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Characterization of *Fusarium* species associated with tobacco diseases in Northwestern Argentina

Lorena A. Berruezo · Guadalupe E. Mercado Cárdenas · Eleonora del M. Harries · Sebastián A. Stenglein · Ramiro N. Curti · Marcela S. Rodriguero · Marta Z. Galván

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Abstract Tobacco (*Nicotiana tabacum* L.) production is centred in the northwestern region of Argentina (NWA), where the incidence of root rot and stem diseases has increased considerably in recent years. This study aimed to analyse the genetic, morphological and pathogenic diversity of the *Fusarium oxysporum* and *F. solani* complexes (hereafter FOSC-FSSC), causing Fusarium wilt and root rot. One hundred tobacco fields were surveyed at six locations during two consecutive seasons, and 130 isolates were recovered from symptomatic tobacco plants. The isolates were characterized by morphological traits, molecular characteristics (EF1-α sequence) and pathogenicity tests. All of the isolates were identified as members of the FOSC or FSSC, exhibiting considerable intra-group variation. Three morphotypes were differentiated based on morphological characters in both complexes. The phylogenetic tree generated from the EF1-α sequences confirmed the isolates' identification. The pathogenicity of the isolates towards tobacco seedlings was assessed in a greenhouse. Considerable variability in pathogenicity was observed among the isolates. Differences in the levels of pathogenicity were recorded. In the FOSC and FSSC, 81% and 60% of the isolates were pathogenic, respectively. In this study, members of FOSC and FSSC exhibited considerable variability in morphological characteristics and virulence, and a portion of them were non-pathogenic for tobacco. To the best of our knowledge, this study is the first to provide information on the variability of the pathogens associated with

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L. A. Berruezo · E. d. M. Harries · R. N. Curti · M. Z. Galván (✉)
Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), CCT-Salta. Leguizamón 366 1er Piso (4400), Salta, Argentina
e-mail: martazgalvan@gmail.com

L. A. Berruezo · G. E. Mercado Cárdenas · E. d. M. Harries · M. Z. Galván
Instituto Nacional de Tecnología Agropecuaria (INTA) EEA Salta, Ruta Nac. 68 km 172 (4403), Cerrillos, Salta, Argentina

G. E. Mercado Cárdenas · E. d. M. Harries · R. N. Curti
Facultad de Ciencias Naturales, Universidad Nacional de Salta, Av. Bolivia 5150 (4400), Salta, Argentina

S. A. Stenglein
Laboratorio de Biología Funcional y Biotecnología (BIOLAB)-CICBA-INBIOTEC-CONICET, Facultad de Agronomía de Azul, Área de Microbiología, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Republica de Italia 780. C.C. 47 (7300), Azul Provincia de Buenos Aires, Argentina

M. S. Rodriguero
Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, IEGEBA (CONICET-UBA), Universidad de Buenos Aires, Intendente Güiraldes y Costanera Norte s/n, 4to. Piso, Pabellón II, Ciudad Universitaria, CI1428 EHA, Ciudad Autónoma de Buenos Aires, Argentina

tobacco wilt and root rot in NWA. This work contributes to the development of sustainable management strategies in tobacco production.

Keywords *Nicotiana tabacum* · Fusarium wilt · Fusarium root rot · EF1-α · Pathogenicity

Introduction

Tobacco (*Nicotiana tabacum* L.) is commercially produced in more than 125 countries worldwide and Argentina is among the ten main producers (FAO 2012). The production of this crop is centred in the northwestern region of the country and represents an important economic activity. Virginia tobacco varieties represent 74% of the national production, and the other 26% corresponds to the types Burley and Tobacco Creole (Miniagri 2016). Approximately 43,815 ha of Virginia tobacco are cultivated in Salta and Jujuy provinces with an average yield of 2000 kg/ha (Miniagri 2016). Root rot and stem diseases, caused by such species as *Rhizoctonia solani*, *Fusarium* spp., *Ralstonia solanacearum* and *Phytophthora nicotianae*, are among the main constraints on tobacco production in the region (Mercado Cárdenas et al. 2015). In Argentina, the incidence of these diseases has increased considerably in recent years, resulting in crop losses of up to 20% (Gimenez Monge et al. 2009).

The *Fusarium oxysporum* and *F. solani* species complexes (hereafter FOSC-FSSC) are an important pathogenic ascomycetous group (Leslie and Summerell 2006). Both saprophytic and pathogenic species are found in soil and rhizosphere (Michielse and Rep 2009). Fusarium wilt, caused by members of FOSC, is a severe disease in tobacco. Slow yellowing and occasional drying of the leaves along one side of the plant are the most common symptoms of the disease (Lucas 1975; Shew and Lucas 1991). Fusarium root rot is another relevant disease in different *Solanaceae* species and is caused by various members of FSSC and FOSC. The more significant symptoms of this disease in tobacco are wilting and chlorosis from the lowest to the higher leaves directly related to root rot. Members of FSSC were associated recently with symptomatic tobacco plants (Chehri et al. 2015).

The pathogenic isolates of FOSC and FSSC are known to contain a considerable number of subspecies/*formae specialis*. Determination of the *formae specialis* is generally performed by testing the fungus for pathogenicity on various plant species. Pathogenic FOSC and FSSC isolates can cause vascular wilt or root rot in over 100 plant species and include morphologically indistinguishable pathogenic and non-pathogenic isolates (Lievens et al. 2008). Previous studies established that differences in virulence in the FOSC are quite important since several *formae specialis* could be involved (Clark et al. 1998). Fusarium wilt of tobacco was attributed to *F. oxysporum* (Schlecht.) Snyd. & Hans. f. sp. *nicotianae* (Johnson) Snyd. & Hans (Johnson 1921). However, later Armstrong and Armstrong (1968) excluded it from the list of *formae speciales* of *F. oxysporum* because they did not obtain evidence of isolates specific to tobacco. Fusarium wilt of tobacco has been attributed to *F. oxysporum* f. sp. *batatas* (Wr.) Snyd. & Hans or to *F. oxysporum* f. sp. *vasinfectum* (Atk.) Snyd. & Hans (Armstrong and Armstrong 1968; LaMondia 1990; Alves-Santos et al. 2007; Rodríguez-Molina et al. 2013).

Several molecular approaches are being developed to complement the pathogenicity tests and morphological characterization for identifying *Fusarium* spp. (Lievens et al. 2008; Huang et al. 2014; Pinaria et al. 2015; Chehri 2016; Koyyappurath et al. 2016). Among these approaches, translation elongation factor 1-alpha (EF1-α) gene sequences have been demonstrated to be a powerful phylogenetic marker within *Fusarium* spp. (O'Donnell et al. 1998). High genetic variability was observed within members of the FOSC and FSSC based on EF1-α sequence analysis, dividing the species into three clades (Nalim et al. 2011; Bueno et al. 2014; Chitrampalam and Nelson 2016). Moreover, based on EF1-α and RPB2 sequence analysis a novel species, *Fusarium paranaense*, causing soybean root rot was described within FSSC (Costa et al. 2015).

In Argentina, there are studies describing the FSSC and FOSC affecting *Dianthus caryophyllus* L., *Glycine max, Citrus limon* and *Latuca sativa* (Lori et al. 2004; Scandiani et al. 2012; Fogliata et al. 2013; Malbrán et al. 2016). However, there are no reports concerning the association between these complexes and tobacco in this region. Therefore, additional works are needed to characterize the genotype and phenotype of isolates in both complexes in association with this crop for the development of efficient management strategies. Therefore, in this study, we characterized *Fusarium* spp. isolates recovered from tobacco fields in northwestern Argentina based on morphological, pathogenic and

molecular traits under the hypothesis that the symptoms of yellowing observed in the field are associated with FSSC and FOSC. The objectives were to: (i) identify the species complexes of *Fusarium* associated with wilt and root rot of tobacco in the northwestern region of Argentina by morphological attributes and molecular markers, and (ii) characterize the isolates of each complex according to their aggressiveness towards tobacco seedlings in the greenhouse.

Materials and methods

Survey area, symptomatology and fungal isolation

One hundred tobacco fields at six locations across northwestern Argentina were surveyed during two consecutive seasons (2013–14, 2014–15). The samples were taken during the crop cycle from tobacco plants exhibiting wilt symptoms. An average of 35 fields was evaluated for each location. A "W" sampling design was performed in each field, including 10 points and evaluating 100 plants per field. One hundred and thirty isolates of *Fusarium* spp. were obtained from the stem and root tissues using peptone PCNB agar (PPA) and potato dextrose agar medium 2% (PDA) acidified to pH 5 with 10% lactic acid (Table 1). Pure cultures were obtained using the single-spore subculturing process described by Leslie and Summerell (2006). The isolates were preserved in freezing spore suspension transferred to cryovials with glycerol (50%) and milk (7%) and also at −4 °C in Spezileller Nahrstoffarmer (SNA). All isolates studied were deposited in the collection of the "Laboratorio de Sanidad Vegetal" INTA-EEA-Salta Microbial Collection, Argentina.

Table 1 Number of *Fusarium* isolates recovered from tobacco plants exhibiting wilt symptoms in 100 fields across northwestern Argentina and tested

Environmental source	FOSC species	FSSC species	Other species	Total number of isolates tested
Stem tissue	22	0	0	22
Root tissue	21	44	3	65
Pathogenic isolates	43	44	0	87
Non-pathogenic isolates	10	30	3	43
Total number of isolates tested	53	74	3	130

Complex identification using morphological attributes

Morphological characteristics of the isolates were described based on macro- and microscopic observations. To study pigmentation and mycelial growth (cm), the isolates were grown on PDA plates under an alternating cycle of 12 h light/12 h darkness at 25 ± 2 °C for 10 days. Colony diameters were measured for each isolate (three reiterations) after three incubation days (Leslie and Summerell 2006). For microscopic observations (conidial characteristics, conidiogenous cells and sporodochia), all isolates were transferred to carnation leaf-pieces agar (CLA) and SNA plates and grown under an alternating cycle of 12 h light/12 h darkness at 25 ± 2 °C for two weeks. Fifteen randomly selected conidia of each septation class (macro-microconidia) were measured. Morphological characterization was performed based on the descriptions reported by Leslie and Summerell (2006) and Koyyappurath et al. (2016) for *F. oxysporum* and descriptions reported by Nalim et al. (2011) and Chehri et al. (2015) without observation of sexual structures for *F. solani*.

Pathogenicity assessment

Pathogenicity tests for each isolate were performed using tobacco plants of cultivar K326, which is susceptible to most of the root diseases and has been evaluated in previous studies (Mercado Cárdenas et al. 2015). Tobacco seeds were seeded under hotbed with equal parts of sterile mulch and sand as substrate (autoclaved for 30 min at 120 °C); the seedlings were grown at 25° ± 2 with a 12 h photoperiod. When the plants reached four true leaves, transplantation was performed. Eight plants were inoculated with the deposition of 1 ml of a suspension of spores (1×10^6 Conidia/ml) of each isolate in the substrate near the base of the stem. Plants inoculated with sterile water served as controls. Plants were maintained for 20 days in a growth chamber with equal conditions mentioned above (Rodríguez-Molina et al. 2013). Severity data were taken weekly following a scale of 0–4. To evaluate the isolates of FOSC, the scale proposed by LaMondia and Taylor (1987) was used (0 = healthy plants, 1 = stunted or off-colour plants, 2 = plants with one symptomatic leaf, 3 = plants with more than one symptomatic leaf, 4 = dead plants). Roots

were rated for Fusarium root rot symptoms following a five class rating scale (0 = no lesions, 1 = small root lesions, 2 = central root lesions, 3 = large root lesions, 4 = dead plant), and the disease severity index (DSI) was calculated for each isolate using the formula: $(n_1) + (n_2x2) + (n_3x3) + (n_4x4)/n_o + n_1 + n_2 + n_3 + n_4$, where n_0 is the number of plants in category 0, n_1 is the number of plants in category 1, n_2 is the number of plants in category 2, n_3 is the number of plants in category 3 and n_4 is the number of plants in category 4 (Fujinaga et al. 2005). Based on the DSI, the isolates were classified into three pathogenicity categories: highly pathogenic (DSI ≥ 2.5), moderately pathogenic (2.5 < DSI < 1.6) and slightly pathogenic (DSI ≤1.5). The plants were cut at the stem to rate the vascular discolouration, placed on PDA and incubated for ten days, and the isolates were later re-isolated and examined morphologically.

DNA extraction, PCR amplification and sequencing

Single spore cultures of pathogenic *Fusarium* isolates were multiplied in 50 ml of complete medium (CM) (Leslie and Summerell 2006) and incubated for 3 days on a rotary shaker (100 rpm) at 25 °C. Mycelia were collected by filtration and stored at −20 °C. Genomic DNA of 71 randomly selected pathogenic FOSC and FSSC isolates was used for amplification of the EF1-α gene. DNA was extracted using a CTAB protocol (Stenglein and Balatti 2006). Amplification of the EF1-α gene was performed with PCR primers EF-1H (5′- ATGGGTAAGGAAGACAAGAC) and EF-2 T (5'GGAAGTACCAGTGATCATGTT (O'Donnell 2000). Each PCR reaction was performed in a 50 ml mixture that contained 20–30 ng of genomic DNA, 100 mM of each dNTP, 10X PCR buffer (100 mM Tris–HCl [pH 9.0], 500 mM KCl, 1% Triton X-100), 25 mM $MgCl_2$, 100 nM of each primer (Genbiotech S.R.L., Argentina) and 0.8 U of *Taq* DNA polymerase (Highway-Inbio, Tandil, Argentina). PCR reactions were performed in a thermocycler (Eppendorf Master Cycler Gradient thermocycler, Hamburg, Germany) programmed with an initial denaturation step at 95 °C for 2 min; 35 cycles at 95 °C for 30 s, 56 °C for 30 s and 72 °C for 45 s; with a final extension at 72 °C for 2 min. Successful amplifications were confirmed by gel electrophoresis. The fragments were purified and subjected to sequencing in both directions using primers EF-1H and EF-2 T in a 3500xL Genetic Analyser sequencer (Applied Biosystems, Foster City, CA, USA) at the Biotechnology Institute of INTA (Castelar, Buenos Aires, Argentina). Forward and reverse sequences were assembled and aligned using BioEdit version 7.2.5 (Hall 1999). The isolates were identified to the species level by conducting Basic Local Alignment Search Tool (BLAST) searches with Fusarium-ID (Geiser et al. 2004) and GenBank sequence data.

Statistical analysis

Analysis of Principal Coordinates (PCoA) applying Gower's distance was carried out for morphotype differentiation based on the morphological data. The morphological characters involved in the PcoA were: colony colour, macroconidia shape and length, microconidia shape and sporodochia colour. A minimum spanning tree (MST) from the corresponding distance matrix was added, contributing to the interpretation of similarity among groups of isolates. A generalized linear mixed model with a multiple comparison test (DGC) was used to determine the aggressiveness of the isolates. Correlation between the phenotypic and genotypic distance matrices was determined using the Mantel test (Mantel 1967). All statistical analysis were performed using INFOSTAT statistical software (Di Rienzo et al. 2017).

DNA sequence alignments were performed using CLUSTAL W (Thompson et al. 1994) and adjusted by eye. Our dataset included 709 and 720 aligned nucleotide positions for FOSC and FSSC, respectively. J-MODELTEST software v.2 (Guindon and Gascuel 2003; Darriba et al. 2012) was used to infer the most appropriate molecular evolution model on the basis of the BIC criterion. HKY85 + I + G and HKY85 + G were selected as the best fit models for nucleotide substitution in each case. Bayesian phylogenetic analyses were performed using the 'metropolis-coupled Markov chain Monte Carlo' (MC3) algorithm implemented in MRBAYES version 3.2.6 (Ronquist and Huelsenbeck 2003; Ronquist et al. 2012). Programme defaults were used for estimation of priors. Two independent analyses were run in both analyses using a random starting tree over 10,000,000 generations sampling every 1000 generations. Tree space was explored using one cold chain and three incrementally heated ones. We assessed the stationarity of the cold Markov chain for all MRBAYES analyses through the standard deviation of the split frequencies. All posterior samples of a run prior to the burn-in point (at 25% of sampled topologies) were discarded. The remaining trees were used to obtain a

50% majority rule consensus tree with mean branch length estimates. Node support was assessed by posterior probability (Huelsenbeck and Ronquist 2001). Maximum likelihood (ML) and maximum parsimony trees were generated to test congruence of tree topology. Maximum likelihood trees were obtained using PhyML 3.0 (Guindon et al. 2010). The robustness of groups in the ML trees was tested by the bootstrap test, with 100 resamplings. Maximum parsimony analyses were run in TNT v. 1.5 under equal weighting (Goloboff and Catalano 2016). Heuristic searches were done under the following parameters: random addition sequence Wagner builds with 1000 replications saving 10 trees per replicate and tree bisection and reconnection (TBR) branch swapping. Branch support was calculated using 1000 bootstrap replicates.

Results

Morphological characterization

One hundred and thirty isolates of *Fusarium* spp. were obtained during the 2013–2014 and 2014–2015 growing seasons, 53 isolates corresponding to FOSC and 74 to FSSC (Tables 1 and 2). Three distinct morphotypes were defined within the FOSC according to ordination analysis: MI, MII and MIII comprising 27%, 61% and 12% of the pathogenic isolates, respectively (Fig. 1). Morphotype characteristics are described in Table 3. The most important differences were observed in colony colour and the shape of macroconidia.

Moreover, three morphotypes were differentiated within the FSSC: MI, MII and MIII comprising 17%, 47% and 36% of the pathogenic isolates, respectively (Fig. 2). The characteristics most distinguishable were macroconidia shape and number of septa, microconidia shape and sporodochia colour (Table 3).

Pathogenicity assessment

A screening for pathogenicity was performed with all 130 isolates. Among the isolates, 67% were pathogenic to tobacco seedlings (cv. 326). The severity of the pathogenic isolates varied among isolates of both complexes (Table 2). Disease severity index (DSI) ranging from 1 to 4 was used to group isolates into three pathogenicity categories: highly pathogenic (HP), moderately pathogenic (MP), and slightly pathogenic (SP) towards tobacco plants. Of the 53 FOSC isolates, 81% were pathogenic to tobacco with a significant difference in pathogenicity (Supplementary Fig. 1). Forty-seven percent of the FOSC isolates were HP, 30% MP and 17% SP. Conversely, of 74 FSSC isolates, 60% were pathogenic to tobacco under the conditions of the test, showing significant differences in aggressiveness among isolates (Supplementary Fig. 2). Thirty-two percent of the FSSC isolates were HP, 40% MP and 23% SP. The isolates of the FOSC proved to be more pathogenic than those of the FSSC. The pathogens were successfully re-isolated from the stem of symptomatic plants; therefore, Koch's postulates were fulfilled.

Molecular characterization

Using the primers EF-1H and EF-2 T, 656–720 bp DNA fragments were generated, and the sequences were deposited in GenBank (MF327596-MF327666). Sequence analysis of the PCR products and comparison with available NCBI and Fusarium-ID sequences showed that all the isolates belonged to FOSC or FSSC with 98–100% similarity.

Independent phylogenetic trees for each complex were generated (Figs. 3 and 4). The FOSC tree showed that 36 isolates were clustered together with clade support of 0.99 (Fig. 3). The isolates were separated into two distinct subgroups (clades 1 and 2) with the exception of five isolate (128, 7, 8, 12 and 15), which grouped separately. Clade 1 included 16 isolates grouped together with *Fusarium oxysporum* f sp. *batatas* (AF008484, AF008848) and *F. oxysporum* f sp. *radicis lycopersici* (HM057329) with clade support of 0.95. Within Clade 2, 16 isolates were grouped with *F. oxysporum* f sp. *batatas* (FJ985353). Considering that the clade support for Clade 2 was 0.88 it would be necessary to include other loci in the analysis to confirm the association of these isolates with *F. oxysporum* f sp. *batatas*. *F. commune* was used as outgroup. ML searches yielded trees topologically congruent with those obtained from the Bayesian analyses (Supplementary Figs. 3 and 4).

In the FSSC phylogenetic tree, three genetic clades (1, 2 and 3) were observed (Fig. 4). All of the FSSC isolates were grouped within clade 3 with clade support of 0.94 (Fig. 4). Of the isolates, 32% formed a cluster with *F. falciforme* (AB817230) and *F. paranaense* (KF597820, KF597813, KF597807) with clade support of 0.92, while 47% were grouped together with *Fusarium falciforme* (DQ247041, KF836701, HF937435).

Table 2 Isolate code, geographic origin, year, symptoms, environmental source, mycelium growth, and disease severity index (DSI) of *Fusarium oxysporum* and *F. solani* complex recovered in northwestern Argentina

Isolate	Geographic origin (province/ location)	Year of collection	Symptoms/ host/ source[a]	Mycelial growth (cm)[b]	DSI[c]	Pathogenicity[d]
FOSC						
1	Salta/La Merced	2013	DO/S/TS	1.7 ± 0.3	2.5	HP
5	Salta/La Merced	2013	DO/S/TR	2.5 ± 0.1	2.5	HP
6	Salta/La Merced	2013	DO/S/TR	2.6 ± 0.1	2.5	HP
7	Salta/Cerrillos	2013	DO/S/TS	3.0 ± 0.1	1.7	MP
8	Salta/Cerrillos	2013	DO/S/TS	3.3 ± 0.1	3.1	HP
9	Salta/Cerrillos	2013	DO/S/TS	2.3 ± 0.5	2.5	HP
11	Salta/Cerrillos	2013	DO/S/TR	3.8 ± 0.1	1.7	MP
12	Jujuy/Ovejería	2013	SY/C/TS	3.6 ± 0.3	3.5	HP
13	Jujuy/La Caravana	2013	SY/C/TS	1.8 ± 0.4	3.3	MP
15	Jujuy/La Caravana	2013	SY/C/TR	3.5 ± 0.2	2.7	HP
16	Jujuy/La Caravana	2013	SY/C/TR	3.5 ± 0.3	2.7	HP
17	Jujuy/San Vicente	2013	SY/C/TS	1.7 ± 0.2	2.0	MP
18	Jujuy/San Vicente	2013	SY/C/TR	2.4 ± 0.1	2.0	MP
19	Jujuy/San Vicente	2013	SY/C/TR	2.0 ± 0.1	0.0	NP
20	Jujuy/Alto Verde	2013	SY/C/TR	3.5 ± 0.6	2.3	HP
22	Jujuy/Alto Verde	2013	SY/C/TR	4.2 ± 0.4	1.8	MP
23	Jujuy/Alto Verde	2013	SY/C/TR	4.0 ± 0.1	1.0	SP
24	Jujuy/C. Arias	2013	SY/C/TR	3.5 ± 0.3	1.2	SP
25	Jujuy/Carambuco	2013	SY/C/TR	3.3 ± 0.2	2.0	MP
27	Jujuy/Alto Verde	2013	SY/C/TS	3.5 ± 0.2	3.0	HP
29	Jujuy/Ovejería	2013	SY/C/TR	3.5 ± 0.3	2.3	HP
30	Jujuy/Ovejería	2013	SY/C/TS	2.4 ± 0.3	2.2	MP
32	Jujuy/Ovejería	2013	SY/C/TS	2.1 ± 0.2	0.0	NP
33	Jujuy/Alto Verde	2013	SY/C/TS	2.9 ± 0.5	3.0	HP
37	Jujuy/Alto Verde	2013	SY/C/TR	2.5 ± 0.1	2.4	MP
49	Salta/La Merced	2013	SY/C/TR	1.7 ± 0.4	1.5	MP
67	Salta/Cerrillos	2013	SY/C/TR	3.1 ± 0.6	2.2	HP
72	Salta/La Merced	2014	SY/C/TR	2.7 ± 0.2	2.0	HP
74	Salta/La Merced	2014	SY/C/TR	1.8 ± 0.2	2.0	MP
82	Salta/Cerrillos	2014	SY/C/TS	2.3 ± 0.1	1.3	SP
90	Salta/Vaqueros	2014	SY/C/TS	2.9 ± 0.3	1.8	HP
96	Salta/Vaqueros	2014	SY/C/TS	2.5 ± 0.9	2.8	HP
100	Salta/La Merced	2014	SY/C/TS	2.5 ± 0.1	2.1	HP
111	Jujuy/Palpala	2014	SY/C/TR	3.5 ± 0.2	2.7	HP
112	Salta/Cerrillos	2014	SY/C/TR	3.5 ± 0.1	2.0	HP
114	Salta/Cerrillos	2015	SY/C/TR	3.8 ± 0.1	2.1	MP
126	Salta/La Viña	2013	SY/C/TS	3.0 ± 0.1	2.5	HP
127	Salta/La Merced	2013	SY/C/TS	3.2 ± 0.1	1.0	SP
128	Jujuy/Coronel Arias	2013	SY/C/TR	3.0 ± 0.1	1.0	SP
129	Salta/Cerrillos	2013	SY/C/TS	1.9 ± 0.2	1.0	SP
130	Salta/Cerrillos	2013	DO/C/TS	2.3 ± 0.2	1.0	SP
131	Jujuy/San Vicente	2013	DO/C/TS	2.8 ± 0.4	1.6	SP

Table 2 (continued)

Isolate	Geographic origin (province/ location)	Year of collection	Symptoms/ host/ source[a]	Mycelial growth (cm)[b]	DSI[c]	Pathogenicity[d]
132	Salta/La Merced	2013	SY/C/TS	3.0 ± 0.1	2.5	HP
133	Jujuy/Coronel Arias	2013	SY/C/TS	2.5 ± 0.2	2.3	MP
134	Jujuy/Coronel Arias	2013	SY/C/TS	2.3 ± 0.6	2.2	MP
FSSC						
26	Jujuy/Alto Verde	2013	RR/C/TR	3.1 ± 0.2	1.5	SP
28	Jujuy/Ovejería	2013	RR/C/TR	2.9 ± 0.1	2.2	MP
31	Jujuy/Ovejería	2013	RR/C/TR	2.0 ± 0.4	1.0	SP
35	Jujuy/Ovejería	2013	RR/C/TR	2.0 ± 0.1	1.9	MP
36	Jujuy/Alto Verde	2013	RR/C/TR	2.6 ± 0.3	2.1	MP
40	Salta/La Merced	2014	RR/C/TR	1.5 ± 0.3	2.0	MP
41	Salta/La Merced	2014	RR/C/TR	2.5 ± 0.1	2.8	HP
42	Salta/La Merced	2014	RR/C/TR	3.1 ± 0.1	2.0	MP
43	Salta/La Merced	2014	RR/C/TR	3.3 ± 0.2	2.5	HP
44	Salta/La Merced	2014	RR/C/TR	3.3 ± 0.3	2.8	HP
45	Salta/La Merced	2014	RR/C/TR	2.5 ± 0.3	1.0	SP
46	Salta/La Merced	2014	RR/C/TR	3.5 ± 0.1	2.8	HP
48	Salta/La Merced	2014	RR/C/TR	1.0 ± 0.1	2.8	HP
50	Salta/Cerrillos	2014	RR/C/TR	1.7 ± 0.1	2.9	HP
51	Salta/Cerrillos	2014	RR/C/TR	1.6 ± 0.1	1.5	SP
52	Salta/Cerrillos	2014	RR/C/TR	3.6 ± 0.3	2.1	MP
53	Salta/La Merced	2014	RR/C/TR	2.7 ± 0.3	2.1	MP
54	Salta/La Merced	2014	RR/C/TR	2.9 ± 0.3	1.9	MP
55	Salta/La Merced	2014	RR/C/TR	1.9 ± 0.6	3.2	HP
56	Salta/La Merced	2014	RR/C/TR	2.0 ± 0.4	1.8	MP
57	Salta/La Merced	2014	RR/C/TR	2.9 ± 0.3	1.6	MP
59	Salta/Cerrillos	2014	RR/C/TR	2.4 ± 0.3	1.5	SP
61	Salta/Cerrillos	2014	RR/C/TR	2.0 ± 0.3	3.5	HP
62	Salta/Cerrillos	2014	RR/C/TR	1.9 ± 0.1	2.0	MP
63	Salta/Cerrillos	2014	RR/C/TR	2.0 ± 0.2	1.5	SP
65	Salta/Cerrillos	2014	RR/C/TR	2.2 ± 0.1	1.5	SP
68	Salta/Cerrillos	2014	RR/C/TR	3.2 ± 0.3	2.8	HP
69	Salta/Cerrillos	2014	RR/C/TR	1.6 ± 0.1	1.5	SP
70	Salta/La Merced	2014	RR/C/TR	1.6 ± 0.1	3.2	HP
71	Salta/La Merced	2014	RR/C/TR	2.0 ± 0.1	2.7	HP
79	Salta/La Merced	2014	RR/C/TR	1.6 ± 0.1	1.0	SP
84	Salta/Cerrillos	2014	RR/C/TR	3.4 ± 0.1	2.0	MP
86	Salta/Cerrillos	2014	RR/C/TR	3.2 ± 0.3	1.0	SP
87	Salta/Chicoana	2014	RR/C/TR	3.0 ± 0.2	1.5	SP
91	Salta/Vaqueros	2014	RR/C/TR	2.3 ± 0.2	2.5	HP
94	Salta/Vaqueros	2014	RR/C/TR	2.5 ± 0.2	3.0	HP
98	Salta/Vaqueros	2014	RR/C/TR	2.4 ± 0.2	2.0	MP
99	Salta/La Merced	2014	RR/C/TR	2.5 ± 0.2	2.0	MP
101	Salta/R. de Lerma	2014	RR/C/TR	3.4 ± 0.2	2.5	HP
102	Salta/R. de Lerma	2014	RR/C/TR	3.4 ± 0.3	1.8	MP

Table 2 (continued)

Isolate	Geographic origin (province/ location)	Year of collection	Symptoms/ host/ source[a]	Mycelial growth (cm)[b]	DSI[c]	Pathogenicity[d]
103	Jujuy/Alto Verde	2014	RR/C/TR	3.2 ± 0.3	2.1	MP
104	Jujuy/Alto Verde	2014	RR/C/TR	3.2 ± 0.1	2.5	HP
107	Jujuy/Alto Verde	2014	RR/C/TR	3.5 ± 0.1	1.5	SP
109	Jujuy/San Vicente	2014	RR/C/TR	2.3 ± 0.2	2.1	MP
117	Salta/R. de Lerma	2015	RR/C/TR	2.0 ± 0.2	1.8	NP
121	Salta/El Carril	2015	RR/C/TR	2.4 ± 0.1	2.0	MP

[a] Symptoms: SY = slow yellowing; DO = damping off. Source: S = seedling; C = crop; TS = stem tissue (vascular); and TR = root tissue

[b] Mycelial radial growth of culture incubated in alternating light and dark at 25 ± 2 °C for 3 days

[c] 0–4 scale for yellowing. Disease severity index was assessed on tobacco cv. "K326" seedlings

[d] Pathogenicity categories: HP: highly pathogenic (DSI ≥ 2.5), MP: moderately pathogenic (2.5 < DSI < 1.6) and SP: slightly pathogenic (DSI ≤1.5)

However, the later were grouped with a low clade support. In this study, EF1-α sequence analysis has insufficient variation to separate *F. paranaense* from *F. falciforme* and *F. keratoplasticum* because this genomic region is too conserved. Four isolates were included in cluster FSSC5 with clade support of 0.99. Three isolates (44, 84 and 121) were grouped separately from the rest. *F. oxysporum* f sp. *vasinfectum* was used as outgroup.

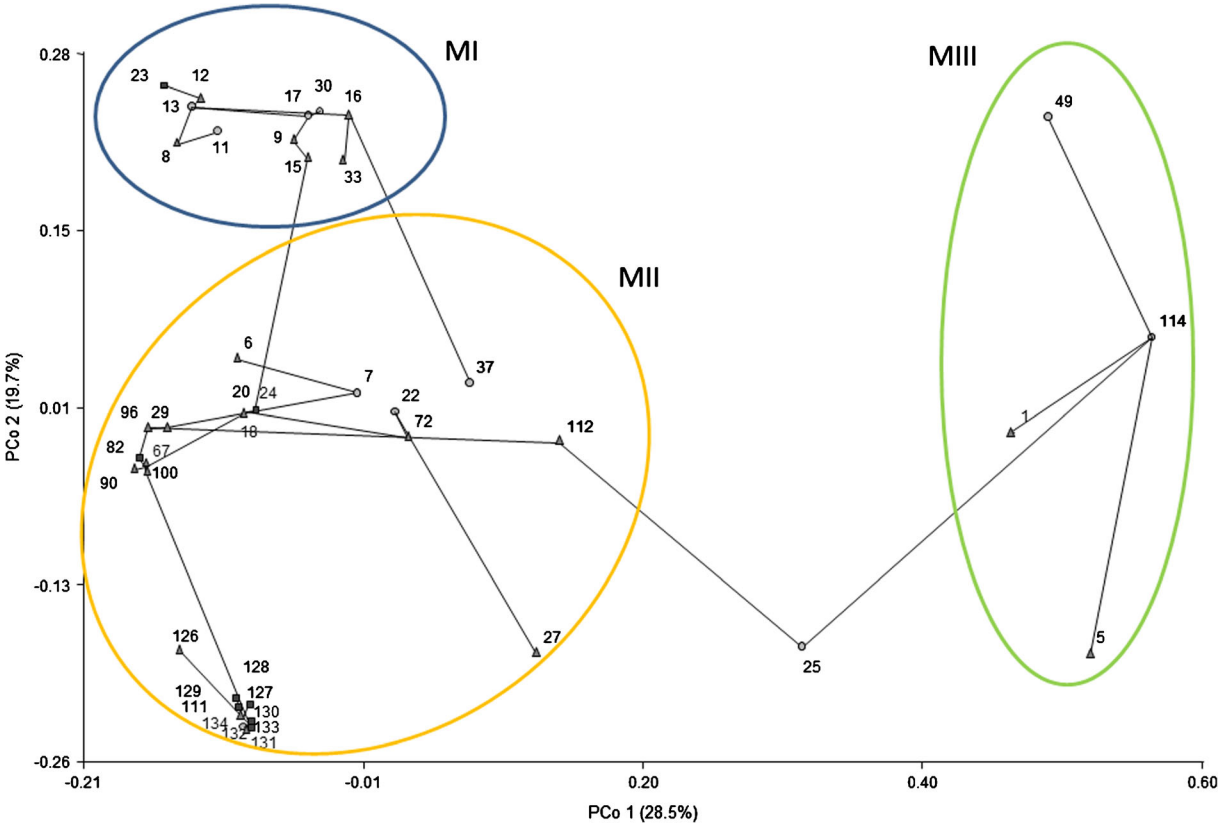

Fig. 1 Ordination of *Fusarium oxysporum* isolates on the first two principal coordinates (PCo) of the principal coordinates analysis and minimum spanning tree superimposed. The three main groups recognised are named and enclosed by circles (MI, MII and MIII: morphotypes I, II and III). The percentage of the variance explained by each principal coordinate is given in parenthesis. ▲ Highly pathogenic isolates, ○ moderately pathogenic isolates and ■ slightly pathogenic isolates

Table 3 Isolate code, geographic origin, mean of mycelium growth, colony colour, macroconidia shape, microconidia shape and sporodochia colour of FOSC and FSSC recovered in northwestern Argentina

Morphotype[a]	Isolates[b]	Mycelial growth (cm)[c]	Colony colour	Macroconidia shape and size	Microconidia	Sporodochia colour
FOSC						
MI	J: 11–12–13-15-16-17-23-30-33 S: 8–9	3.2 ± 0.7	Pink, white to pink	Slightly curved, 4–5 septate. 38.8 ± 3.0 μm	Ovals without septa	Strong cream
MII	J: 131–18–111-29-27-134-133-128-24-22-20 S: 96–90–126-132-127-100-72-67-37-130-129-112-82-7-6	2.4 ± 0.8	White to violet	Slightly straight, 4–5 septate. 42.5 ± 4.5 μm	Ovals without septa	Orange
MIII	J: 25 S: 1–5–49-114	2.6 ± 0.5	White to violet	Slightly curved commonly 4 septate. 40.5 ± 4.4 μm	Oval and elliptical	Strong cream
FSSC						
MI	J: 109 S: 55–59–61-71-98-117-121	2.2 ± 0.35	White to cream	Falcate-slightly straight, commonly 5 septate. 46.5 ± 6.3 μm	Ovals, elongated oval	Cream or blush green
MII	J: 26–31–35-36-45-103-107 S: 40–42–50-51-52-57–62-63-65-69-70-79-87-102	2.9 ± 0.7	White	Falcate and dorsiventral curved, commonly 4 septate. 39.1 ± 3.9 μm	Ovals, elongated oval (1 septa)	Cream
MIII	J: 28–104 S: 41–43–44-46-48-53-54-56-68-70-84-86-91-94-99-101	2.6 ± 0.7	White to green	Falcate and dorsiventral curved, commonly 5 septate.48.3 ± 5.1 μm	Oval, reniform, oval elongated without septa	Cream or blush green

[a] Morphotypes differentiated within the FOSC and the FSSC

[b] Isolate code; Geographic origin: S: Salta province, J: Jujuy province

[c] Mean of mycelium radial growth of culture incubated in alternating light and dark at 25 ± 2 °C for 3 days

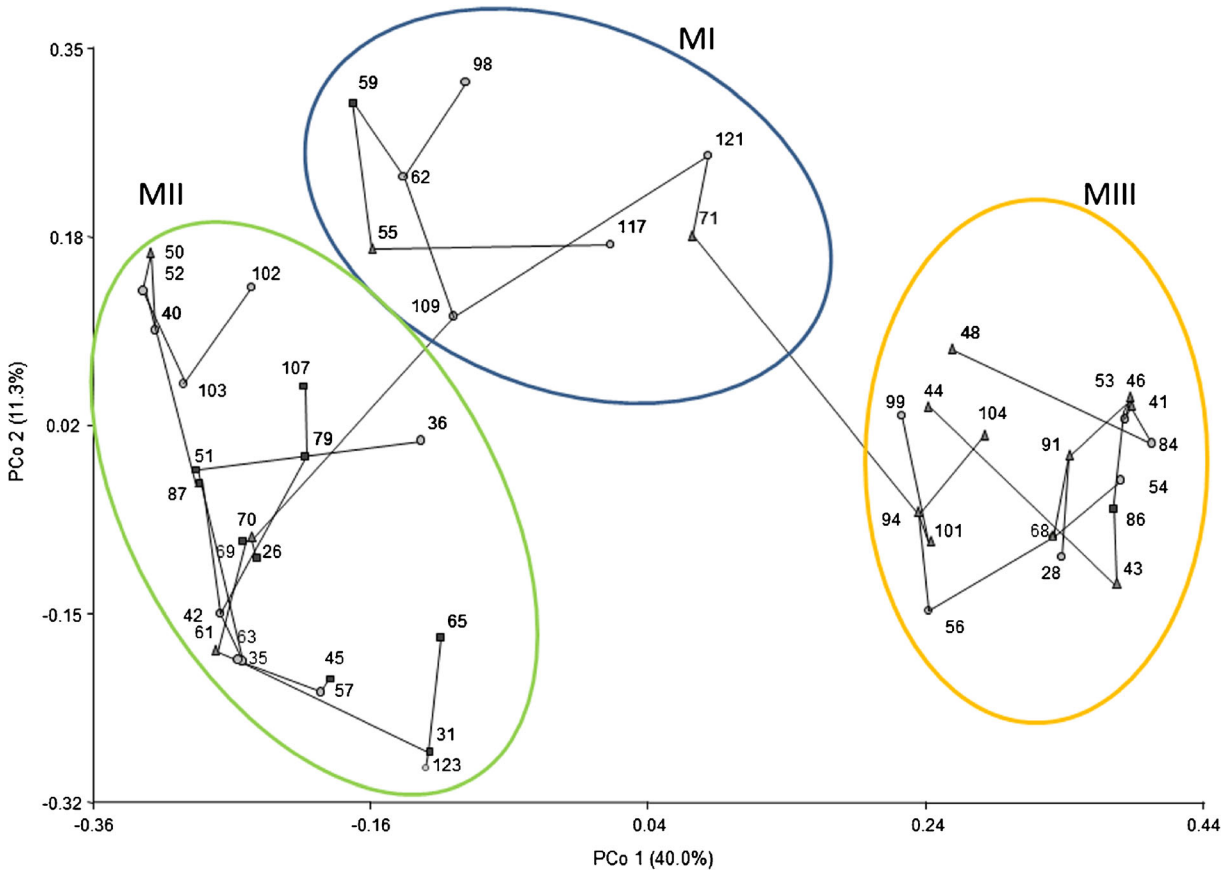

Fig. 2 Ordination of *Fusarium solani* isolates on the first two principal coordinates (PCo) of the principal coordinates analysis and minimum spanning tree superimposed. The three main groups recognised are named and enclosed by circles (MI, MII and MIII: morphotypes I, II and III). The percentage of the variance explained by each principal coordinate is given in parenthesis. ▲ Highly pathogenic isolates, ○ moderately pathogenic isolates and ■ slightly pathogenic isolates

Discussion

The primary objective of this study was to identify and characterize the species of *Fusarium* associated with Virginia tobacco in Argentina. Accordingly, 43 FOSC and 45 FSSC pathogenic isolates were recovered from tobacco fields in different locations in the northwestern region of the country. Given that the *Fusarium* spp. are difficult to study because isolates with similar morphology represent different biological groups that include endophytes (Leslie et al. 1990), saprophytes (Fracchia et al. 2000), and plant pathogens (Chandra et al. 2011), it was necessary to identify the pathogen by combining morphological, molecular and pathogenicity data. In this study, the *Fusarium* spp. isolates exhibited appreciable variability in morphological characteristics and virulence, and a portion of them were non-pathogenic to tobacco.

Based on the EF1-α locus the FOSC isolates were grouped into different clades in agreement with previous reports (O'Donnell et al. 1998, 2009; Laurence et al. 2014; Pinaria et al. 2015), manifesting two subgroups where most of the isolates were related to *F. oxysporum* f. sp. *batatas*. These results are in accordance with previous reports of *F. oxysporum* f. sp. *batatas* isolated from tobacco fields in Spain where the molecular analysis showed two different clusters with different physiological characteristics (Alves-Santos et al. 2007). The molecular differentiation of *F. oxysporum* isolates below the species level is complicated because of the polyphyletic nature of many *forma specialis* (Kistler 1997; Lievens et al. 2008). A thorough study including the analysis of other loci would be necessary to resolve the taxonomic status of the FOSC isolates analyzed. Differences in the levels of pathogenicity were recorded with 47% of the isolates highly

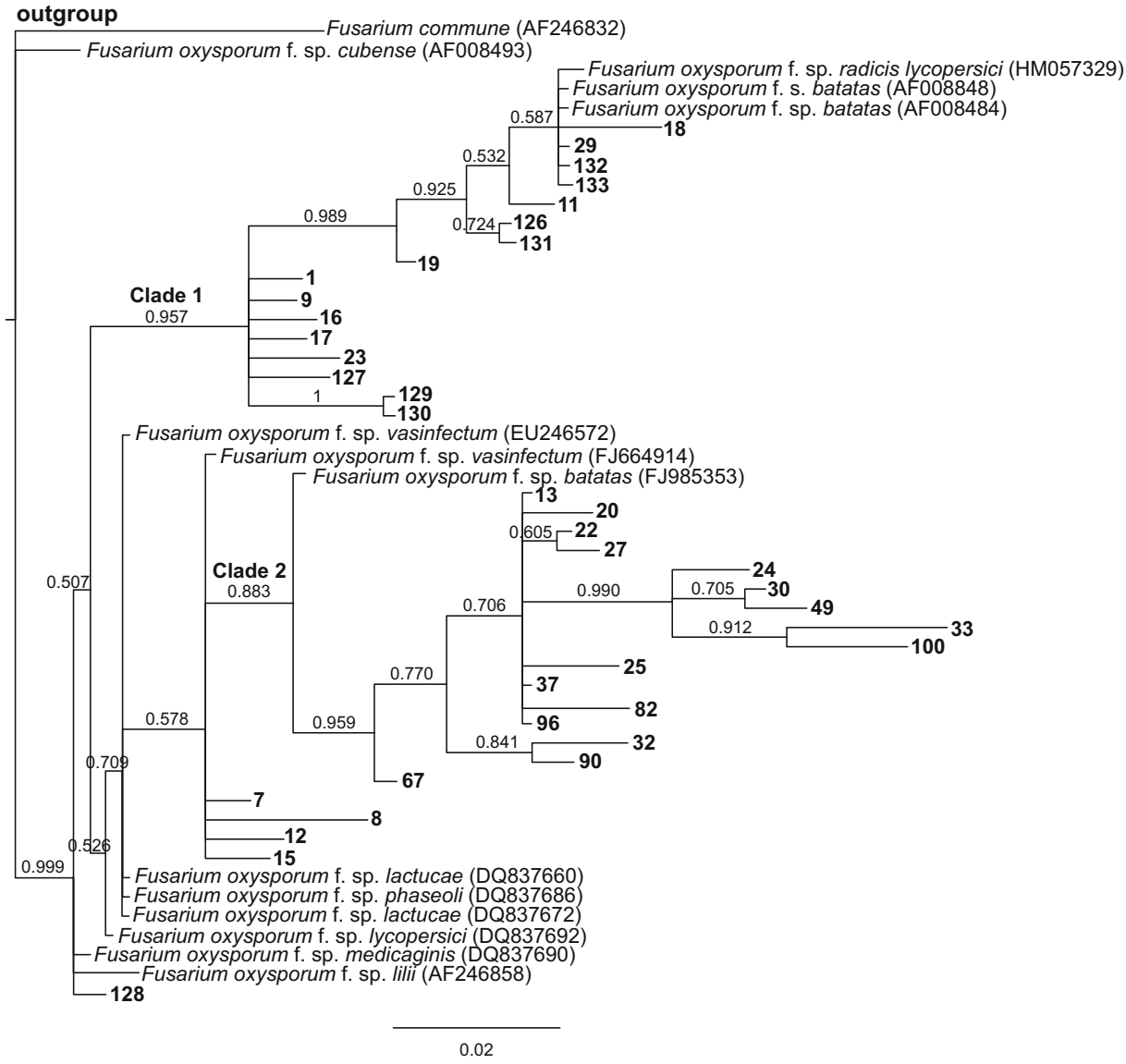

Fig. 3 Phylogenetic tree based on 36 EF1-α sequences recovered from *Fusarium oxysporum* isolates and 14 control sequences retrieved from GenBank. The tree was constructed using the HKY + G + I substitution model for Bayesian analysis. The numbers above the branches indicate node support (posterior probability). The scale bar represents the number of substitutions per site. Sequences obtained in this study are shown in bold. *Fusarium commune* was used as outgroup

pathogenic. Different authors suggest the existence of close relations between isolates of *F. oxysporum* from tobacco, sweet potato (*Ipomea batatas*) and cotton (*Gossypium hirsutum*) with variable aggression within each species (Shew and Lucas 1991; LaMondia 2015). Clark et al. (1998) suggested the existence of three evolutionary lineages among isolates of *F. oxysporum* from sweet potato and tobacco based on pathogenic characteristics, RAPD markers and VCG analyses, and they observed differences in host-pathogen interactions. Moreover, the name of *F. oxysporum* f. sp. *nicotianae* was suggested for the first time by Tjamos et al. (2006) based on an analysis of isolates from tobacco fields in Greece using pathogenicity tests. However, there are no EF1-α sequences from this *formae specialis* available in GenBank. Great variability was also observed on the bases of qualitative and quantitative characters with the differentiation of three morphotypes. Most of the highly pathogenic isolates were included within a single morphotype (Morphotype I). However, no association

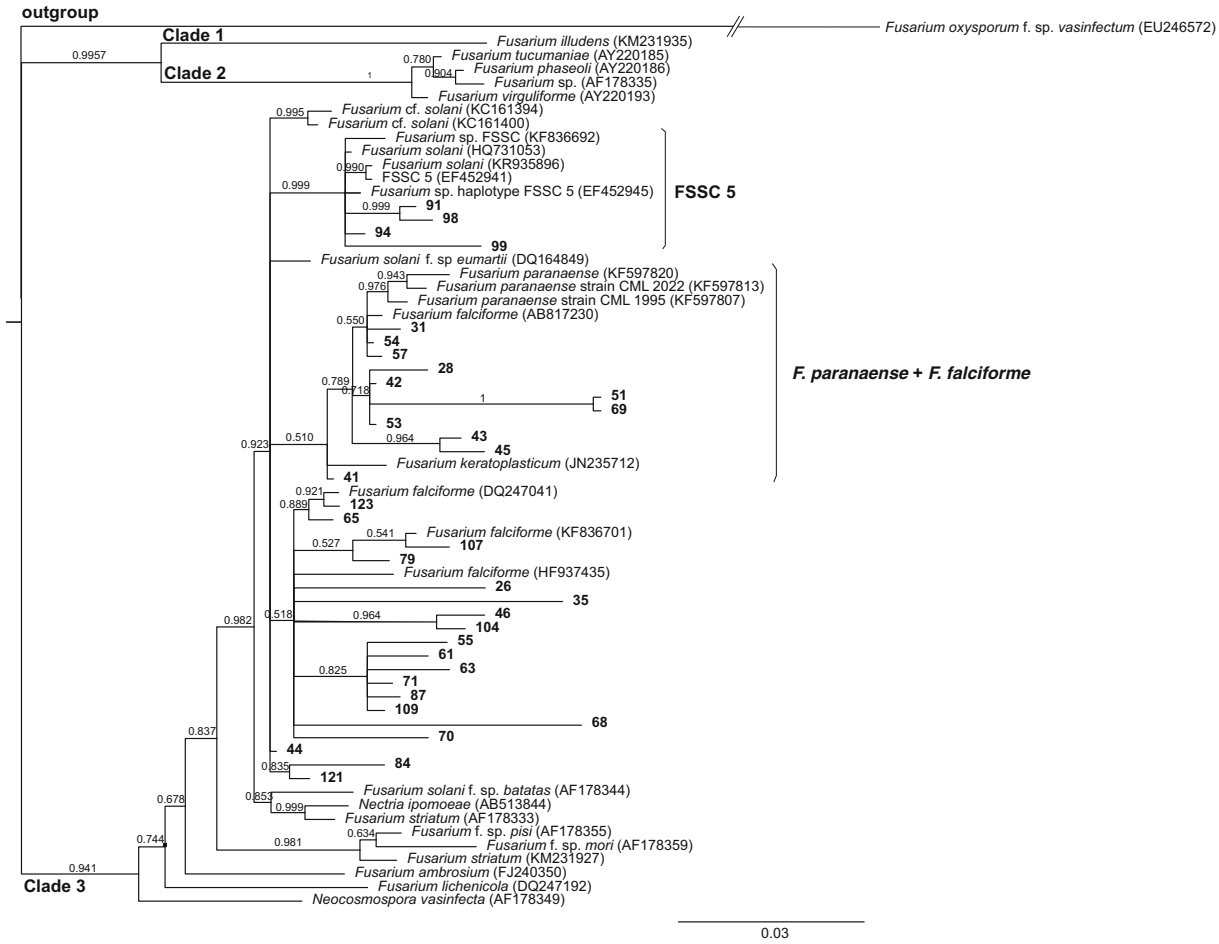

Fig. 4 Phylogenetic tree based on 34 EF1-α sequences recovered from *Fusarium solani* isolates and 30 control sequences retrieved from GenBank. The tree was constructed using the HKY + G substitution model for Bayesian analysis. The numbers above the branches indicate node support (posterior probability). The scale bar represents the number of substitutions per site. Sequences obtained in this study are shown in bold. *Fusarium solani* f. sp. *vasinfectum* was used as outgroup

based on the Mantel test was observed between morphological and molecular data, suggesting that the approaches revealed different levels of genetic diversity.

All of the isolates identified as FSSC had morphological traits in accordance with previous reports (Booth 1971; Nalim et al. 2011; Chehri et al. 2014; Costa et al. 2015; Schroers et al. 2016). Of the 74 FSSC isolates obtained from tissue, only 45 (60%) were pathogenic to tobacco plants. Three morphotypes we differentiate based on morphological characters, and most of the highly pathogenic isolates were included within morphotypes I and III. The most distinctive characters of both morphotypes were macroconidia shape and number of septa, microconidia shape and sporodochia colour. We assume that morphotype II belongs to *Fusarium falciforme* according to previous reports (Chehri et al. 2015). However, the molecular characterization of the isolates does not support this assumption and it would be necessary to include other loci to test this hypothesis.

Based on EF1-α sequence analysis, all of the FSSC isolates were included in the same cluster (clade 3) in agreement with previous reports (O'Donnell et al. 2008). Clade 3 was initially described as including clinically important species (Zhang et al. 2006; O'Donnell et al. 2008). However, the inclusion of soil and plant isolates, such as tree bark, rice, watermelon and mango, within this clade was later reported (O'Donnell et al. 2008; Short et al. 2013). This association of clinically important species with agricultural environments was also found in soybean (Chitrampalam and Nelson 2016) and

tobacco (Chehri et al. 2015). Given the great aggressiveness of the isolates included in clade 3, since they are associated with different hosts and/or environments, they were described as opportunistic or facultative pathogens (Chitrampalam and Nelson 2016). Many species of *Fusarium* are opportunistic or weak pathogens capable of attacking only plants that were weakened previously by certain other stress factors, such as those induced by the environment, such as poor soil drainage and presence of nematodes. The FSSC isolates collected in this study were able to induce root rot in tobacco plants and might be endemic soil microbiota in tobacco fields and possibly in most fields in this area. However, a more thorough study including isolates obtained from other hosts showing root rot symptoms would be necessary to confirm this hypothesis. In addition, a more structured sampling of the isolates in different regions would make it possible to identify the effect of edaphic and climatic conditions on the population dynamics of the pathogen and to elucidate the relationship of *Fusarium* spp. inoculum density with the incidence of tobacco root rot and wilt in the fields of the studied region.

The information generated in the present study concerning FOSC and FSSC provides, for the first time, information on the variability of the pathogen associated with tobacco wilt and rot root in northwestern Argentina. The generation of specific molecular markers for *formae specialis* would enable the early and selective detection of the pathogen, enhancing the disease's prevention at all stages of tobacco production. This work contributes to the development of sustainable management strategies, such as plant resistance, sanitation, rotation, plant nutrition, nematode management, and fumigation or biofumigation in tobacco production.

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Compliance with ethical standards

Conflict of interest The authors state that there are no conflicts of interest.

Human and animal rights This article does not contain any studies with human or animal subjects.

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