc., Cotati, CA, USA); the mobile phase consisted of a binary gradient applied as follows: the initial composition of the mobile phase 57% (A) water–acetic acid (99/1, v/v)/43% (B) acetonitrile–acetic acid (99/1, v/v) was kept constant for 5 min, then solvent B was linearly increased to 54% in 21 min, then up to 58% at 25 min and kept constant for 5 min. The flow rate of the mobile phase was 0.8 ml/min. The fluorometric detector was set at wavelengths, ex = 335 nm, and em = 440 nm. The retention times of FB_1, FB_2 and FB_3 were about 19, 28 and 29 min, respectively.

Fumonisins (FB_1 and FB_2) were quantified by measuring peak areas, and comparing them with a calibration curve obtained with standard solutions. FB_3 was quantified by comparing with the calibration curve of FB_2. Detection limit for fumonisins was 0.05 μg/g.

Certified calibrant solutions of FB_1 and FB_2 in acetonitrile–water (1:1, v/v) at a concentration of 50 μg/ml were purchased from Biopure (Romer Labs Diagnostic GmbH, Tulln, Austria). Dilutions of standard solutions were made with the same mix of solvents to obtain final concentrations of 25, 50, 100; 250; 500; 1000; 5000 ng/ml of fumonisin B_1 and fumonisin B_2. All standard solutions were stored at −18 °C until use. A recovery experiment was performed in triplicate by spiking 1 g of ground fumonisin-free wheat samples with FB_1 and FB_2 at level of 1 μg/g. Spiked samples were left overnight at room temperature to allow solvent evaporation prior to proceed with the extraction step. Mean recoveries for FB_1 and FB_2 were 109.7% and 91.8%, respectively.

3. Results and discussion

3.1. Characterization of Fusarium strains

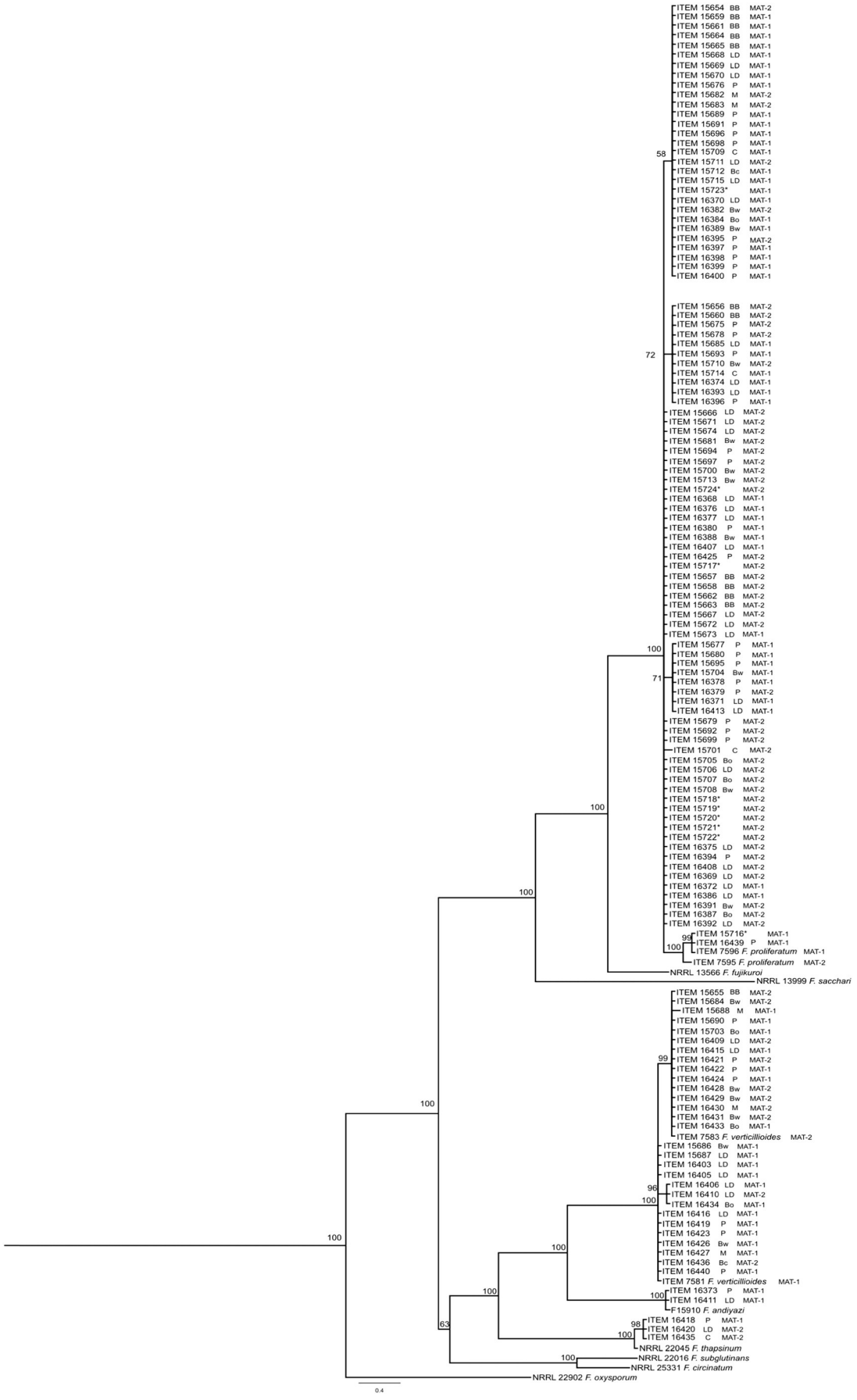
3.1.1. Morphological and molecular identification of Fusarium strains isolated from durum wheat grains

One hundred and twenty one *Fusarium* strains were isolated and morphologically identified as members of the FFSC. Based on the EF-1α gene sequences subjected to BLAST analysis at the *Fusarium*-ID database, the strains were identified as *F. proliferatum* (87 strains), *F. verticillioides* (29 strains) and 3 strains were identified as *F. thapsinum* while 2 strains were identified as *F. andiyazi*. *F. thapsinum* and *F. andiyazi* are common sorghum pathogens, however *F. thapsinum* has also been associated with banana, maize, peanut (Klittich et al., 1997) and native grasses in the USA (Leslie et al., 2004), while recently *F. andiyazi* has also been associated with bakanae disease of rice (Wulff et al., 2010). The relatively high occurrence of *F. verticillioides* (24%, of *Fusarium* strains collected) together with *F. proliferatum* (72%) shows that the risk associated with fumonisin occurrence in wheat in Argentina is due to both species, although *F. proliferatum* is the prevailing one. This makes it more difficult to avoid fumonisin contamination of kernels, since the control strategies must deal with two different biological entities.

3.1.2. Mating type determination

The MAT allele was determined for all strains isolated (Table 1). Both alleles, 1 and 2, were present in *F. proliferatum* (45:42, respectively) and *F. verticillioides* (19:10, respectively). The ratio of mating type alleles

Fig. 2. Bayesian inferred tree based on partial sequences of CaM and EF-1α genes. Values at branch nodes indicate branch support with posterior probabilities (PP × 100); values ≥50 are shown. *Fusarium oxysporum* strain NRRL 22902 was used as the outgroup. Asterisks (*) indicate *Fusarium proliferatum* strains isolated from maize. Abbreviations used for localities: Bw: Barrow, Bc: Balcarce, Bo: Bordenave, BB: Bahia Blanca, C: Cabildo, LD: La Dulce, M: Miramar, P: Pieres. Mating type allele (1 or 2) is indicated next to each strain.


ITEM 15654 BB MAT-2
ITEM 15659 BB MAT-1
ITEM 15661 BB MAT-1
ITEM 15664 BB MAT-1
ITEM 15665 BB MAT-1
ITEM 15668 LD MAT-1
ITEM 15669 LD MAT-1
ITEM 15670 LD MAT-1
ITEM 15676 P MAT-1
ITEM 15682 M MAT-2
ITEM 15683 M MAT-2
ITEM 15689 P MAT-1
ITEM 15691 P MAT-1
ITEM 15696 P MAT-1
ITEM 15698 P MAT-1
ITEM 15709 C MAT-1
ITEM 15711 LD MAT-2
ITEM 15712 Bc MAT-1
ITEM 15715 LD MAT-1
ITEM 15723* MAT-1
ITEM 16370 LD MAT-1
ITEM 16382 Bw MAT-2
ITEM 16384 Bo MAT-1
ITEM 16389 Bw MAT-1
ITEM 16395 P MAT-2
ITEM 16397 P MAT-1
ITEM 16398 P MAT-1
ITEM 16399 P MAT-1
ITEM 16400 P MAT-1
ITEM 15656 BB MAT-2
ITEM 15660 BB MAT-2
ITEM 15675 P MAT-2
ITEM 15678 P MAT-2
ITEM 15685 LD MAT-1
ITEM 15693 P MAT-1
ITEM 15710 Bw MAT-2
ITEM 15714 C MAT-1
ITEM 16374 LD MAT-1
ITEM 16393 LD MAT-1
ITEM 16396 P MAT-1
ITEM 15666 LD MAT-2
ITEM 15671 LD MAT-2
ITEM 15674 LD MAT-2
ITEM 15681 Bw MAT-2
ITEM 15694 P MAT-2
ITEM 15697 P MAT-2
ITEM 15700 Bw MAT-2
ITEM 15713 Bw MAT-2
ITEM 15724* MAT-2
ITEM 16368 LD MAT-1
ITEM 16376 LD MAT-1
ITEM 16377 LD MAT-1
ITEM 16380 P MAT-1
ITEM 16388 Bw MAT-1
ITEM 16407 LD MAT-1
ITEM 16425 P MAT-2
ITEM 15717* MAT-2
ITEM 15657 BB MAT-2
ITEM 15658 BB MAT-2
ITEM 15662 BB MAT-2
ITEM 15663 BB MAT-2
ITEM 15667 LD MAT-2
ITEM 15672 LD MAT-2
ITEM 15673 LD MAT-1
ITEM 15677 P MAT-1
ITEM 15680 P MAT-1
ITEM 15695 P MAT-1
ITEM 15704 Bw MAT-1
ITEM 16378 P MAT-1
ITEM 16379 P MAT-2
ITEM 16371 LD MAT-1
ITEM 16413 LD MAT-1
ITEM 15679 P MAT-2
ITEM 15692 P MAT-2
ITEM 15699 P MAT-2
ITEM 15701 C MAT-2
ITEM 15705 Bo MAT-2
ITEM 15706 LD MAT-2
ITEM 15707 Bo MAT-2
ITEM 15708 Bw MAT-2
ITEM 15718* MAT-2
ITEM 15719* MAT-2
ITEM 15720* MAT-2
ITEM 15721* MAT-2
ITEM 15722* MAT-2
ITEM 16375 LD MAT-2
ITEM 16394 P MAT-2
ITEM 16408 LD MAT-2
ITEM 16369 LD MAT-2
ITEM 16372 LD MAT-1
ITEM 16386 LD MAT-1
ITEM 16391 Bw MAT-2
ITEM 16387 Bo MAT-2
ITEM 16392 LD MAT-2
ITEM 15716* MAT-1
ITEM 16439 P MAT-1
ITEM 7596 F. proliferatum MAT-1
ITEM 7595 F. proliferatum MAT-2
NRRL 13566 F. fujikuroi
NRRL 13999 F. sacchari
ITEM 15655 BB MAT-2
ITEM 15684 Bw MAT-2
ITEM 15688 M MAT-1
ITEM 15690 P MAT-1
ITEM 15703 Bo MAT-1
ITEM 16409 LD MAT-2
ITEM 16415 LD MAT-1
ITEM 16421 P MAT-2
ITEM 16422 P MAT-1
ITEM 16424 P MAT-1
ITEM 16428 Bw MAT-2
ITEM 16429 Bw MAT-2
ITEM 16430 M MAT-2
ITEM 16431 Bw MAT-2
ITEM 16433 Bo MAT-1
ITEM 7583 F. verticillioides MAT-2
ITEM 15686 Bw MAT-1
ITEM 15687 LD MAT-1
ITEM 16403 LD MAT-1
ITEM 16405 LD MAT-1
ITEM 16406 LD MAT-1
ITEM 16410 LD MAT-2
ITEM 16434 Bo MAT-1
ITEM 16416 LD MAT-1
ITEM 16419 P MAT-1
ITEM 16423 P MAT-1
ITEM 16426 Bw MAT-1
ITEM 16427 M MAT-1
ITEM 16436 Bc MAT-2
ITEM 16440 P MAT-1
ITEM 7581 F. verticillioides MAT-1
ITEM 16373 P MAT-1
ITEM 16411 LD MAT-1
F 15910 F. andiyazi
ITEM 16418 P MAT-1
ITEM 16420 LD MAT-2
ITEM 16435 C MAT-2
NRRL 22045 F. thapsinum
NRRL 22016 F. subglutinans
NRRL 25331 F. circinatum
NRRL 22902 F. oxysporum
0.4

obtained for both populations suggests possible occurrence of sexual reproduction in the wheat fields, although this seems more frequent in *F. proliferatum*.

Sexual reproduction enhances adaptation in changing environments by conferring genetic variability to populations. This is important for plant pathogen control since variability could influence the response of the fungus to new agricultural practices, the introduction of new cultivars with different resistance levels or the application of fungicides or biocontrol agents.

3.2. Phylogenetic analysis

Bayesian phylogenetic analysis was performed on the combined dataset for the calmodulin gene (CaM) and the translation elongation factor alpha gene (EF-1α) of the 130 strains (Fig. 2). The inferred tree shows three main clades (PP = 100). The first clade (PP = 100) included the reference strains of *F. sacchari*, and *F. fujikuroi* and all *F. proliferatum* strains; the second clade (PP = 100) included all the *F. verticillioides* strains, *F. andiyazi* and *F. thapsinum* strains and the third clade (PP = 100) contains *F. subglutinans* and *F. circinatum* reference strains. *F. proliferatum* strains separated into four groups and two subgroups but there was no observed correlation to origin, host or harvest year as the strains isolated from maize were distributed throughout the four groups of the clade. This division into groups and subgroups indicates high intraspecific variability in *F. proliferatum*. *F. verticillioides* strains also separated into two groups and no correlation with origin or harvest year was observed.

These results are in good agreement with a previous study on genetic variability of *F. proliferatum* and *F. verticillioides* isolated from pea, in which the *F. proliferatum* population was observed to be more variable than that of *F. verticillioides*, although this study did not analyze conserved genes, but a fumonisin biosynthetic gene (Waśkiewicz et al., 2013). Furthermore, Jurado et al. (2010) performed a phylogenetic analysis based on partial sequences of EF-1α gene of several *F. proliferatum* strains isolated from diverse hosts and origins and observed a high variability between them supported by the number of groups and subgroups obtained in the dendrogram. However, no correlation to origin and host was observed. In addition, von Bargen et al. (2009) carried out a genetic fingerprinting study of *F. proliferatum* strains obtained from asparagus plantings from Austria and Germany and observed 14 different fingerprinting groups which indicate genetic heterogeneity of this species, but no association was found between fingerprint group and origin of the strains. In the *F. proliferatum* population from wheat here analyzed, the genetic variability reported can be related to the possible high level of sexual recombination occurring in the field, as the ratio of mating types alleles scored suggests.

3.3. Fumonisin production by Fusarium strains

The analysis of the capability of the 121 *Fusarium* strains isolated from durum wheat to produce fumonisins on autoclavated durum wheat grains revealed that 97% of the *F. proliferatum* strains isolated were able to produce FBs in variable levels (Table 1, Fig. 3). Total fumonisin production varied from 0.9 to 2072 μg/g (mean = 145.11 μg/g), with FB_1 being the most abundant. All positive strains produced FB_1 in levels ranging from 0.05 to 1616 μg/g. Seventy-seven of 84 strains produced FB_2 in a range from 0.3 to 761 μg/g, and 72 of 84 strains produced FB_3 in levels ranging from 0.2 to 152 μg/g. There were 18 strains that produced more FB_2 than FB_1, 13 strains that produced more FB_3 than FB_2 and 5 strains that produced more FB_3 than FB_1. Of the *F. verticillioides* strains 93% were fumonisin producers in levels ranging from 0.05 to 317 μg/g (mean = 56.91 μg/g). Twenty-four out of 27 strains produced FB_1 in concentrations between 0.05 and 175 μg/g. Twenty-three out of 27 strains produced FB_2 in a range from 0.05 to 62 μg/g and 21 out of 27 strains were FB_3 producers in levels ranging from 0.1 to 160 μg/g. There were 8 strains that produced more FB_2 than FB_1, 17 strains that produced more FB_3 than FB_2 and 13 strains that produced more FB_3 than FB_1. *F. thapsinum* and *F. andiyazi* strains did not produce these toxins. *Fusarium proliferatum* strains were capable of producing more fumonisins than *F. verticillioides* strains on durum wheat grains. These results are similar to those obtained by Waśkiewicz et al. (2013) who found that most *F. proliferatum* strains isolated from pea produced higher amounts of fumonisins than *F. verticillioides* strains on rice cultures. Several authors have also reported high variability of fumonisin production by *F. proliferatum* isolated from diverse host plants (Jurado et al., 2010; Moretti et al., 1999; Stępień et al., 2011). The fumonisin levels produced by *F. proliferatum* on durum wheat grains are consistent with those reported by other authors for this species isolated from wheat but grown on maize kernels (Busman et al., 2012; Desjardins et al., 2007).

It is noteworthy that atypical *F. proliferatum* and *F. verticillioides* strains producing more FB_2 or FB_3 than FB_1 were isolated from durum wheat; similar results were observed for strains isolated from maize, pea and also durum wheat (Chulze et al., 1998; Palacios et al., 2011; Reynoso et al., 2004; Waśkiewicz et al., 2013).

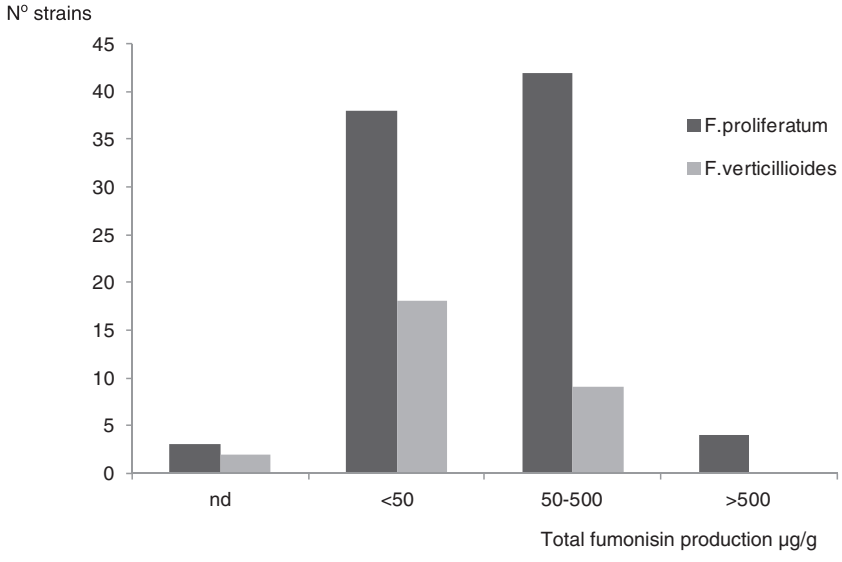


Fig. 3. Total fumonisin production ($FB_1 + FB_2 + FB_3$) by *Fusarium proliferatum* and *Fusarium verticillioides* strains isolated from durum wheat grains.

4. Conclusions

Durum wheat grains were contaminated by *F. proliferatum* and *F. verticillioides*. The phylogenetic analysis revealed that *F. proliferatum* strains were more diverse genetically and they were also capable of producing greater amounts of fumonisins on autoclaved durum wheat grains than *F. verticillioides*. Since wheat is an important cereal in the human diet, its contamination with fumonisin-producing *Fusarium* species is relevant and should be taken into account when determining fumonisin intake, especially in those countries where wheat consumption is high.

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