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Phylogenetic analyses of the *Fusarium graminearum* species complex isolated from soybean in Argentina and Brazil

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Abstract Soybean is one of the most economically important crops in Argentina and Brazil. However, there is limited information on the biodiversity of the FGSC from soybean as compared to other crops of large-scale growing such as wheat and maize. A phylogenetic recognition of the *Fusarium graminearum* species complex (FGSC) isolated from soybean in Argentina and Brazil was performed in order to identify species responsible for trichothecene production. Sequences of genes encoding for the partial translation elongation factor, the 3-O-acetyltransferase and a putative reductase were analysed by the Maximum Parsimony method. Although the present study has focused on a limited number of isolates, this is the first report that provides evidence of the presence of at least four species within the FGSC associated with soybean in Argentina: *F. graminearum* sensu stricto, *F. cortaderiae*, *F. meridionale* and *F. boothii*. In addition, *F. graminearum* sensu stricto was detected for the first time among Brazilian isolates from soybean.

Keywords *Fusarium graminearum* species complex · Phylogeny · Soybean · Trichothecenes

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Members of the *Fusarium graminearum* species complex (FGSC) are economically important pathogens that cause significant yield and quality losses in the production of cereal crops. Before the year 2000 this complex was considered a single species, *Fusarium graminearum*. Since then, DNA-based analyses have resolved the FGSC into at least sixteen lineages (O'Donnell, 2004; Starkey et al. 2007; O'Donnell et al. 2008; Yli-Mattila et al. 2009; Sarver et al. 2011). Among them, *Fusarium graminearum* sensu stricto has long been considered the main fungal pathogen from this complex. *Fusarium graminearum* causes the Fusarium head blight (FHB) disease in wheat and barley and the ear rot of maize (Goswami and Kistler 2004). Recently, this and other species within the FGSC were identified as pathogens of soybean in South America, producing pod discoloration, seed decay and root rot (Martinelli et al. 2004; Pioli et al. 2004; Barros et al. 2014). However, there is limited information on the biodiversity of the FGSC from soybean, as compared to other crops of large-scale growing such as wheat and maize. Within the FGSC, three strain-specific B-trichothecene chemotypes have been identified: the NIV, the 3-ADON and the 15-ADON. Trichothecene toxin differences appear to be adaptive since three strain-specific profiles have been maintained by balancing selection over multiple speciation events (Ward et al. 2002). Based on this context, the aims of the present study were: i) to evaluate the biodiversity of species within the FGSC isolated from soybean in Argentina and Brazil through phylogenetic studies, using Maximum Parsimony analyses; ii) to correlate the phylogenetic species with the trichothecene chemotypes of the isolates.

In this study, a total of 32 isolates from soybean were included (Table 1). The isolates were morphologically identified within the FGSC according to Leslie and Summerell (2006). This set included 24 isolates (21 with the 15-ADON chemotype and 3 with unusual ability to produce both DON and NIV)

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Table 1 *Fusarium graminearum* species complex isolates for *EF-1α RED Tri101* sequenced as part of this study

Name collection	Source/State/Country	Identification	GenBank accession number		
			EF-1α gene	*RED* gene	*Tri101* gene
B2299	Seed/Paraná/Brazil	*F. meridionale*	KT179785	KT188371	KT188404
B2301	Seed/Paraná/Brazil	*F. graminearum*	KT179786	KT188372	KT188405
B2302	Seed/Paraná/Brazil	*F. graminearum*	KT179787	KT188373	KT188406
B2300	Seed/Paraná/Brazil	*F. meridionale*	KT179788	KT188374	KT188407
B2304	Seed/Paraná/Brazil	*F. meridionale*	KT179789	KT188375	KT188408
B2305	Seed/Paraná/Brazil	*F. graminearum*	KT179790	KT188376	KT188409
B2306	Seed/Paraná/Brazil	*F. meridionale*	KT179791	KT188377	KT188410
B2307	Seed/Paraná/Brazil	*F. meridionale*	KT179792	KT188378	KT188411
F5001	Pod/Córdoba/Argentina	*F. graminearum*	KT179793	KT188379	KT188412
F5024	Pod/Córdoba/Argentina	*F. graminearum*	KT179794	KT188380	KT188413
F5028	Pod/Córdoba/Argentina	*F. graminearum*	KT179795	KT188381	KT188414
F5030	Flower/Córdoba/Argentina	*F. meridionale*	JQ740897	KT188382	KT188415
F5031	Pod/Córdoba/Argentina	*F. graminearum*	KT179796	KT188383	KT188416
F5036	Seed/Córdoba/Argentina	*F. cortaderiae*	JQ740894	KT188384	KT188417
F5038	Pod/Córdoba/Argentina	*F. graminearum*	KT179797	KT188385	KT188418
F5043	Seed/Córdoba/Argentina	*F. meridionale*	JQ740895	KT188386	KT188419
F5048	Pod/Córdoba/Argentina	*F. meridionale*	JQ740896	KT188387	KT188420
F5049	Pod/Córdoba/Argentina	*F. graminearum*	KT179798	KT188388	KT188421
F5050	Pod/Córdoba/Argentina	*F. graminearum*	JQ740892	KT188389	KT188422
F5051	Flower/Córdoba/Argentina	*F. graminearum*	JQ740893	KT188390	KT188423
F5053	Seed/Córdoba/Argentina	*F. graminearum*	KT179799	KT188391	KT188424
F5054	Pod/Córdoba/Argentina	*F. graminearum*	KT179800	KT188392	KT188425
F5057	Pod/Córdoba/Argentina	*F. graminearum*	KT179801	KT188393	KT188426
F5059	Pod/Córdoba/Argentina	*F. graminearum*	KT179802	KT188394	KT188427
F5184	Pod/Córdoba/Argentina	*F. graminearum*	KT179803	KT188395	KT188428
F5185	Seed/Córdoba/Argentina	*F. graminearum*	KT179804	KT188396	KT188429
F5187	Seed/Córdoba/Argentina	*F. boothii*	KT179805	KT188397	KT188430
F5221	Seed/Córdoba/Argentina	*F. graminearum*	KT179806	KT188398	KT188431
F5222	Pod/Córdoba/Argentina	*F. graminearum*	KT179807	KT188399	KT188432
F5223	Pod/Córdoba/Argentina	*F. graminearum*	KT179808	KT188400	KT188433
F5225	Pod/Córdoba/Argentina	*F. graminearum*	KT179809	KT188401	KT188434
F5227	Pod/Córdoba/Argentina	*F. graminearum*	KT179810	KT188402	KT188435
F5228	Seed/Córdoba/Argentina	*F. graminearum*	KT179811	KT188403	KT188436

obtained from soybean plants collected in two fields in the Province of Córdoba, Argentina (Barros et al. 2012). The remaining 8 strains were isolated from soybean seeds in the Province of Parana, Brazil (5 isolates with the NIV chemotype and 3 with the 15-ADON chemotype). The isolates were grown in complete medium (CM) for DNA extraction. DNA was extracted by means of the cetyltrimethylammnonium bromide (CTAB) method (Leslie and Summerell 2006).

Amplification of the partial translation elongation factor (*EF-1α*, 725 bp), 3-O-acetyltransferase (*Tri101*, 1329 bp), and putative reductase (*RED*, 993 bp) genes sequences was performed using the E1/E2, AT1/AT2 and RED1d/RED2 primers, respectively (O'Donnell et al. 2004, 2008). PCR were carried out in a 1060 PTC-200 thermal cycler (MJ Research Inc., Watertown, MA, USA). The amplified products were purified using a Wizard® SV Gel and a PCR Clean-Up System kit (Promega, WI, USA), according to the manufacturer's instructions. Sequences were analyzed by the Sanger sequencing method using an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems).

For Maximum Parsimony, the sequences from the 32 strains isolated from soybean were analysed together with the sequences from most of the species included in the FGSC to assess the evolutionary relationships. *Fusarium pseudograminearum* was selected as the outgroup based on results from previous studies (Starkey et al. 2007; O'Donnell

et al. 2008). Editing of the sequences was carried out manually by using the BioEdit Sequence Alignment Editor 1997–2011. The ambiguously aligned regions in each alignment were removed by using Gblocks *V* 0.91b. The test of substitution saturation was performed by working on the third and the first-second codon position and by using the Data Analysis in Molecular Biology and Evolution (DAMBE) *V* 5.3.64. Three single-gene and concatenated trees were constructed by using Maximum Parsimony analyses. The different trees were compared by visual inspection and a clade was considered as an independent "phylogenetic taxon" when its basal node was well supported (bootstrap higher than 70 %) in the concatenated trees, and was not contradicted by any single-gene tree. The Maximum Parsimony (MP) analyses were conducted with the TNT program *V* 1.1, using tree-bisection-reconnection (TBR) branch-swapping algorithms and 10,000 random sequence additions per replicate, and saving 100 trees per replicate. The consistency index (CI), as well as the retention index (RI), was calculated using scrip *stats.run* to obtain the amount of homoplasy in the dataset.

The Maximum Parsimony results showed that the alignment of the *EF-1α*, *Tri101* and *RED* genes sequences contained 222, 148 and 257 parsimony informative characters, respectively. The tree obtained from the analysis of *Tri101* sequences supported the highest number of clades ($n = 4$) compared to the sequences trees from *EF-1α* and *RED* ($n = 3$ and $n = 2$, respectively). The combined dataset *EF-1α - Tri101 - RED* consisted of 2431 aligned nucleotide positions, of which 527 were parsimony informative. The parsimony analysis of these informative characters resulted in 5260 most parsimonious trees of 131 steps. The CI and the RI of the generated trees were 0.70 and 0.89, respectively. Four lineages were identified within the FGSC once the phylogenetic tree was obtained (Fig. 1). Cluster I included the largest number ($n = 23$) of tested strains, which were DON-15ADON producers and grouped with the *F. graminearum* sensu stricto NRRL 38383 reference

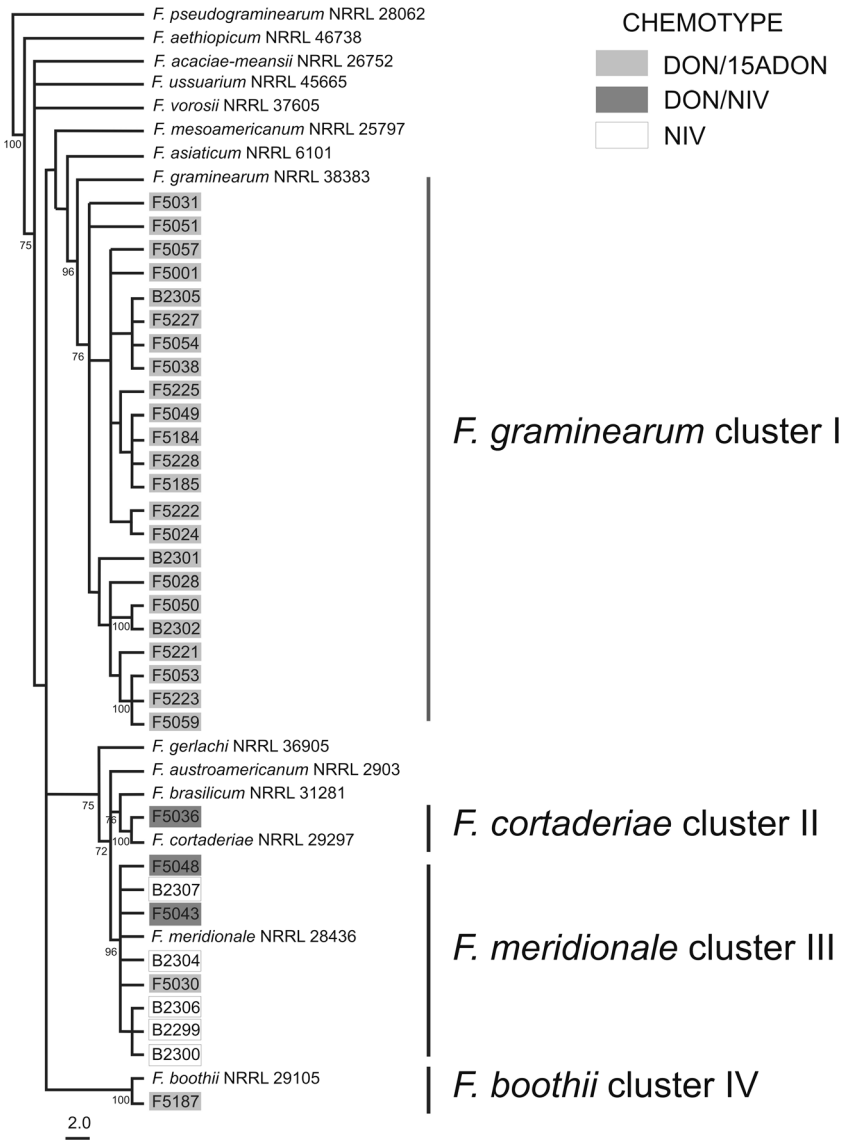

Fig. 1 Consensus tree of the *Fusarium graminearum* species complex inferred by Maximum Parsimony from a combined data set of 3-O-acetyltransferase (*Tri101*), reductase (*RED*) and translation elongation factor 1α (*EF-1α*) genes. Numbers within the tree represent the bootstrap values, with values lower than 70 % not shown

strain (bootstrap of 96 %). Cluster II included only one DON/NIV producing strain, which clustered with the *F. cortaderiae* NRRL 29297 reference strain (bootstrap of 100 %). Cluster III was represented by 8 strains grouped with the *F. meridionale* NRRL 28436 reference strain (bootstrap of 96 %). All of the strains isolated from Brazil were producers of NIV, while all of the Argentinean strains were producers of DON/NIV. Cluster IV included only one DON-15ADON producing strain, which clustered together with the *F. boothii* NRRL 29105 reference strain (bootstrap of 100 %).

Although the present study has focused on a limited number of strains, this is the first report that provides evidence of the presence of at least four species within the FGSC associated with soybean in Argentina. This group of strains was previously analysed using $AFLP_s$ markers (Barros et al. 2012) and only two phylogenetic species, *F. graminearum* and *F. meridionale*, were detected. This study allowed resolving the identities of two new species within the FGSC, *F. cortaderiae* (F5036) and *F. boothii* (F5187), all of which was strongly supported by the MP bootstrap values.

The species composition of the FGSC appears to be host and location dependent. In Argentina, *F. graminearum* sensu stricto was the only phylogenetic species isolated from wheat in different subregions of the main wheat production area (Ramirez et al. 2007; Alvarez et al. 2011), but *F. meridionale* and *F. boothii* were the most important on maize in the Northwest area of Argentina (Sampietro et al. 2011). In Brazil, surveys of FGSC isolates from wheat showed that *F. graminearum* sensu stricto was the dominant species (Astolfi et al. 2012), while *F. meridionale* represented an 80 % of the isolates from maize kernels in the Central and Southern maize growing regions of Brazil (Tessmann et al. 2011). In soybean, a preliminary report showed the presence of three species, *F. austroamericanum*, *F. meridionale* and *F. cortaderiae* in soybean samples from Brazil (Martinelli et al. 2004). Additionally, the present study detected *F. graminearum* sensu stricto among the Brazilian soybean strains. Based on the limited surveys to date in South America and on the results obtained in this work, we could infer that the diversity of species in the FGSC from soybean is higher than those previously reported for wheat and maize from Argentina and Brazil.

In summary, the phylogeny of the FGSC carried out in the present study allowed the identification of previously unidentified species in soybean from Argentina and Brazil. The most likely reasons could be: a) few studies conducted on this crop, b) the incomplete recognition of characterized species by conventional phenotypic identification, C) the use of unsuitable molecular tools. Thus, it would be essential to continue working on systematic molecular studies to characterize the FGSC populations from soybean isolated from Argentina and Brazil and to determine the role of soybean as a reservoir of several species within the FGSC.

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