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Phylogenetic analyses and toxigenic profiles of *Fusarium equiseti* and *Fusarium acuminatum* isolated from cereals from Southern Europe

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ABSTRACT

Fusarium equiseti and *Fusarium acuminatum* are toxigenic species that contaminate cereal crops from diverse climatic regions. They are common in Spanish cereals. The information available on their phylogenetics and toxigenic profiles is, however, insufficient to assist risk evaluation. In this work, phylogenetic analyses were performed using partial sequences of the translation elongation factor gene (*EF-1α*) of *F. equiseti* and *F. acuminatum* strains isolated from barley and wheat from Spain and other countries. The Northern and Southern European *F. equiseti* strains largely separated into two phylogenetically distinct clusters. This suggests the existence of two distinct populations within this species, explaining its presence in these regions of markedly different climate. Production of type A and B trichothecenes by the Spanish strains, examined in wheat cultures using a multitoxin analytical method, indicated that *F. equiseti* could produce deoxynivalenol and nivalenol and other trichothecenes, at concentrations that might represent a significant risk of toxin contamination for Southern European cereals. *F. acuminatum* showed low intraspecific genetic variability and 58% of the strains could produce deoxynivalenol at low level. Neither species was found to produce T-2 or HT-2 toxins. The present results provide important phylogenetic and toxigenic information essential for the accurate prediction of toxigenic risk.

1. Introduction

Cereals are a dietary staple in most temperate regions. Unfortunately, they can become colonised by *Fusarium*, often resulting in severe crop disease, strongly reduced yields, and the accumulation of secondary metabolites toxic to humans and animals. Fusarium head blight (FHB) of small grain cereals is a disease complex that involves several *Fusarium* species causing largely indistinguishable symptoms. The species predominantly associated with FHB in Europe are *Fusarium graminearum*, *Fusarium avenaceum* and *Fusarium poae* (Nicholson et al., 2003; Somma et al., 2010; Xu et al., 2008). Less frequently isolated species are *Fusarium tricinctum*, *Fusarium sporotrichioides*, *Fusarium equiseti*, *Fusarium langsethiae* and *Fusarium culmorum* (Kosiak et al., 2003; Logrieco et al., 2003; Xu et al., 2005). Other species encountered sporadically include *Fusarium acuminatum*, *Fusarium subglutinans*, *Fusarium solani*, *Fusarium oxysporum*, *Fusarium semitectum*, *Fusarium verticillioides* and *Fusarium proliferatum* (Logrieco et al., 2003). Climate change scenarios predict increasing temperatures and variations in water availability could induce changes in the profile of FHB species on cereals. Since each species has a characteristic mycotoxin profile, the risk of mycotoxin contamination of cereals might also change (Miraglia et al., 2009).

F. equiseti is a cosmopolitan fungus distributed across regions with cool through to hot and arid climates (Leslie and Summerell, 2006). It behaves as a soil saprophyte associated with rotting fruit and other decaying plant material, and as a pathogen of a wide range of crops. This species is often detected in Norwegian cereals (Kosiak et al., 2005), but it is also common in Southern Europe (Logrieco et al., 2003), particularly in Spain (Jurado et al., 2006a; Marín, 2010; Soldevilla et al., 2005). Further, it belongs to the so-called *Fusarium incarnatum–F. equiseti* species complex, a genetically highly diverse group (O'Donnell et al., 2009) the members of which are associated with human disease.

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F. acuminatum is widely distributed around the world, although mainly in temperate regions. It behaves as a soil saprophyte but is also found associated with the roots and crowns of plants (Leslie and Summerell, 2006; Pitt and Hocking, 2009). Its presence has recently been reported in Southern Europe, particularly Spain (Marín, 2010).

Both *F. equiseti* and *F. acuminatum* have been reported trichothecene producers (Adejumo et al., 2007; Kosiak et al., 2005; Logrieco et al., 1992). Trichothecenes are potent inhibitors of protein synthesis in eukaryotic cells (Brown et al., 2001), a consequence (mainly) of their interfering with peptidyl transferase activity. They cause different acute and severe diseases in humans and animals depending on the type of trichothecene ingested (Trenholm et al., 1989). *F. equiseti* produces trichothecenes such as T-2 toxin, 4-acetylnivalenol (FUS-X), deoxynivalenol (DON), nivalenol (NIV) and scirpentriol or its mono- and diacetyl derivatives (MAS and DAS) (Kosiak et al., 2005; Leslie and Summerell, 2006). It has been also reported to produce butenolide, beauvericin, equisetin (EQ), fusarochromanone (FUSCHR) and zearalenone (ZEA) (Leslie and Summerell, 2006). *F. acuminatum* has been reported to produce trace levels of trichothecene toxins such as diacetoxyscirpenol (DAS), monoacetoxyscirpenol (MAS), neosolaniol (NEO) and HT-2 toxin (HT-2) (Adejumo et al., 2007; Wing et al., 1993, 1994). Additionally, it produces enniatin B, steroids and moniliformin (Leslie and Summerell, 2006).

The identification of *Fusarium* species traditionally relies on the detection of morphological and physiological features. However, discrimination among similar species is often difficult. Neither do such methods detect intraspecific variability. Fortunately, phylogenetic analyses that make use of DNA sequence data have made significant contributions to our understanding of the systematics of *Fusarium*, providing species boundaries that are essential for establishing inter- and intraspecific relationships with respect to toxin profiles (Jurado et al., 2006a; Kristensen et al., 2005; Mirete et al., 2004; O'Donnell et al., 2009). Additionally, these techniques have provided the basis necessary for developing rapid, specific and accurate diagnostic methods based on PCR. These can be used to predict mycotoxin risk, providing the information necessary for early control strategies to be adopted (Jurado et al., 2005, 2006b; Knutsen et al., 2004; Konstantinova and Yli-Mattila, 2004). Several genomic sequences have been used to analyse intraspecific variability in *Fusarium*, including intron regions of histone coding genes, the β-tubulin gene (*βTUB*), the calmodulin gene (O'Donnell et al., 1998a; Steenkamp et al., 2002), and the translation elongation factor gene *EF*-1α (O'Donnell et al., 1998b, 2000). *EF*-1α gene has been used as a single-locus identification tool and is a suitable genetic marker for discriminating between *Fusarium* species (Geiser et al., 2004).

The literature contains little information regarding the phylogenetics of *F. acuminatum*. However, several reports suggest the existence of intraspecific diversity within *F. equiseti* (Kosiak et al., 2005; Kristensen et al., 2005; Jurado et al., 2006a). Kosiak et al. (2005) reported the existence of two groups with differences in morphological features and toxin production. However, the strains used in their study were basically all from Northern Europe. Later, a preliminary phylogenetic analysis conducted with *F. equiseti* strains from both Southern Europe (mostly isolated from Spanish cereals) and Northern Europe showed them to occupy one of two different clusters (Jurado et al., 2006a). The toxigenic profiles of the Spanish isolates were not analysed.

The aim of the present work was: (i) to examine, using partial *EF*-1α gene sequences, the phylogenetics of *F. equiseti* and *F. acuminatum* strains isolated from cereal-producing regions in Spain, and (ii) to analyse the toxin profile of these Spanish strains in relation to their phylogenetics and those of Northern European strains.

2. Material and methods

2.1. Fusarium strains

Eight *F. equiseti* and 36 *F. acuminatum* strains were isolated from barley cultivated in two different regions of Spain (Castilla y Léon and Castilla-La Mancha) in 2006 (Table 1). Fungal cultures were maintained on potato dextrose agar medium (PDA) (Scharlau Chemie, Barcelona, Spain) at 4 °C and stored as spore suspensions in 15% glycerol in our collection at the Complutense University of Madrid (UCM).

2.2. DNA extraction and PCR amplification of a partial sequence of *EF-1α*

Genomic DNA extractions of pure cultures of eight strains of *F. equiseti* and 36 strains of *F. acuminatum* were undertaken using three mycelium disks excised from 5 to 7 day-old PDA plate cultures, and making use of the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A partial sequence of the *EF*-1α gene was obtained by PCR using the primers and protocol described elsewhere (O'Donnell et al., 1998b). PCR-amplified fragments were purified using the UltraCleanTM PCR Clean-UpTM kit (MoBio Laboratories Inc., USA) according to the manufacturer's instructions. Automated sequencing of both DNA strands was performed with the *EF*-1α pair of primers using a 3730 DNA Analyzer and the Big Dye® Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, USA) at the UCM Genomic Unit. The sequences were corrected using Chromas v 1.43 software (Brisbane, Australia) and analysed and edited using Bioedit Sequence Alignment Editor v 7.0.9.0 software (Hall, 1999).

2.3. Phylogenetic analyses

Using PAUP v 4.0 b10 software (Swofford, 2003), individual maximum-parsimony (MP) phylogenetic analyses were performed for *F. equiseti* and *F. acuminatum* using the partial sequences of the *EF*-1α gene obtained. Additional sequences previously obtained in our laboratory (Jurado et al., 2006a) were included, as were others retrieved from databases, along with sequences for species closely related to *F. equiseti* and *F. acuminatum* (*Fusarium scirpi* and *F. avenaceum* respectively). A total of 70 strains for *F. equiseti* and 63 for *F. acuminatum* were employed in the phylogenetic analyses (Table 1). An *F. graminearum* strain (AF212461) was used as an outgroup in both the *F. equiseti* or *F. acuminatum* MP analyses. Gaps were coded as missing data and were excluded from analyses. Unweighted parsimony analyses were performed on the individual data sets using the heuristic search option with 1000 random additional sequences with tree bisection-reconnection (TBR) branch swapping. Clade stability was assessed via 1000 bootstrap replications (Hillis and Bull, 1993). Additionally, phylogenetic analyses based on Neighbor Joining were performed using the Jukes–Cantor model (Jukes and Cantor, 1969).

Nucleotide diversities estimated as the average number of differences per site between two homologous sequences (π) were calculated using DnaSp v 4.50.3 software (Rozas et al., 2008), employing Eq. (10.5) of Nei (1987). Molecular diversities were calculated in each species for both the total sequence dataset and for the sequence dataset of each cluster. Gaps present in the alignment were excluded from analysis.

To determine the proportion of the total genetic variance attributable to inter-population differences, Wright's Fst statistic was determined via analysis of molecular variance (AMOVA) using the Arlequin v 3.01 software (Excoffier et al., 2005). The Wright's Fst statistic for the different groups was estimated using DNAsp v

Table 1
Fusarium strains used in this study, indicating host, origin and accession number of the *EF*-1α partial sequences. The same isolate name, followed by a number, were given to different strains grouped in the same cluster. Strains in bold were obtained in the present work. The same accession number was given to identical *EF*-1α partial sequences for the strains obtained in this work, followed by a further identification number.

Isolate name	Strain	Host	Origin	Accession number
F. equiseti				
EQU1	**H3SA.042**	Barley	Spain	JF496568[j]
EQU2	**C1SA.060**	Barley	Spain	JF496569[j]
EQU3	**C1SA.063**	Barley	Spain	JF496570[j]
EQU4	**C3RA.065**	Barley	Spain	JF496571[j]
EQU5	**C1SA.073**	Barley	Spain	JF496568-1[j]
EQU6	**D24SZ.090**	Barley	Spain	JF496572[j]
EQU7	**C1SA.102**	Barley	Spain	JF496573[j]
EQU8	**C3SH.103**	Barley	Spain	JF496574[j]
EQU9	H2-2-5B (Type II)	Durum wheat	Spain	JF496575[a,j]
EQU10	L1-2-2 (Type II)	Durum wheat	Spain	JF496575-1[a,j]
EQU11	L3-1-2J (Type II)	Durum wheat	Spain	JF496576[a,j]
EQU12	U6-1-1 (Type II)	Durum wheat	Spain	JF496577[a,j]
EQU13	VI01066 (Type II)	Soil	Malta	AJ543571[b]
EQU14	VI01067 (Type I)	Beet	Denmark	AJ543558[b]
EQU15	VI01068 (Type I)	Barley	Sweden	AJ543557[b]
EQU16	VI01069 (Type I)	Onion	Denmark	AJ543561[b]
EQU17	VI01070 (Type I)	Onion	Denmark	AJ543562[b]
EQU18	VI01071 (Type I)	Wheat	Denmark	AJ543563[b]
EQU19	VI01072 (Type I)	Barley	Denmark	AJ543559[b]
EQU20	VI01079 (Type I)	Wheat	Norway	AJ543564[b]
EQU21	VI01084 (Type I)	Oats	Norway	AJ543565[b]
EQU22	VI01087 (Type II)	Wheat	Norway	AJ543570[b]
EQU23	VI01093 (Type I)	Barley	Norway	AJ543566[b]
EQU24	VI01095 (Type I)	Barley	Norway	AJ543560[b]
EQU25	VI01096 (Type I)	Barley	Norway	AJ543567[b]
EQU26	VI01104 (Type I)	Wheat	Norway	AJ543568[b]
EQU27	VI01105 (Type I)	Oats	Norway	AJ543569[b]
EQU30	11_ZP_2	Soil	Canada	DQ842055
EQU31	34/2.1.1	Esparto grass	Spain	DQ854854[c]
EQU31-1	45/1.2.1	Esparto grass	Spain	DQ854855[c]
EQU33	DAOM194187	Wheat	Canada	DQ842084
EQU35	DAOM232362	Barley	Canada	DQ842096
EQU36	DAOM236361	Wheat	Canada	DQ842099
EQU38	G4_2_QC_ND_3_2_1_2	Soybean	Canada	DQ842101
EQU43	11_ZP_1	Ginseng soil	Canada	DQ842054
EQU44	16_ZP_2	Wheat	Canada	DQ842058
EQU46	22_ZP_2	Straw	Canada	DQ842061
EQU48	2_ZP_2	Straw	Canada	DQ855945
EQU48-1	5_ZP_1	Straw	Canada	DQ842077
EQU48-2	6_ZP_2	Straw	Canada	DQ860274
EQU48-3	9_ZP_2	Straw	Canada	DQ842080
EQU48-4	19_ZP_2	Straw	Canada	DQ842059
EQU48-5	27_ZP_2	Straw	Canada	DQ842063
EQU49	7_ZP_1	Ginseng root	Canada	DQ842078
EQU49-1	45_ZP_2	Ginseng root	Canada	DQ842076
EQU50	60	Sugar beet	France	FJ939674[d]
EQU52	113	Sugar beet	US	FJ939678[d]
EQU53	90	Sugar beet	Sweden	FJ939675[d]
EQU53-1	115	Sugar beet	Sweden	FJ939679[d]
EQU58	157	Sugar beet	Germany	FJ939684[d]
EQU60	149	Sugar beet	Germany	FJ939680[d]
EQU60-1	150	Sugar beet	Germany	FJ939681[d]
EQU60-2	151	Sugar beet	Germany	FJ939682[d]
EQU60-3	159	Sugar beet	Germany	FJ939685[d]
EQU60-4	160	Sugar beet	Germany	FJ939683[d]
EQU61	174	Sugar beet	Italy	FJ939686[d]
EQU62	DAOM194188	Wheat	Canada	DQ842085
EQU64	DAOM215463	Corn	Canada	DQ842094
EQU65	DAOM232364	Wheat	Canada	DQ842098
EQU66	GLS2	Rice	Italy	GQ848542[e]
EQU68	NRRL20697	Beet	Chile	GQ505594[f]
EQU69	NRRL26419	Soil	Germany	GQ505599[f]
EQU70	NRRL36136	–	–	GQ505644[f]
EQU71	NRRL36321	Soil	Netherlands	GQ505647[f]
EQU72	NRRL36466	Potato peel	Denmark	GQ505356[f]
EQU73	NRRL43636	Dog	US	GQ505663[f]
F. scirpi				
SCI1	NRRL36478	Pasture soil	Australia	GQ505654[f]
SCI2	NRRL29134	Pasture soil	Australia	GQ505605[f]
SCI3	NRRL26922	Soil	France	GQ505601[f]
SCI4	NRRL13402	Pine soil	Australia	GQ505592[f]
F. graminearum				
GRA1	NRRL29169	Wheat	US	AF212461[g]
F. acuminatum				
ACU1	**C1RA.007**	Barley	Spain	JF496578[j]
ACU2	**C1RA.038**	Barley	Spain	JF496578-1
ACU3	**C1RA.045**	Barley	Spain	JF496579
ACU4	**C1RA.066**	Barley	Spain	JF496580
ACU5	**C1RA.067**	Barley	Spain	JF496580-1
ACU6	**C1RA.079**	Barley	Spain	JF496578-2
ACU7	**C1SA.047**	Barley	Spain	JF496580-2
ACU8	**C2SA.024**	Barley	Spain	JF496578-3[j]
ACU9	**C2SA.035**	Barley	Spain	JF496578-4
ACU10	**C3RA.022**	Barley	Spain	JF496580-3[j]
ACU11	**C3RA.032**	Barley	Spain	JF496581[j]
ACU12	**C3RA.037**	Barley	Spain	JF496581-1
ACU13	**C3RA.075**	Barley	Spain	JF496582
ACU14	**C3SA.013**	Barley	Spain	JF496580-4[j]
ACU15	**C3SA.048**	Barley	Spain	JF496578-5
ACU16	**C3SA.049**	Barley	Spain	JF496583
ACU17	**H1SA.014**	Barley	Spain	JF496578-6[j]
ACU18	**H1SA.017**	Barley	Spain	JF496581-2[j]
ACU19	**H1SA.052**	Barley	Spain	JF496578-7
ACU20	**H1SA.053**	Barley	Spain	JF496584
ACU21	**H2RA.033**	Barley	Spain	JF496585[j]
ACU22	**H2RA.054**	Barley	Spain	JF496585-1
ACU23	**H2SA.019**	Barley	Spain	JF496585-2[j]
ACU24	**H2SA.020**	Barley	Spain	JF496585-3[j]
ACU25	**H2SA.043**	Barley	Spain	JF496580-5
ACU26	**H2SA.044**	Barley	Spain	JF496585-4
ACU27	**H2SA.077**	Barley	Spain	JF496580-6
ACU28	**H2SA.059**	Barley	Spain	JF496580-7
ACU29	**H3RA.031**	Barley	Spain	JF496578-8[j]
ACU30	**H3RA.039**	Barley	Spain	JF496580-8
ACU30-1	DAOM170306	Rice	India	DQ855948
ACU30-2	DAOM194173	–	Canada	EF521137
ACU30-3	DAOM194174	–	Canada	EF521138
ACU30-4	DAOM213304	Grass	Canada	DQ842086
ACU30-5	DAOM215457	Corn	Canada	DQ842091
ACU30-6	DAOM230348	–	Canada	EF521139
ACU30-7	DAOM238674	–	Canada	EF521134
ACU30-8	DAOM238675	–	Canada	EF521136
ACU30-9	DAOM238676	–	Canada	EF521135
ACU30-10	DAOM238678	–	Canada	EF521133
ACU30-11	F30	*Astragalus racemosus*	–	EF531698
ACU30-12	R-2165	Lavender	France	FJ154733[h]
ACU30-13	R-6678	*Hibiscus*	Egypt	FJ154735[h]
ACU30-14	R-7408	Morning glory	USA	FJ154737[h]
ACU30-15	NRRL 54218	–	–	HM068316
ACU30-16	NRRL 36147	Human bronchial secretion	–	GQ505420[f, i]
ACU30-17	NRRL 45994	Cloaca	USA	GQ505432[e]
ACU31	**H3RA.040**	Barley	Spain	JF496585-5
ACU31-1	E5	–	–	GQ922925
ACU31-2	NRRL 54210	–	–	HM068308
ACU31-3	NRRL 54211	–	–	HM068309
ACU31-4	NRRL 54212	–	–	HM068310
ACU31-5	NRRL 54214	–	–	HM068312
ACU31-6	NRRL 54215	–	–	HM068313
ACU31-7	NRRL 54217	–	–	HM068314
ACU32	**H3RA.056**	Barley	Spain	JF496578-9
ACU33	**H3RA.078**	Barley	Spain	JF496578-10
ACU34	**H3SA.018**	Barley	Spain	JF496578-11[j]
ACU35	**H3SA.050**	Barley	Spain	JF496578-12
ACU36	**H3SA.051**	Barley	Spain	JF496578-13

(*continued on next page*)

Table 1 (*continued*)

Isolate name	Strain	Host	Origin	Accession number
ACU50	R-6934	Soil	Australia	FJ154736[h]
ACU52	R-9382	Chrysanthemum	China	FJ154738[h]
ACU59	NRRL 54216	–	–	HM068314
F. avenaceum				
AVE1	VI01057	Wheat	Norway	AJ543518[b]

[a] Jurado et al., 2006a.
[b] Kristensen et al., 2005.
[c] Maciá-Vicente et al., 2008.
[d] Nitschke et al., 2009.
[e] Amatulli et al., 2010.
[f] O'Donnell et al., 2009.
[g] O'Donnell et al., 2000.
[h] Nalim et al., 2009.
[i] O'Donnell et al., 2010.
[j] Isolates tested for trichothecene production.

4.50.3 software (Rozas et al., 2008) using Eq. (3) of Hudson et al. (1992) to take into account the genetic distances between the groups in both species.

To show more clear dendrograms for each species, the MP analyses were repeated eliminating some of the isolates that occurred in the same cluster in the first analyses, and which either had identical sequences or had differences of up to five singletons (non-parsimonious informative sites). These isolates were given the same name followed by different numbers. In both species, the dendrograms obtained in both the first and second MP analyses showed identical topologies. The same was also recorded when using the Jukes-Cantor model.

2.4. Growth conditions for toxin detection

Twelve strains of *F. equiseti*, including four isolated in a previous study (Jurado et al., 2006a) and 12 strains of *F. acuminatum* isolated in the present work were tested for trichothecene production (Table 1). Fifty gram samples of autoclaved wheat kept at a moisture content of 45% for one night were inoculated with a small quantity of each strain. Cultures were incubated at 25 °C under fluorescent light (12 h photoperiod) for four weeks, then dried at 48 °C for 24 h and ground to a fine powder. Control (non-inoculated) wheat was treated in the same way.

2.5. Chemical analysis

A multitoxin analytical method, combining high-performance liquid chromatography (HPLC), atmospheric pressure chemical ionisation (APCI) and triple quadrupole tandem mass spectrometry (LC-MS/MS) under the selected reaction monitoring (SRM) mode, was used to detect the following mycotoxins: NIV, DON and its derivatives, FUS-X, NEO, HT-2, T-2 and DAS. Standards of these toxins were purchased from Sigma–Aldrich (Milan, Italy) and stored at 4 °C in the dark. The details of the procedure have been previously described (Somma et al., 2010). The limits of detection for NIV, DON, FUS-X, NEO, HT-2, T-2, DAS were 0.0033, 0.0005, 0.0015, 0.0033, 0.0033, 0.001, 0.0025 and 0.001 μg/g respectively.

3. Results

3.1. Phylogenetic analyses

The amplification of *EF*-1α produced a sequence of 616 bp and 612 bp for *F. equiseti* and *F. acuminatum* respectively. In *F. equiseti*, the total number of nucleotides analysed, excluding indels, was 584. Of these, 95 nt were polymorphic sites and 60 were parsimony-informative sites. In the case of *F. acuminatum*, excluding indels, the total number of nucleotides analysed was 589. Of these, 11 nt were polymorphic sites and 9 nt were parsimony-informative sites. Nucleotide diversities per site (π) were 0.02846 ± 0.00197 (standard deviation) and 0.00433 ± 0.00073 for the *F. equiseti* and *F. acuminatum EF*-1α sequences respectively.

Results from the first (data not shown) and second MP phylogenetic analyses (Figs. 1 and 2) generated the same *EF*-1α genealogies for *F. equiseti* and for *F. acuminatum*. Figs. 1 and 2 show the bootstrap 50% majority consensus trees based on MP analysis plus the consistency (CI), retention (RI) and rescaled consistency (CR) indices. For *F. equiseti*, both types of phylogenetic analysis (MP and Jukes-Cantor model [data not shown for the latter]) revealed three distinct clusters of isolates corresponding to *F. equiseti* types I and II and *F. scirpi*. The genetic distance, in terms of Fst estimated using DNAsp software, between *F. equiseti* type I and type II was 0.87166, between *F. equiseti* type II and *F. scirpi* it was 0.56589, and between *F. equiseti* type I and *F. scirpi* it was 0.63569. The Fst fixation index value, determined by AMOVA for the three groups as a whole was 0.90646. The *F. equiseti* type I and type II groups were homogeneous and showed low intra-group variability. The nucleotide diversity per site within these groups was $\pi = 0.00359 \pm 0.00104$ and $\pi = 0.00607 \pm 0.00110$ respectively. Variability was higher among the strains of the *F. scirpi* group ($\pi = 0.03026 \pm 0.00343$). The results did not support any influence of the host. However, the Northern European and Southern European *F. equiseti* strains largely separated into the type I and type II phylogenetic clusters (Fig. 1), with 16 of the 17 Spanish strains in the type II cluster and all 20 Northern European strains in the type I cluster. Strains from other locations (Table 1) fell into either the type I, type II or *F. scirpi* clusters.

The phylogenetic analyses revealed three defined groups for *F. acuminatum* (A, B and C) (Fig. 2) plus a set of sequences that did not belong to any of the above clusters. The Fst fixation index obtained for this species was 0.84570. The intraspecific variability for the *EF*-1α sequences was very low, with π values between groups A, B and C less than 0.002 (data not shown).

3.2. Toxin production

To characterize the chemical profile of *F. equiseti* and *F. acuminatum* toxin production, 12 isolates of each species were examined (Table 2). None of the 12 isolates of *F. equiseti* tested produced 3/15Ac-DON, and none of the 12 *F. acuminatum* isolates produced NIV, 3/15Ac-DON, DON derivatives, NEO or DAS. T-2 and HT-2 were absent in all cultures of both species. In *F. equiseti*, NIV, DON, DON derivatives, FUS-X, NEO and DAS were produced by 3 (25%), 10 (83.33%), 4 (33.33%), 5 (41.67%), 3 (25%) and 3 (25%) isolates respectively (Table 3). Production levels were very variable, ranging from 227 to 7005 μg/kg for NIV, from 46 to 1035 μg/kg for DON, from 367.5 to 10,150 μg/kg for DON derivatives, from 32.5 to 16,750 μg/kg for FUS-X, from 38.1 to 1665 μg/kg for NEO, and from 9.65 to 197 μg/kg for DAS. In *F. acuminatum* the production of DON and FUS-X was detected in 7 (58.33%) and 1 (8.33%) isolate respectively. The production level was constant, ranging from 45.1 to 61.1 μg/kg for DON, and from 32.5 to 16,750 μg/kg for FUS-X (Table 3). Three isolates of *F. equiseti* produced both type A (NEO and DAS) and type B (NIV, DON, 3/15Ac-DON, DON derivatives and FUS-X) trichothecenes, whereas seven produced only type B trichothecenes. Only two isolates produced no type of trichothecene. In *F. acuminatum*, seven isolates produced only DON, and only one of these produced FUS-X. Five isolates did not produce any of the trichothecenes analysed (Table 2).

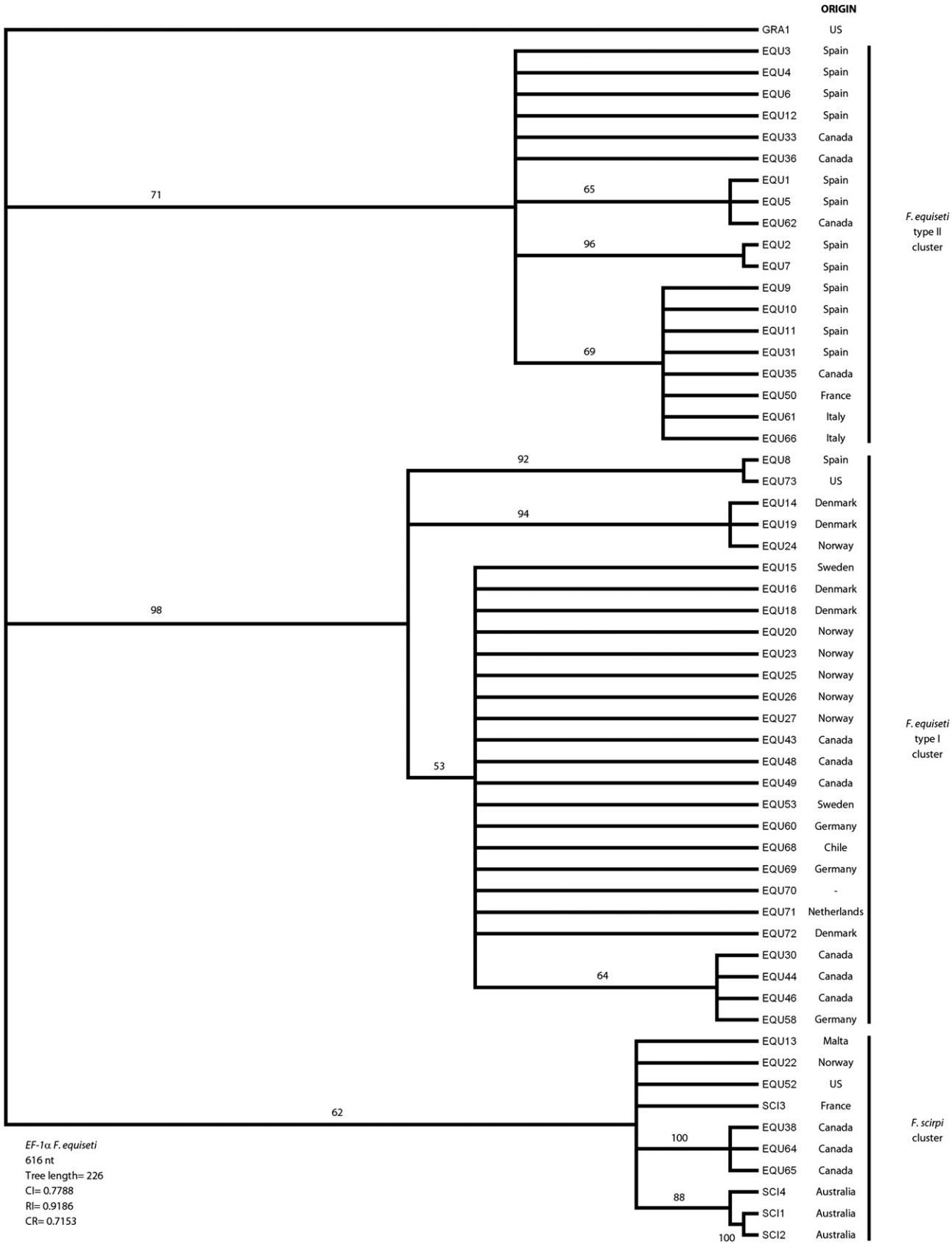

Fig. 1. Bootstrap 50% majority-rule consensus tree based on MP analysis of *F. equiseti* isolates. CI: Consistency index, RI: Retention index, CR: Rescaled consistency index.

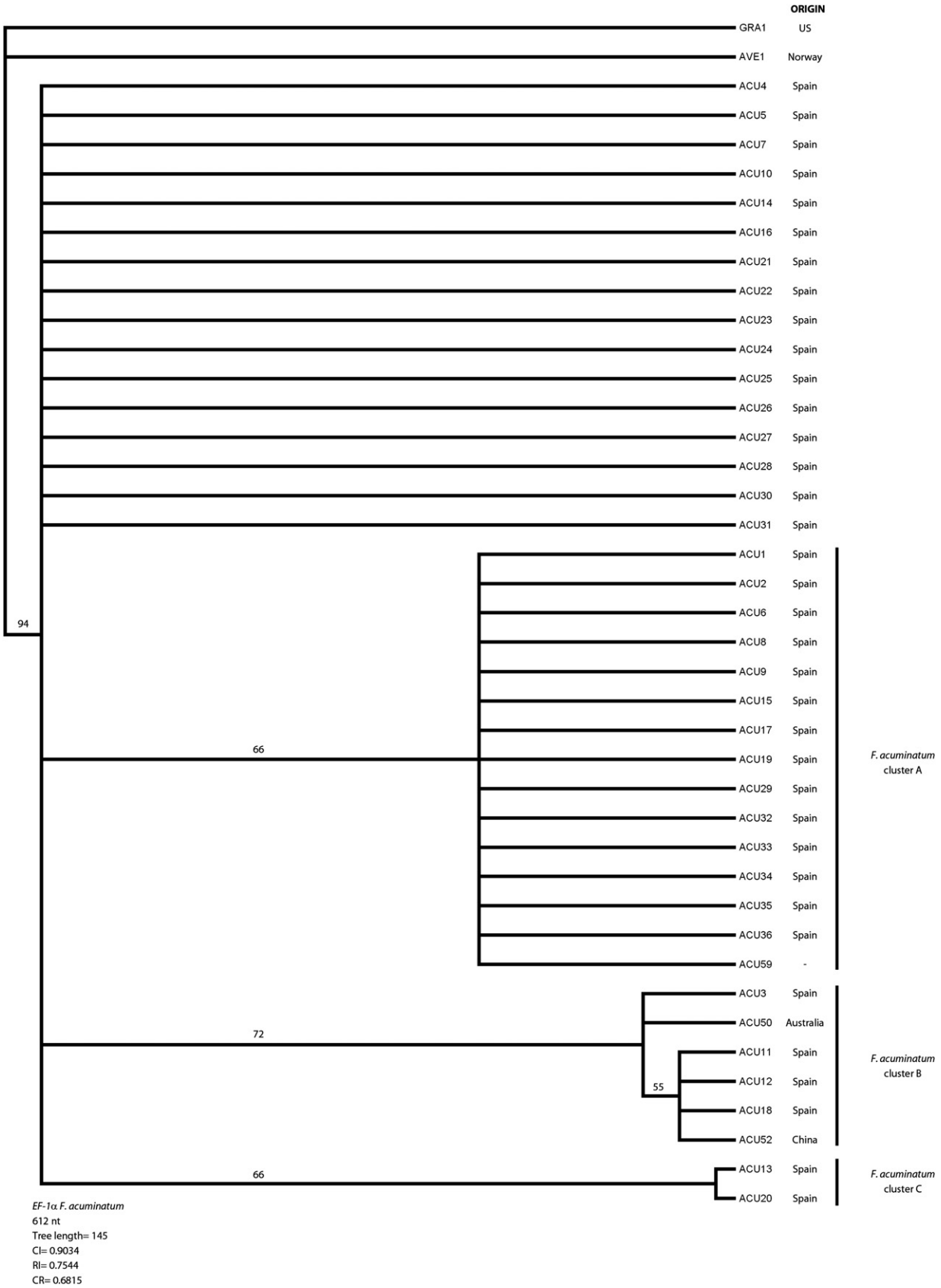

Fig. 2. Bootstrap 50% majority-rule consensus tree based on MP analysis of *F. acuminatum* strains. CI: Consistency index, RI: Retention index, CR: Rescaled consistency index.

Table 2
Toxin production by the *F. equiseti* and *F. acuminatum* strains isolated from Spanish cereals. NIV: nivalenol; DON, deoxynivalenol; FUS-X, 4-acetylnivalenol; NEO, neosolaniol; DAS, diacetoxyscirpenol; DON derivatives; n.d., not detected; 1, type A (NEO and DAS) and type B (NIV, DON, 3/15Ac-DON, DON derivatives and FUS-X) trichothecene producer; 2, type A trichothecene producer; 3, type B trichothecene producer; 4 non-trichothecene producer.

Isolate Name	Toxin production (μg/kg)							Toxin profile
	Type B trichothecene					Type A trichothecene		
	NIV	DON	3/15Ac-DON	DON derivatives	FUS-X	NEO	DAS	
F. equiseti								
EQU1 (type II)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4
EQU2 (type II)	n.d.	46	n.d.	n.d.	n.d.	n.d.	n.d.	3
EQU3(type II)	n.d.	62.4	n.d.	n.d.	n.d.	n.d.	n.d.	3
EQU4 (type II)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4
EQU5 (type II)	119.5	67.01	n.d.	118.5	121	n.d.	n.d.	3
EQU6 (type II)	n.d.	61.5	n.d.	n.d.	n.d.	n.d.	n.d.	3
EQU7 (type II)	7005	1035	n.d.	10150	16750	1665	197	1
EQU8 (type I)	n.d.	52.1	n.d.	n.d.	n.d.	n.d.	n.d.	3
EQU9 (type II)	n.d.	116.5	n.d.	578.5	2320	60.65	15.9	1
EQU10 (type II)	227	62.3	n.d.	367.5	1115	38.1	9.65	1
EQU11(type II)	n.d.	56.55	n.d.	n.d.	n.d.	n.d.	n.d.	3
EQU12 (type II)	n.d.	60.35	n.d.	n.d.	32.5	n.d.	n.d.	3
F. acuminatum								
ACU1	n.d.	58.65	n.d.	n.d.	n.d.	n.d.	n.d.	3
ACU8	n.d.	48.4	n.d.	n.d.	n.d.	n.d.	n.d.	3
ACU10	n.d.	60.11	n.d.	n.d.	46.7	n.d.	n.d.	3
ACU11	n.d.	61.1	n.d.	n.d.	n.d.	n.d.	n.d.	3
ACU14	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4
ACU17	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4
ACU18	n.d.	58.2	n.d.	n.d.	n.d.	n.d.	n.d.	3
ACU21	n.d.	45.1	n.d.	n.d.	n.d.	n.d.	n.d.	3
ACU23	n.d.	58.35	n.d.	n.d.	n.d.	n.d.	n.d.	3
ACU24	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4
ACU29	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4
ACU34	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4

4. Discussion

Accurate predictions of mycotoxigenic risk basically rely on the correct identification of the fungal species in agrofood products and the determination of the toxigenic profiles of strains from different origins that might adequately represent the species. Conventional methods for identifying fungal species may overlook diversity at intraspecific level, i.e., the existence of cryptic populations. Phylogenetic analyses, however, provide a useful tool for revealing such populations, they can efficiently assist in the identification of fungal strains, and permit toxigenic profiles to be associated with particular populations or species. In the present work this approach was used to investigate a sample of isolates from two *Fusarium* species, *F. equiseti* and *F. acuminatum*, which often occur in cereals grown in Spain, and which have not been characterized to date. Their phylogenetic analysis also included a representative sample of isolates of different origin in order to situate them within a wider geographical context, particularly that of Europe.

Table 3
Percentage of *F. equiseti* and *F. acuminatum* strains producing any of the toxins tested in this study, and the minimum and maximum quantities produced. NIV: nivalenol; DON, deoxynivalenol; FUS-X, 4-acetylnivalenol; NEO, neosolaniol; DAS, diacetoxyscirpenol; DON derivatives; n.d., not detected.

Toxin	Producer isolates (%)	Toxin production (μg/kg)	
		Minimum	Maximum
F. equiseti			
NIV	25	227	7005
DON	83.33	46	1035
DON derivatives	33.33	367.5	10150
FUS-X	41.67	32.5	16750
NEO	25	38.1	1665
DAS	25	9.65	197
F. acuminatum			
DON	58.33	75.1	61.1
FUS-X	8.33	46.7	46.7

The phylogenetic results for *F. equiseti* reveal the existence of wide genetic variability and two different clusters, type I and type II, that predominantly group the Northern and Southern European *F. equiseti* strains respectively. These results agree with those of a previous study by our group (Jurado et al., 2006a). Further, four out of five strains from Northern Europe described by Kosiak et al. (2005) fell into the type I cluster, while the other fell into the *F. scirpi* cluster. The existence of two distinct populations within this species might explain its presence in these two regions of markedly different climate. The genetic isolation of these two populations (which would depend greatly on the amount of sexual reproduction practised) may have occurred and genetic differences providing better adaptation to ecophysiological factors may have arisen in both. Although the life cycle of *F. equiseti* includes the perfect stage *Gibberella intrincans* (Leslie and Summerell, 2006), perithecia of *G. intrincans* have only been observed in laboratory experiments. Therefore, the predominant mode of reproduction of *F. equiseti* is considered to be asexual. If no sexual events occur between the two proposed populations, the different features and toxigenic profiles of each will persist. Additional studies would be useful for characterizing these two populations, including an examination of the ecophysiological characteristics of individuals from both populations and their relationship with their climatologically different origins.

Several studies indicate that *F. equiseti* strains can produce a wide array of toxins (Adejumo et al., 2007; Kosiak et al., 2005). In the present study, the toxigenic profile of the type II Spanish strains showed differences with respect to previously reported

strains from Northern Europe (Kosiak et al., 2005), which fell into the type I cluster. In neither set of strains, however, was the production of toxins T-2 nor HT-2 detected. Kosiak et al. (2005) reported *F. equiseti* strains to produce higher quantities of type A trichothecenes, no detectable levels of DON nor DON derivatives, but significant amounts of NIV and FUS-X. In the present study, the Spanish *F. equiseti* strains *did* produce DON, DON derivatives and the highly toxic NIV at higher levels. Over 80% of the strains produced at least DON and 25% produced NIV as well. Further, the type II population showed diversity among individuals regarding the set of toxins produced and the relative quantities manufactured. In any event, the low level of non-toxigenic strains (less than 20%), the importance of some of the toxins produced, and their wide occurrence in cereals, highlight the potential contribution of *F. equiseti* to the toxin risk associated with the consumption of Spanish cereals, as well as the need to design early detection and control strategies for this species. The PCR-based *F. equiseti* detection protocol (Jurado et al., 2005, 2006b) may be useful in this respect.

The topology of the dendrogram obtained, which included the closely related species *F. scirpi* as a reference, suggests that further studies are needed for clear species distinctions to be made. Indeed, the present results, and those of a recent study on the clinically important *F. incarnatum–F. equiseti* species complex involving the use of multilocus DNA sequence data (O'Donnell et al., 2009), show the strong variability of *F. equiseti* and *F. scirpi*. O'Donnell et al. (2009) concluded that the *F. incarnatum–F. equiseti* complex contained 28 phylogenetically distinct species in which both *F. equiseti* and *F. scirpi* were represented in two distinct clusters. These *F. equiseti* and *F. scirpi* strains were also included in the present phylogenetic study, and they clustered within *F. equiseti* type I and *F. scirpi* respectively. Unfortunately, it would seem that no isolate of *F. equiseti* type II was included in the study performed by O'Donnell et al. (2009). Further studies should examine a fully representative sample of the diversity of *F. equiseti* in order to obtain a reliably robust phylogeny.

Diversity was also found for *F. acuminatum*, with different clusters detected, although no relationship with host or geographic origin could be established (all these groups contained *F. acuminatum* strains isolated from Spanish barley). However, analyses of the *EF*-1α genomic sequence revealed less intraspecific variability than that recorded for *F. equiseti*, with a low number of parsimony-informative sites and little nucleotide diversity despite the different origins of the strains analysed. Thus, these results are suggestive of a quite homogenous population. This species is clearly phylogenetically distinct from other related species with similar morphological features within the *F. avenaceum*/*F. acuminatum*/*F. tricinctum* species complex (Harrow et al., 2010; Leslie and Summerell, 2006). Harrow et al. (2010) suggest that *F. acuminatum* may not be as abundant as *F. avenaceum*, probably as a consequence of biogeographic limitation, narrow host preferences and/or competitive disadvantages restricting its presence in different environments, and indicate that this might be related to the scant variability it shows. As mentioned above, a number of authors have reported different toxigenic profiles for *F. acuminatum*. However, to our knowledge, the production of DON by *F. acuminatum* has not been previously reported. In the present study seven out of 12 strains analysed were able to produce DON, although at a very low level. This suggests that this species should not be considered a DON non-producing species, although additional studies are needed to confirm this. The potential toxin risk of DON contamination should not, therefore, be dismissed. A study including both phylogenetic and toxin analyses are essential for accurate predictions of toxin risk since this could detect intraspecific variability that otherwise might be overlooked, and associate toxigenic profiles to groups/lineages/species.

In summary, the present results suggest the existence of two phylogenetically distinct populations of *F. equiseti* apparently associated with two different geographical/climatic regions: Northern and Southern Europe (type I and type II respectively). Toxin production by the type II population indicates that its members could pose a risk of cereal contamination in Southern Europe, particularly involving DON and NIV. In the case of *F. acuminatum*, although the potential DON risk is probably small, it should not be disregarded.

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