International Journal of Food Microbiology 199 (2015) 86–92

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Fusarium temperatum and *Fusarium subglutinans* isolated from maize in Argentina

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A R T I C L E I N F O

Article history:
Received 26 September 2014
Received in revised form 29 December 2014
Accepted 12 January 2015
Available online 20 January 2015

Keywords:
Fusarium temperatum
Fusarium subglutinans
Maize
Beauvericin
Fusaproliferin
Fumonisins

A B S T R A C T

Fusarium temperatum and *Fusarium subglutinans* isolated from the Northwest region (NOA region) of Argentina were characterized using a polyphasic approach based on morphological, biological and molecular markers. Some interfertility between the species was observed. The phylogenetic analysis showed that the two species represented two clades strongly supported by bootstrap values. The toxigenic profile of the strains was also determined. *F. temperatum* strains were fusaproliferin and beauvericin producers, and only some strains were fumonisin B_1 producers. All *F. subglutinans* strains produced fusaproliferin but none produced beauvericin, indicating a potential toxicological risk from maize harvested in the NOA region of Argentina. This study provides new information about *F. temperatum* isolated from maize in Argentina.

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1. Introduction

Maize (*Zea mays* L.) is, after wheat, the second most important cereal crop in human and animal diets worldwide (FAO, 2011). In Argentina maize is cultivated in the main maize growing region (Buenos Aires, Santa Fe and Cordoba provinces) and in the NOA region (SIIA, 2012). In the latter, there are also native maize races, which are cultivated in small farms and are used for domestic consumption (ILSI, 2006).

Several maize diseases are caused by *Fusarium* species, leading to significant yield losses and potential risk of mycotoxin contamination. *Fusarium subglutinans,* a member of the *Fusarium fujikuroi* species complex (FFSC), is a globally distributed pathogen causing stalk and ear rot of maize (Leslie and Summerell, 2006). Several studies in Argentina showed that the most prevalent species isolated from this cereal were *Fusarium verticillioides, Fusarium proliferatum* and *F. subglutinans* (Chulze et al., 2000; Reynoso, 2002; Torres et al., 2001) depending on geographical and climatic conditions. *F. subglutinans* was the predominant species in cold and temperate zones such as the NOA region which has an average annual temperature ranging from 18 to 24 °C (SIGA INTA, 2014; Torres et al., 2001).

Species within the FFSC are able to produce a wide range of mycotoxins such as fumonisins, and other toxins such as fusaproliferin, beauvericin and moniliformin (Jestoi, 2008). *F. subglutinans* has been reported to produce moniliformin, fusaproliferin and beauvericin (Logrieco et al., 1996, 1998; Marasas et al., 1986; Moretti et al., 1995), although no fumonisin production was observed (Proctor et al., 2004).

In the FFSC, morphological, biological and molecular phylogenetic studies have revealed that this complex includes 50 phylogenetically distinct species that comprise three biogeographically structured clades. This complex also includes 13 biological species (Aoki et al., 2014; Geiser et al., 2013).

Steenkamp et al. (2002) found two major groups in populations of *F. subglutinans* isolated from maize. The groups, called group 1 and group 2, showed some interfertility between the strains under laboratory conditions (Desjardins et al., 2000; Srobarova et al., 2002). The phylogenetic concordance analysis indicated that these two groups were reproductively isolated, representing cryptic species (Steenkamp et al., 2002). Population studies on *F. subglutinans* isolated from maize from various regions of the world have shown that the strains belonging to both groups were taxonomically divergent (Moretti et al., 2008; O'Donnell et al., 2000; Steenkamp, et al., 1999; Viljoen et al., 1997). Scauflaire et al. (2011) using a polyphasic approach, described a new species within the FFSC naming it *Fusarium temperatum* corresponding to the one previously classified as *F. subglutinans* group 1.

F. temperatum was recently reported from maize in Belgium (Scauflaire et al., 2012) and from sorghum in Serbia (Lević et al., 2013), and later this species was reported from maize in Spain and China too, causing seedling malformation and maize stem rot (Pintos et al., 2013; Wang et al., 2013). The ability of *F. temperatum* to produce diverse mycotoxins such as moniliformin, beauvericin, enniatins and fumonisin B_1 has also been observed (Scauflaire et al., 2012; Wang et al., 2013).

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http://dx.doi.org/10.1016/j.ijfoodmicro.2015.01.011
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The aims of this study were to characterize *F. temperatum* and *F. subglutinans* isolates collected from maize harvested in the NOA region of Argentina, using a polyphasic approach based on: identification of the isolates by morphological markers; identification at the biological species level by sexual crosses with tester strains; determination of the mating type and female fertility of the isolates; assessment of the cross-fertility between *F. temperatum* and *F. subglutinans;* molecular identification by sequencing of translation elongation factor 1α (EF-1α); determination of the toxigenic profile of the isolated strains; and determination of the phylogenetic relationships among the Argentinean, Belgian and Chinese *F. temperatum* isolates from maize based on EF-1α and β-tubulin combined genes, and determination of the phylogenetic relationships among our isolates with FFSC strains by EF-1α, β-tubulin and *RPB2* combined genes.

Our working hypothesis was that some of the strains previously reported as *F. subglutinans* isolated from maize from the NOA region of Argentina were *F. temperatum* and could be misidentified as *F. subglutinans,* and some interfertility among these strains could occur.

2. Materials and methods

2.1. Strain isolation and identification

F. temperatum and *F. subglutinans* were isolated from native and commercial maize harvested in the NOA region of Argentina. Native maize was collected from a region located at an elevation ranging from 1260 to 3300 m.a.s.l., with an annual mean temperature of 16 °C and an annual relative humidity of 53% (region 1); commercial maize was collected from one region located at an elevation of 450 m.a.s.l., with an annual mean temperature of 21 °C and an annual relative humidity of 71% (region 2) (SIGA INTA, 2014).

Morphological identification was done from monosporic cultures plated on potato dextrose agar (PDA), carnation leaf agar (CLA) and Spezieller Nahrstoffarmer agar (SNA) and incubated 10–14 days at 25 °C under cycles of 12 h white light–12 h black light. Morphology was observed on PDA, and conidiogenous cells, conidial characteristics and sporodochia were observed on CLA and SNA. Monosporic cultures were cryopreserved in sterile 15% glycerol (Leslie and Summerell, 2006). Strains were maintained in the culture collection at the Department of Microbiology and Immunology, UNRC (National University of Rio Cuarto), as RCFS and RCFT, corresponding to *F. subglutinans* and *F. temperatum* respectively.

2.2. Identification of the isolates at the biological species level

2.2.1. Mating type specific PCR and crossing procedures

Crosses to determine biological species were done on carrot agar as described in Leslie and Summerell (2006). Tester strains used were *F. subglutinans* KSU 0990 (MATE1-1) and *F. subglutinans* KSU 2192 (MATE1-2) from Kansas State University, (Kansas, U.S.), and *F. temperatum* ITEM 16196 (MAT1-1) and *F. temperatum* ITEM 16190 (MAT1-2) from the Institute of Sciences of Food Production (Bari, Italy).

Prior to making the crosses, mating type (MAT1-1 or MAT1-2) was determined by PCR as described by Steenkamp et al. (2000) in order to reduce the number of crosses. Crosses were done in triplicate and fertility was confirmed by observation of a cirrhus on the top of perithecia and by microscopic observation of mature asci with ascospores, within 4–5 weeks of incubation. Female fertility was determined as described above, but using the field isolates as female parents and tester strains as male parents.

2.2.2. Recombinant progeny from the interfertile crosses

Isolates that produced fertile crosses with tester strains of *F. temperatum* and *F. subglutinans* were evaluated to determine the presence of recombinant progeny. Interfertile isolates and tester strains were marked with different types of nitrate nonutilizing (*nit*) mutations. *Nit* mutants were obtained as fast-growing sectors on minimal medium amended with 2% chlorate. The *nit* phenotypes were determined on basal medium amended with different nitrogen sources. Sexual crosses were performed between *nit* complementary mutants of field isolates and tester strains on carrot agar as described in Leslie and Summerell (2006) with tester strains as female parents and the field isolates as male parents. Crosses were tested in triplicate and fertility was confirmed by observation of cirrhus on the top of perithecia. Cirrhi were carefully removed with a sterile needle and were placed in a tube containing 4.5 ml of sterile 2.5% Tween 60 solution. The tube was mixed for 5 to 10 s with a Vortex, after which 300 μl was spread on MMTS medium (minimal medium is amended with 0.05% (vol/vol) tergitol type NP-10 and 2% (wt/vol) L-sorbose instead of 3% sucrose). After 5 to 7 days of incubation, characteristics of the colonies were observed. Each cross was made in triplicate and from each replication three perithecia were randomly selected for the account. Colonies growing thin, with little or no aerial mycelium were considered mutants; in contrast, dense button-like colonies with cottony white aerial mycelium were considered wild type colonies. Prototrophic wild types were assumed to be the result of sexual recombination (Bowden and Leslie, 1999).

2.3. Identification of the isolates at the phylogenetic species level

2.3.1. DNA isolation, PCR amplification and sequencing

The strains were grown in 50 ml of complete medium (CM) (Leslie and Summerell, 2006) and incubated on an orbital shaker (150 r.p.m.) for 3 days at 25 °C. Fresh mycelia were collected by vacuum filtration using a Millipore system and stored at −20 °C. Frozen mycelia were ground to a powder under liquid nitrogen with a mortar. The ground mycelia were transferred to a 1.5 ml microcentrifuge tube. Fungal DNA was extracted by using the cetyl-trimethylammonium bromide (CTAB) method (Leslie and Summerell, 2006). DNA was quantified in a 0.8% agarose gel with ethidium bromide, and diluted to achieve a concentration of 1–10 ng/μl. Amplification of the translation elongation factor 1α (EF-1α) gene was carried out with PCR primers EF1 and EF2 using the amplification conditions of O'Donnell et al. (1998). Amplification of β-tubulin was carried out with PCR primers T1 and T2 using the amplification conditions of O'Donnell and Cigelnik (1997). Amplification of RNA polymerase II beta subunit (*RPB2*) was carried out with PCR primer pairs 5f2–7cf and 7cf–11ar using the amplification conditions of O'Donnell et al. (2007). PCR products were purified with the DNA Wizard-Clean up purification kit (Promega, Madison, WI., USA) according to the manufacturer's instructions and sequenced in both directions in a ABI Prism 3100 (Applied Biosystem, USA) sequencer. Sequences were edited with BioEdit Sequence Alignment Editor Version 7.1.3.0 (Hall, 1999) and compared with FUSARIUM-ID (Geiser et al., 2004) and GenBank data bases for identification of the field isolates.

2.3.2. Phylogenetic analysis

Sequences were aligned using the software ClustalX2 (Larkin et al., 2007). Phylogenetic analyses were performed using the combined sequences of EF-1α and β-tubulin, and based on *RPB2* sequence. Maximum parsimony analyses were made with PAUP*4.0 (Swofford, 1998) with 1000 bootstrap replications to test clade support. Consistency index (CI) and retention index (RI) were calculated. *F. proliferatum* NRRL 22944 was used as outgroup. Sequences included in the analysis were obtained from GenBank and they are listed in Table 1.

2.4. Mycotoxin profile of the isolates

F. temperatum and *F. subglutinans* isolates were cultured on 50 g of yellow maize kernels sterilized by gamma irradiation in a ^{60}Co source (National Commission of Atomic Energy, Buenos Aires, Argentina), with a dose of 1200 kRad (Chulze et al., 1999). Grains were adjusted to 40% moisture in 500-ml Erlenmeyer flasks and inoculated with 2 ml

Table 1
Characteristics of *Fusarium temperatum* and *Fusarium subglutinans* isolated from maize in the Northwest region of Argentina.

Isolate number	Origin	Biological species	MAT allele	Female fertility[(c)]	GenBank acc. numbers		
					EF-1α	β-tub	*RPB2*
RCFT 488	Region 1[(a)]	*F. temperatum* [(d)]	MAT-2	−			
RCFT 672	Region 1	*F. temperatum*	MAT-2	−			
RCFT 684	Region 1	*F. temperatum*	MAT-2	−			
RCFT 780	Region 1	*F. temperatum*	MAT-1	−			
RCFT 792	Region 1	*F. temperatum*	MAT-1	−			
RCFT 801	Region 1	*F. temperatum*	MAT-1	−	KP270935	KP270964	
RCFT 866	Region 1	*F. temperatum*	MAT-1	−			
RCFT 881	Region 1	*F. temperatum*	MAT-2	−	KP270936	KP270965	
RCFT 892	Region 1	*F. temperatum*	MAT-2	−			
RCFT 895	Region 1	*F. temperatum*	MAT-2	−			
RCFT 900	Region 1	*F. temperatum*	MAT-1	−	KP270937	KP270966	
RCFT 903	Region 1	*F. temperatum*	MAT-1	+	KP270938	KP270967	KP270985
RCFT 906	Region 1	*F. temperatum*	MAT-1	−	KP270939	KP270968	
RCFT 907	Region 1	*F. temperatum*	MAT-1	−			
RCFT 912	Region 1	Cross-fertile	MAT-1	−	KP270940	KP270969	
RCFT 913	Region 1	*F. temperatum*	MAT-1	+	KP270941	KP270970	
RCFT 914	Region 1	*F. temperatum*	MAT-2	+	KP270942	KP270971	
RCFT 919	Region 1	*F. temperatum*	MAT-2	−			
RCFT 921	Region 1	*F. temperatum*	MAT-2	−	KP270943	KP270972	
RCFT 925	Region 1	*F. temperatum*	MAT-1	−	KP270944	KP270973	
RCFT 926	Region 1	*F. temperatum*	MAT-2	+	KP270945	KP270974	
RCFT 928	Region 1	*F. temperatum*	MAT-2	−	KP270946	KP270975	
RCFT 934	Region 1	*F. temperatum*	MAT-2	+	KP270947	KP270976	
RCFT 937	Region 1	*F. temperatum*	MAT-2	−	KP270948	KP270977	
RCFT 956	Region 1	*F. temperatum*	MAT-2	−			
RCFT 977	Region 1	*F. temperatum*	MAT-1	−			
RCFT 983	Region 1	*F. temperatum*	MAT-2	−	KP270949	KP270978	KP270986
RCFT 986	Region 1	*F. temperatum*	MAT-2	−			
RCFT 991	Region 1	*F. temperatum*	MAT-1	−			
RCFT 997	Region 1	*F. temperatum*	MAT-1	−	KP270950	KP270979	KP270987
RCFT 998	Region 1	*F. temperatum*	MAT-1	−			
RCFT 1002	Region 1	*F. temperatum*	MAT-2	−			
RCFT 1004	Region 1	*F. temperatum*	MAT-2	−			
RCFT 1016	Region 1	*F. temperatum*	MAT-1	−	KP270951	KP270980	
RCFT 1018	Region 1	*F. temperatum*	MAT-1	−	KP270952	KP270981	
RCFT 1047	Region 1	*F. temperatum*	MAT-1	−			
RCFT 1051	Region 1	*F. temperatum*	MAT-1	−			
RCFT 1076	Region 1	*F. temperatum*	MAT-1	−			
RCFS 297	Region 2[(b)]	Cross-fertile	MAT-2	−	KP270924	KP270953	
RCFS 426	Region 2	*F. subglutinans*[(e)]	MAT-1	−	KP270925	KP270954	
RCFS 491	Region 2	*F. subglutinans*	MAT-1	−	KP270926	KP270955	
RCFS 502	Region 2	*F. subglutinans*	MAT-1	−	KP270927	KP270956	
RCFS 517	Region 2	*F. subglutinans*	MAT-1	−	KP270928	KP270957	
RCFS 521	Region 2	*F. subglutinans*	MAT-1	+	KP270929	KP270958	KP270982
RCFS 528	Region 2	*F. subglutinans*	MAT-1	−	KP270930	KP270959	KP270983
RCFS 639	Region 2	Cross-fertile	MAT-2	−	KP270931	KP270960	KP270984
RCFS 694	Region 2	*F. subglutinans*	MAT-1	−	KP270932	KP270961	
RCFS 872	Region 2	*F. subglutinans*	MAT-2	−	KP270933	KP270962	
RCFS 885[e]	Region 1	*F. subglutinans*	MAT-1	−	KP270934	KP270963	
RCFS 1079	Region 2	*F. subglutinans*	MAT-1	+			

[(a)] Region 1. Elevation ranging 1260 to 3300 m.a.s.l; annual mean temperature of 16 °C; annual relative humidity of 53%.
[(b)] Region 2. Elevation of 450 m.a.s.l; annual mean temperature of 21 °C; annual relative humidity of 71%.
[(c)] (+) hermaphrodite strains; (−) female sterile strains.
[(d)] All *Fusarium temperatum* were isolated from Andean maize.
[(e)] All *Fusarium subglutinans* were isolated from commercial maize, except the strain RCFS 885, which was isolated from Andean maize.

of a suspension containing approximately 10^7 conidia/ml. Cultures were incubated at 25 °C for 4 weeks. The harvested culture material was dried in a forced draft oven at 60 °C for 48 h, finely ground and stored at 4 °C until use. Controls were treated in the same way, except that they were not inoculated. For beauvericin (BEA) and fusaproliferin (FUS) extraction, the protocols of Munkvold et al. (1998) were followed, according to which 10 g of each sample was homogenized for 30 min with 15 ml of methanol, then samples were filtered through Whatman no. 4 filter paper, and methanol was removed under reduced pressure. An aliquot of 100 μl of methanol extracts was filtered before the high performance liquid chromatography (HPLC) injection. The amounts of BEA and FUS were determined by HPLC with UV detection. For FUS, the HPLC system was set up with a constant flow rate of 1.0 ml/min and with an acetonitrile: water (65:35 *v*/*v*) eluent system. The retention time of the standard of FUS was 6 min. Quantification by HPLC procedures was carried out by comparison of the peak areas of the samples with the calibration curve of the authentic standard. FUS was detected at 261 nm. The detection limit for FUS was 0.03 μg/g. For BEA a gradient system was used (Monti et al., 2000) which started at a constant flow of 1.5 ml/min with acetonitrile:water (65:35 *v*/*v*) as starting eluent system. The starting ratio was constant for 5 min and then linearly modified to 70% acetonitrile in 10 min. After 1 min, the mobile phase was taken back to the starting conditions in 4 min. The retention time of the standard of BEA was 12.5 min. Quantification by HPLC procedures was carried out by comparison of the peak areas of the samples with the calibration curve of the authentic standard. BEA was detected at 205 nm. The detection limit for BEA was 0.10 μg/g. Fumonisin (FUM) analysis was mainly based on the method originally reported by Shephard et al. (1990) modified

by Doko et al. (1995). 15 g of the ground subsamples was shaken with 50 ml of methanol/water (3:1) for 30 min and filtered through Whatman No. 4 filter paper. An aliquot of the eluate (50 μl) was derivatized with 200 μl of o-phthaldialdehyde (OPA) solution obtained by adding 5 ml of 0.1 M sodium tetraborate and 50 μl of 2-mercaptoethanol to 1 ml of methanol containing 40 mg of OPA. The fumonisin OPA derivatives (50 μl solution) were analyzed using a reversed-phase HPLC/fluorescence detection system. Methanol: 0.1 M sodium dihydrogen phosphate (75:25) solution adjusted to pH 3.35 with orthophosphoric acid was used as mobile phase, at a flow rate of 1.5 ml/min. Fluorescence of the fumonisin OPA derivatives was recorded at excitation and emission wavelengths of 335 and 440 nm respectively. Fumonisin quantification was performed by peak area measurements and compared with reference standard solutions. The limit of detection of the analytical method was 0.02 μg/g for the toxins.

3. Results

3.1. Strain identification

Using morphological characters 175 strains isolated from maize harvested in two zones from the NOA region of Argentina were identified as *F. subglutinans*/*F. temperatum* based on the absence of chlamydospores and on the production of microconidia in false heads on monophialides and polyphialides without microconidial chains in the aerial mycelium. From these isolates, 50 were selected for further characterization. At the biological species level, all were successfully identified by sexual crosses as *F. temperatum* or *F. subglutinans*. Only three isolates were able to produce fertile perithecia with tester strains belonging to *F. temperatum* and *F. subglutinans*. The identification at the phylogenetic species level showed that 38 isolates were *F. temperatum* and 12 were *F. subglutinans*. Table 1 shows data on the analyzed strains.

All strains identified as *F. temperatum* and one strain identified as *F. subglutinans* were isolated from the region 1 with the coldest temperature (16 °C), whereas from region 2 with the warmest temperature (21 °C) all the isolated strains were *F. subglutinans*. Similar results were obtained by other authors in relation to climate conditions for occurrence of *F. temperatum* or *F. subglutinans* (Moretti et al., 2008; Scauflaire et al., 2011).

Isolates of both species showed the two mating type idiomorphs. Among *F. temperatum*, 18 isolates were MAT1-2 and 20 isolates were MAT1-1; among *F. subglutinans*, 3 isolates were MAT1-2 and 9 isolates were MAT1-1. Five *F. temperatum* strains were hermaphrodites while only 2 *F. subglutinans* strains were hermaphrodites.

Among the 50 isolates evaluated, 3 were able to produce fertile perithecia with the tester strains of *F. temperatum* and *F. subglutinans*. The fertile cross between *F. temperatum* RCFT 912 (MAT1-1) and *F. subglutinans* KSU 2192 (MAT1-2) produced an average of 94 prototrophic colonies per plate; the fertile cross between *F. subglutinans* RCFS 639 (MAT1-2) and *F. temperatum* ITEM 16196 (MAT1-1) produced an average of 42 prototrophic colonies per plate; and the fertile cross between *F. subglutinans* RCFS 297 (MAT1-2) and *F. temperatum* ITEM 16196 (MAT1-1) produced an average of 62 prototrophic colonies per plate (Table 1).

3.2. Phylogenetic analysis

In the phylogenetic analysis we included 18 strains of *F. temperatum* and 11 strains of *F. subglutinans* isolated from maize harvested in the NOA region of Argentina, 6 strains of *F. temperatum* and one strain of *F. subglutinans* isolated from Chinese maize described in Wang et al. (2013); 3 strains isolated from Belgian maize described by Scauflaire et al. (2011) and a reference strain *F. subglutinans* NRRL 22016 isolated from maize in USA. *F. proliferatum* NRRL 22944 was included as outgroup. The GenBank accession numbers of the sequences used in this study are listed in Table 1.

We examined the genetic relatedness of *F. subglutinans* and *F. temperatum* isolated from NOA region in Argentina analyzing the nucleotide sequences of partial β-tubulin and EF-1α genes. Several subgroups were observed amongst the isolates of *F. temperatum* and *F. subglutinans*. The diversity of sequences among the *F. subglutinans* strains led us to identify 7 different groups (haplotypes) of sequences in EF-1α and 6 in β-tubulin. Among the *F. temperatum* strains, we identified 14 haplotypes considering EF-1α and 9 according to β-tubulin sequence. In the combined dataset, *F. subglutinans* strains grouped in 8 different haplotypes and *F. temperatum* grouped in 17 haplotypes (Table 2, Fig. 1). In relation to nucleotide diversity, the results showed minimal differences of the nucleotide composition between the two species studied.

The first phylogenetic analysis was conducted on partial sequences of two protein coding nuclear genes (EF-1α and β-tubulin), individually and combined, of *F. subglutinans* and *F. temperatum* strains isolated from maize in Argentina. Data on tree statistics are summarized in Table 2.

Alignment of EF-1α and β-tubulin partial sequences included respectively 550 and 539 nucleotide positions, totaling 1089 aligned nucleotide positions. There were 997 constant characters, 57 parsimony-uninformative variable characters and 35 parsimony-informative characters. Of these 35 parsimony informative characters, 31 were from EF-1α and 4 from β-tubulin. Maximum parsimony heuristic analysis of the combined dataset yielded 96 most parsimonious trees (MPTs, length 137 steps, CI: 0.7153, RI: 0.8904) in which the *F. subglutinans* and *F. temperatum* clades were strictly conserved. The EF-1α and β-tubulin individual (data not shown) and combined MPTs are topologically concordant. The maximum parsimony bootstrap values for the *F. temperatum* clades were 99% and 67%, and they were 100% and 87% for the *F. subglutinans* clades, in the EF-1α and β-tubulin individual trees respectively (Fig. 1).

The second phylogenetic analysis was conducted on partial sequences of three protein-encoding nuclear genes (EF-1α, β-tubulin and RPB2), individually and combined. In the analysis were included *F. subglutinans* strains (RCFS 0521, RCFS 0528 and RCFS 0639) and *F. temperatum* strains (RCFT 0903, RCFT 0983 and RCFT 0997) with those of representative species within FFSC. The *F. subglutinans* and *F. temperatum* haplotypes were located within the American clade of

Table 2
Tree statistics.

Locus	Characters (bp)	Trees	Parsimony tree length	CI/RI[a]	Informative characters	Aut/syn[b]	Bootstrap support (%)		Haplotypes[c]	
							F. t.[d]	*F. s.*[e]	*F. t.*	*F. s.*
EF-1α	550	41	44	0.7955/0.9721	31	23/8	99	100	14	7
β-tub	539	4	4	1/1	4	4/0	67	87	9	6
Combined data	1089	96	137	0.7153/0.8904	35	19/16	100	100	17	8

[a] CI = consistency index, RI = retention index.
[b] Aut = autapomorphies, syn = synapomorphies (calculated with PAUP*4.0 (Swofford, 1998)).
[c] The number of haplotypes was calculated using the software DNAsp (Rozas et al., 2003).
[d] *F. t.*: *Fusarium temperatum*.
[e] *F. s.*: *Fusarium subglutinans*.

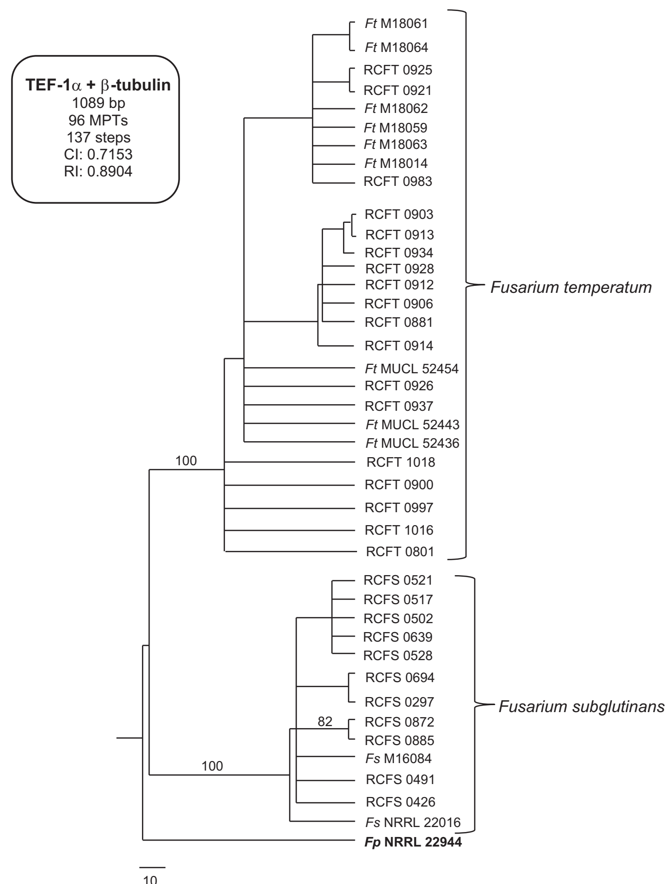

Fig. 1. One of the 96 most parsimonious trees constructed with PAUP*4.0 (Swofford, 1998) based on the combined analysis of EF-1α and β-tubulin sequences of the *F. subglutinans (Fs)/F. temperatum (Ft)* isolates from maize. *Fusarium proliferatum* NRRL 22944 (*Fp*) was used as outgroup. Bootstrap values (1000 replications) of 70% and higher are indicated above internodes. We included sequences of Belgian strains of *F. temperatum* ("MUCL" strains), Chinese strains of *F. temperatum* and *F. subglutinans* ("M" strains), *F. subglutinans* reference strains ("NRRL" strains) and Argentinean strains of *F. temperatum* and *F. subglutinans* ("RCFT": Rio Cuarto, *F. temperatum;* "RCFS": Rio Cuarto, *F. subglutinans*).

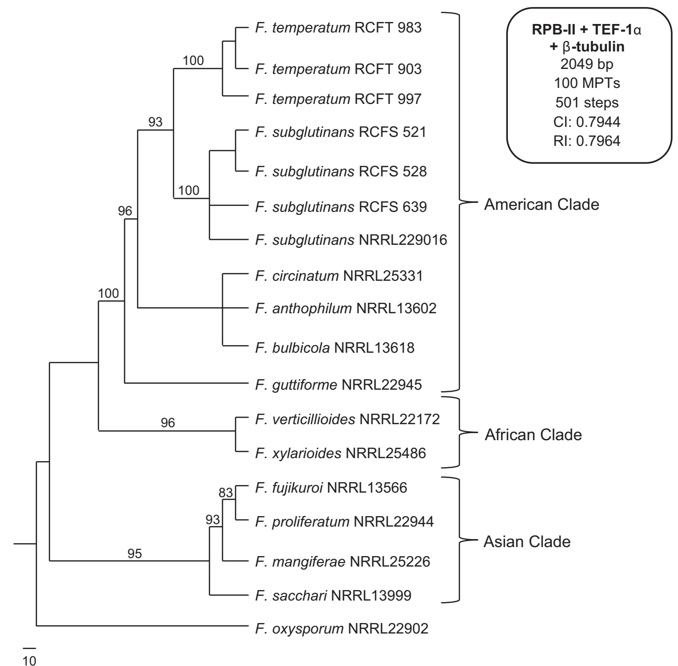

Fig. 2. One of the 100 most parsimonious trees constructed with PAUP*4.0 (Swofford, 1998) based on the combined analysis of *RPB2,* EF-1α and β-tubulin sequences of species within FFSC and isolates of *F. temperatum* and *F. subglutinans* from the NOA region in Argentina. *Fusarium oxysporum* NRRL 22902 was used as outgroup. Bootstrap values (1000 replications) of 70% and higher are indicated above internodes. Sequences of reference strains were obtained from NRRL (ARS, USDA, USA) collection from GenBank database. RCFT: Rio Cuarto, *F. temperatum.* RCFS: Rio Cuarto, *F. subglutinans* (Argentinean isolates).

the FFSC, as they were described in previous studies (Scauflaire et al., 2011; Wang et al., 2013), and the monophyletic origin of the corresponding *F. subglutinans* and *F. temperatum* clades was strongly supported within the combined gene tree, with 100% maximum parsimony bootstrap value (Fig. 2).

3.3. Mycotoxin profile

Of 38 strains identified as *F. temperatum*, 23 (61%) were fusaproliferin (FUS) producers at levels ranging from 40 to 1700 μg/g (mean: 316 μg/g ± 85). Among the strains identified as *F. subglutinans*, 9 of 12 (75%) were FUS producers at levels ranging from 200 to 1600 μg/g (mean: 605 μg/g ± 67). Among the strains identified as *F. temperatum,* 24 of 38 (63%) were beauvericin (BEA) producers at levels ranging from 10 to 1000 μg/g (mean: 201 ± 60 μg/g), whereas no *F. subglutinans* strains produced BEA. In relation to fumonisin B_1 production, 10 of 38 (26%) *F. temperatum* were FB_1 producers (range: 1 to 130 μg/g; mean: 34 μg/g ± 12) and no *F. subglutinans* were FB_1 producers (Table 3).

4. Discussion

This study showed the presence of *F. temperatum* and taxonomic relationship with *F. subglutinans* isolated from maize harvested in Argentina. A polyphasic evaluation using morphological, biological and molecular data showed that both *F. temperatum* and *F. subglutinans* co-occurred in maize harvested from the region 1 of the Argentinean NOA region; but from region 2 only *F. subglutinans* was isolated. Climatic conditions could be the factor selecting for the occurrence of *F. temperatum* and/or *F. subglutinans*. While *F. temperatum* was isolated from the coldest region (region 1), *F. subglutinans* was isolated from the warmest region (region 2), as was shown also by Moretti et al. (2008) and Scauflaire et al. (2012). This climatic hypothesis for the separation of the two species postulates that differences in temperature and humidity may influence fungal physiology and produce changes within the strains that reflect the climate characteristics of the geographic origins (Moretti et al., 2008).

Phylogenetic analyses based on combined sequences of EF-1α, β-tubulin and *RPB2* genes showed that *F. subglutinans* and *F. temperatum* strains isolated from maize in Argentinean NOA region clustered into two well supported groups, as it was demonstrated previously by Scauflaire et al. (2011) with strains isolated from maize in Belgium. Although the single-locus phylogenetic tree topologies did not show any contradiction between them and with the combined tree, the bootstrap value for the *F. temperatum* clade was low (<70%) for β-tubulin in the single-locus analysis.

Strain RCFS 885 isolated from Andean maize region 1 and the strain RCFS 872 isolated from commercial maize from region 2, clustered together in the phylogenetic tree of the combined genes (EF-1α and β-tubulin) with a bootstrap of 82%, and were *F. subglutinans*. This result indicates the occurrence of some degree of genetic difference between them in comparison with the other strains which clustered together in the tree (Fig. 2). Geographical isolation and adaptation to climatic conditions could explain this difference observed between the strains.

Both mating types were present among the isolates of *F. temperatum* and the ratio MAT1-1:MAT1-2 was close to 1:1. This MAT1-1:MAT1-2 ratio and the presence of hermaphrodite strains, indicate the possibility

Table 3
Toxin production by *Fusarium temperatum* and *Fusarium subglutinans* isolated from maize in the Northwest region of Argentina.

Isolate number	Toxin production (μg/gr)		
	BEA [(a)]	FUS [(b)]	FB1 [(c)]
Fusarium temperatum			
RCFT 488	ND[(d)]	ND	ND
RCFT 672	ND	ND	ND
RCFT 684	ND	ND	ND
RCFT 780	ND	ND	ND
RCFT 792	ND	ND	ND
RCFT 801	ND	ND	ND
RCFT 866	ND	ND	ND
RCFT 881	111 ± 72	145 ± 84	ND
RCFT 892	ND	ND	43 ± 17
RCFT 895	107 ± 45	187 ± 61	7 ± 3
RCFT 900	140 ± 72	434 ± 85	2 ± 0,5
RCFT 903	1040 ± 133	84 ± 23	ND
RCFT 906	492 ± 35	ND	1 ± 0,8
RCFT 907	106 ± 5	191 ± 22	ND
RCFT 912	896 ± 95	145 ± 15	ND
RCFT 913	231 ± 106	493 ± 126	ND
RCFT 914	36 ± 2	333 ± 84	2 ± 1,3
RCFT 919	91 ± 21	265 ± 57	ND
RCFT 921	103 ± 33	455 ± 38	ND
RCFT 925	ND	ND	ND
RCFT 926	91 ± 21	187 ± 89	88 ± 27
RCFT 928	63 ± 22	161 ± 67	ND
RCFT 934	1151 ± 350	129 ± 37	ND
RCFT 936	ND	ND	ND
RCFT 956	ND	47 ± 21	27 ± 11
RCFT 977	ND	ND	ND
RCFT 983	132 ± 20	ND	ND
RCFT 986	ND	ND	112 ± 36
RCFT 991	115 ± 13	140 ± 35	ND
RCFT 997	101 ± 17	1456 ± 336	55 ± 21
RCFT 998	119 ± 24	126 ± 37	8 ± 3
RCFT 1002	118 ± 59	682 ± 206	ND
RCFT 1004	ND	ND	ND
RCFT 1016	605 ± 113	80 ± 29	ND
RCFT 1018	80 ± 15	53 ± 9	ND
RCFT 1047	263 ± 66	1131 ± 395	ND
RCFT 1051	156 ± 77	353 ± 34	ND
RCFT 1076	109 ± 34	453 ± 77	ND
Fusarium subglutinans			
RCFS 297	ND	492 ± 50	ND
RCFS 426	ND	226 ± 43	ND
RCFS 491	ND	ND	ND
RCFS 502	ND	1612 ± 169	ND
RCFS 517	ND	388 ± 51	ND
RCFS 521	ND	393 ± 72	ND
RCFS 528	ND	390 ± 28	ND
RCFS 639	ND	1091 ± 131	ND
RCFS 694	ND	ND	ND
RCFS 872	ND	348 ± 35	ND
RCFS 885	ND	ND	ND
RCFS 1079	ND	244 ± 28	ND

[(a)] BEA beauvericin, detection limit: 0.10 μg/g.
[(b)] FUS fusaproliferin, detection limit: 0.03 μg/g.
[(c)] FB1 fumonisin B1, detection limit: 0.02 μg/g.
[(d)] ND not detected, <detection limit.

of sexual reproduction in the population isolated from these regions in Argentina.

Although phylogenetic analysis showed that *F. temperatum* and *F. subglutinans* formed two robust and separated clades, three isolates were interfertile and produced viable progeny. Interfertility was also described between *F. subglutinans* group 1 and group 2 by Steenkamp et al. (1999, 2002) and also between *F. proliferatum* and *F. fujikuroi* by Leslie et al. (2004), which supports our data. The cross-fertility observed between *F. temperatum* and *F. subglutinans* could indicate that these species isolated from Argentinean maize could be in the process of divergence into two reproductively isolated linages.

Our results and those obtained in previous studies by Moretti et al. (2008), Munkvold et al. (2009) and Scauflaire et al. (2012) showed that *F. temperatum* and *F. subglutinans* can also be distinguished based on the BEA production. Among the Argentinean isolates of *F. temperatum*, 63% were BEA producers whereas *F. subglutinans* strains did not produce BEA.

F. subglutinans have lost their fumonisin-production capacity because of the excision of the fumonisin biosynthetic genes (Proctor et al., 2004). The *F. subglutinans* strains analyzed in the present study were not fumonisin producers. *F. temperatum* strains were low fumonisin producers (max: 130 μg/g) in comparison with the main fumonisin producers isolated from maize in Argentina, *F. verticillioides* (mean: 2500 μg/gr) and *F. proliferatum* (mean: 2000 μg/gr) (Etcheverry et al., 2002; Reynoso, 2002). The low fumonisin levels produced by *F. temperatum* agree with Scauflaire et al. (2012), who reported the production of low levels of this toxin by *F. temperatum* isolates from Belgium.

Recent reports have focused on the study of non-traditional mycotoxins because of their importance in many food commodities, including maize (Jestoi, 2008; Zinedine et al., 2011). Production of FB_1, FUSA and BEA by *F. temperatum* in Argentina provides new information on the toxigenic profile and toxicological risk of this species isolated from maize. Further studies are ongoing on *F. temperatum* and *F. subglutinans* related to the ecophysiology of these species in relation to mycotoxin production.

Acknowledgments

This study was supported by grants from CONICET PIP 112-201101-00297 and ANPCyT PICT 2012-1436. Maria Veronica Fumero is a fellow from CONICET and Sofia Chulze and Maria M. Reynoso are members of the Research Career from CONICET.

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