European Journal of Plant Pathology **110:** 515–523, 2004.

Differentiation of *Fusarium verticillioides* from banana fruits by IGS and EF-1α sequence analyses

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Key words: EF-1α, fumonisins, Gibberella fujikuroi, IGS, maize

Abstract

Fusarium verticillioides (*Gibberella moniliformis, G. fujikuroi* mating population A) is an important pathogen of maize and produces several mycotoxins, including fumonisins, which cause diseases in humans and animals. The partial sequences of the IGS region (Intergenic Spacer of rDNA units) and the translation elongation factor EF-1α gene of a representative sample (48 strains) of *F. verticillioides* isolated from diverse hosts, geographical origins and with different levels of fumonisin production were analyzed. A phylogenetic approach by PAUP was used to evaluate the genetic variability in this species and to detect the occurrence of lineages which could be associated with different hosts or produced different toxin profiles within this species. Genetic variability detected by both sequences was high, especially with the IGS sequence which showed a high number of parsimony-informative sites and nucleotide diversity. The results of the phylogenetic analysis indicated that *F. verticillioides* occurs as (i) a major fumonisin-producing population with a wide geographical distribution, wide host preferences (cereals), showing variability and considerable incidence of sexual reproduction and (ii) a minor fumonisin non-producing population, with restricted host preference (banana), low variability and clonal reproductive strategy.

Introduction

Fusarium verticillioides (*Gibberella moniliformis, G. fujikuroi* mating population A) is a pathogen of agriculturally important crops, especially maize, and it is considered one of the most important worldwide sources of fumonisin contamination of food and feed products. The most abundant fumonisin produced in nature is fumonisin B1 (FB_1) which causes several chronic and acute diseases in humans and animals (Gelderblom et al., 1988; Nelson et al., 1993; Moss, 1998). Fumonisins are produced by the fungus during colonization of host plants and often produce no visible disease symptoms (Bacon and Hinton, 1996). This prevents early recognition and subsequent control of the pathogen.

In order to devise effective strategies to control pathogen growth, to reduce the impact of diseases and to minimize fumonisin production in host plants it is necessary to have knowledge about the genetic variability and the population structure at an intraspecific level and to be able to detect populations and lineages that show interesting features in terms of toxin profile or differential host preferences. The use of DNA markers in combination with phylogenetic methods of data analysis provide the basis for developing accurate, rapid and specific diagnostic protocols for early detection of the pathogen. Several DNA sequences have been used to analyze intraspecific variability, including intron regions of histone, the β-tubulin and calmodulin genes (O'Donnell et al., 1998a, 2000b; Steenkamp et al., 2002) and the translation elongation factor EF-1α (O'Donnell et al., 1998b, 2000a,b). The Intergenic Spacer of the rDNA units (IGS) is a highly variable non-coding sequence which appears to be the most rapidly

evolving spacer region (Hillis and Dixon, 1991) presumably due to relative lack of selective constraints (Appel and Gordon, 1996; Mirete et al., 2003).

In this work, a sample of *F. verticillioides* which represents different geographical origins, different hosts and different levels of fumonisin production was analyzed using the partial genomic sequences of the IGS region and the EF-1α gene. A phylogenetic approach was used in order to evaluate the genetic variability present in this species and to detect the occurrence of lineages or populations that exhibit differential host preferences or toxin profiles.

Materials and methods

Fungal isolates and cultural conditions

Forty-eight isolates of *Fusarium verticillioides* and one of *F. proliferatum* (Table 1) were analyzed for their ability to produce fumonisins B_1 and B_2 (FB_1 and FB_2). Fumonisin production of the isolates (denoted by + in Table 1) was determined by HPLC (Mirete et al., 2003). Limits of detection (signal-to-noise ratio 3:1) were 0.05 mg/kg dried culture for each fumonisin.

Single-spore cultures were maintained on potato dextrose-agar (PDA) medium at 4 °C and stored as spore suspensions in 15% glycerol at −80 °C. The isolates were cultured in 100 ml Erlenmeyer flasks containing 20 ml Sabouraud liquid medium. Cultures were inoculated with mycelial disks cut from the margins of seven-day old colonies and incubated at 25 °C under static conditions. Mycelia from six-day old cultures were harvested by filtration through Whatman No. 1 paper and kept at −80 °C for DNA isolation.

DNA isolation, PCR amplification and sequencing

Mycelia were ground to a fine powder under liquid nitrogen. Total genomic DNA was isolated using the genomic DNA Extraction Kit (Genomix, Talent, Trieste, Italy) following the manufacturer's instructions.

For PCR amplification of the entire IGS region (2.5 kb), primers CNL12 (5′ CTGAACGCCTCTAAGTCAG 3′) and CNS1 (5′ GAGACAAGCATATGACTACTG 3′) (Appel and Gordon, 1995) were used. The PCR reaction was performed in a Eppendorf Mastercycler Gradient using 50 ng of genomic DNA. The amplification program used was as follows: 1 cycle of 85 s at 94 °C, 35 cycles of 35 s at 95 °C (denaturalization), 55 s at 58 °C (annealing), 2 min at 72 °C (extension) and finally 1 cycle of 10 min at 72 °C. Each reaction mixture contained 50 ng of template DNA in 3 μl, 1.25 μl of each primer (20 μM), 0.2 μl of Taq DNA polymerase (5 Uμl-1), 2.5 μl of 10x PCR buffer, 1 μl of $MgCl_2$ (50 mM) and 0.25 μl of dNTPs (100 mM) supplied by the manufacturer (Ecogen, Barcelona, Spain) up to a total volume of 25 μl.

A set of internal primers was designed from the complete IGS sequence for phylogenetic analyses based on the high variability found in this shorter region. These primers were I960U (5′ GTTAAGAGGCGCGGTGTCGGTGTG 3′) and V1455L (5′ TCTCAAACGTGCTCCCACCCGCTC 3′) for *F. verticillioides* isolates. In the case of *F. proliferatum*, primer P1455L (5′ TCCCAAACGTGCCCAAATCTGCTC 3′) was used instead of V1455L. The conditions of PCR amplifications were as follows: 1 cycle of 1 min 25 s at 94 °C, 35 cycles of 35 s at 95 °C (denaturalization), 55 s at 65 °C (annealing), 1 min at 72 °C (extension) and finally 1 cycle of 10 min at 72 °C.

The partial PCR amplification of the EF-1α gene was carried out with primers EF1T (5′ ATGGGTAAGGAGGACAAGAC 3′) and EF2T (5′ GGAAGTACCAGTGATCATGTT 3′) (O'Donnell et al., 2000a). The conditions were the same as above, except for a reduction of the annealing temperature to 57 °C and an increase of the extension time to 30 s.

For the identification of the mating types MAT-1 and MAT-2 alleles the following primers were used: MAT-1(F) (5′ GACCAACTCAAACCTCGTGGCG 3′) and MAT-1(R) (5′ TCATCAAAGGGCAAGCGATACC 3′) for MAT-1 and MAT-2(F) (5′ ACCGTAAGGAGCGTCACCATT 3′) and MAT-2(R) (5′ GGGGTACTGTCGGCGATGTT 3′) for MAT-2 (Kerényi et al., 1999) using the following PCR conditions: 1 cycle of 1 min 25 s at 94 °C, 25 cycles of 35 s at 95 °C (denaturalization), 30 s at 64 °C (annealing), 30 s at 72 °C (extension) and finally 1 cycle of 10 min at 72 °C.

Both partial IGS region and EF-1α gene amplification products were isolated using the

Table 1. Fusarium verticillioides strains analysed indicating host, origin and ability to produce fumonisins

Strain	Host	Origin	FB_1 μg g^{-1}	FB_2 μg g^{-1}	MAT
CB 109[a]	Zea mays	Lisbon (Portugal)	+	NA	2
CB 184[a]	Zea mays	Lisbon (Portugal)	43.7	42.02	2
GF 8[b]	*Zea mays*	Valencia (Spain)	−	−	1
GF 11[b]	*Zea mays*	Castellón (Spain)	−	−	1
GF 2[b]	*Zea mays*	Valencia (Spain)	2678.3	185.2	1
GF 24[b]	*Zea mays*	Valencia (Spain)	+	NA	2
GF 20[b]	*Zea mays*	Lérida (Spain)	1515.6	569.2	1
FV 1501[c]	*Zea mays*	Italy	359.34	243.25	2
FV 1370[c]	*Zea mays*	Sesia (USA)	2386.3	410.7	2
FV 1372[c]	*Zea mays*	Asgrow (USA)	4774.7	1055.3	2
FV 674[c]	*Zea mays*	Peru	4713.5	476.8	1
FV 2172[c]	*Zea mays*	USA (Dekalb)	839.18	436.6	2
FV 1838[c]	*Zea mays*	China	1588.0	532.0	1
FV 1859[c]	*Zea mays*	China (Kashan area)	889.2	273.1	2
FV 2242[c]	*Zea mays*	Russia	141.4	61.5	2
FV 3209[c]	*Zea mays*	South Africa	15.60	5.7	1
FV 1848[c]	*Zea mays*	China (non Kashan)	979.0	273.1	1
FV 2056[c]	*Zea mays*	South Africa	2808.5	652.9	1
FV 1917[c]	*Zea mays*	Argentina	+	+	2
FV 2006[c]	*Ostrinia nubilalis*	Italy	1358.0	262.5	1
FV 2012[c]	*Ostrinia nubilalis*	Italy	331.1	139.6	1
FV 1939[c]	*Triticum*	Spain	114.6	100.7	2
FV 1259[c]	*Triticum*	Italy	2057.1	634.0	1
FV 2025[c]	*Cucumis melo*	Italy	525.7	210.1	2
FV 1142[c]	*Musa sapientum*	Ecuador	−	−	1
GF 16[b]	*Musa sapientum*	Ecuador	−	−	1
GF 1[b]	*Musa sapientum*	Ecuador	−	−	2
GF 10[b]	*Musa sapientum*	Ecuador	−	−	2
GF 15[b]	*Musa sapientum*	Ecuador	−	−	1
FV 1146[c]	*Musa sapientum*	Ecuador	−	−	1
FV 1143[c]	*Musa sapientum*	Ecuador	−	−	2
FV 1250[c]	*Musa sapientum*	Canary Islands (Spain)	−	−	1
FV 1249[c]	*Musa sapientum*	Canary Islands (Spain)	−	−	1
FV 1251[c]	*Musa sapientum*	Canary Islands (Spain)	−	−	2
FV 1149[c]	*Musa sapientum*	Panama	−	−	1
FV 1150[c]	*Musa sapientum*	Panama	−	−	1
FV 1243[c]	*Musa sapientum*	Canary Islands (Spain)	−	−	2
FV 1245[c]	*Musa sapientum*	Canary Islands (Spain)	−	−	2
FV 1145[c]	*Musa sapientum*	Ecuador	−	−	1
FV 1139[c]	*Musa sapientum*	Panama	−	−	1
FV 1144[c]	*Musa sapientum*	Ecuador	−	−	1
FV 1252[c]	*Musa sapientum*	Canary Islands (Spain)	−	−	1
M-7451[d]	*Zea mays*	Kathmandu (Nepal)	+	+	2
M-7442[d]	*Zea mays*	Bhaniya (Nepal)	+	+	2
M-5500[d]	*Zea mays*	Kathmandu (Nepal)	77.6	6.13	2
M-5507[d]	*Zea mays*	Kathmandu (Nepal)	11.85	1.75	2
M-5515[d]	*Zea mays*	Kathmandu (Nepal)	Trace	Trace	1
A 0999[e]	*Zea mays*	Indiana (USA)	3622.2	986.43	2
FP 2287[c]		Italy	+	+	2

[a] Strains supplied by C. Tavora (Lisbon, Portugal).
[b] Strains supplied by M. Jiménez (UV, Valencia, Spain).
[c] Strains supplied by A. Logrieco (ISPA, Bari, Italy).
[d] Strains from the Fusarium Research Center (Penn State University, USA).
[e] *G. fujikuroi* mating population A (Leslie et al., 1992).
(+): fumonisin production, (−): fumonisin non-production, (NA): not analyzed. FP 2287 (*F. proliferatum*) was used to root the trees.

High Pure PCR Product Purification Kit (Roche, Germany). Sequencing of both strands was performed in the ABI 3700 DNA Sequencer in the Sequencing Service Unit of the Centro Investigaciones Biológicas, CSIC (Madrid, Spain). Sequences were edited and aligned by the Clustal method using Dnastar (Lasergene, Wisconsin, USA).

Phylogenetic analyses

All phylogenetic analyses were performed using PAUP 4.0 b10 (Swofford, 2002). Partial DNA sequences of the IGS region and the EF-1α gene were used, both as individuals and as a combined data set for the 48 strains of *F. verticillioides,* with an isolate of *F. proliferatum* FP2287 used as an outgroup element. Gaps were coded as missing data and were excluded from the analyses. Unweighted parsimony analyses were performed on the individual and combined data sets using the heuristic search option with 1000 random addition sequences with tree bisection-reconnection (TBR) branch swapping. Clade stability was assessed by 1000 bootstrap replications (Hillis and Bull, 1993). The partition homogeneity test (PHT) (Farris et al., 1995) was performed on parsimony-informative sites only with 1000 randomized data sets, using heuristic searches with simple addition of sequences. Additionally, phylogenetic analyses based on neighbour joining were performed using the Jukes–Cantor model (Jukes and Cantor, 1969).

Nucleotide diversities or the average number of differences per site between two homologous sequences (π) were calculated using DnaSp 3.5 (Rozas and Rozas, 1999) following equation 10.5 of Nei (1987). Molecular diversities were calculated separately for each sequence dataset both for the global population as a whole as well as for one of the clades. Gaps present in the alignment were excluded from the analysis.

In order to know the proportion of the total genetic variance in both sequences attributable to inter-population differences, the statistic Wright's Fst was calculated. The sequences were classified into haplotypes and were divided in two groups (fumonisin-producing and fumonisin non-producing isolates). Fst was calculated by the Molecular Analysis of the Variance (AMOVA) using the programme ARLEQUIN (Excoffier et al., 1992; Schneider et al., 1997).

Results

Fumonisin and mating type analyses

The ability of the isolates to produce FB1 and FB2 is indicated in Table 1. A group of 20 isolates was unable to produce fumonisins at detectable levels (fumonisin non-producing group, FNP). All other strains produced FB1 and FB2 at significant levels (fumonisin-producing group, FP). Table 1 also shows the mating type, MAT-1 or MAT-2, of the isolates deduced from the amplification pattern of all the strains obtained using primers MAT-1(F)/MAT-1(R) and MAT-2(F)/MAT-2(R). The ratio of MAT-1 vs. MAT-2 was 11:17 in the FP group of isolates while it was 14:6 in the FNP group.

Phylogenetic analysis

A sequence of 451 bp was amplified with IGS primers and a sequence of 648 bp long was amplified with the EF-1α gene. The amplified region of the EF-1α gene contained three introns and four exons. Excluding indels, the total number of nucleotides (nt) analyzed for IGS sequence was 451 nt and for the EF-1α sequence was 647 nt. Of the 451 nt of the IGS sequence, 29 nt were polymorphic sites and 23 parsimony-informative sites. Of the 647 nt analyzed for the EF-1α gene, 17 nt were polymorphic sites and 12 parsimony-informative sites, all of them localized in introns. Nucleotide diversities per site (π) were 0.02127 ± 0.0007 (standard deviation) and 0.00779 ± 0.00028 for IGS and EF-1α sequences, respectively. Phylogenetic analysis generated unique gene genealogies for each of the two individual data sets (Figures 1 and 2). Figure 1 shows one out of 19 most parsimonious trees for IGS data set and Figure 2 shows one out of two most parsimonious trees for the EF-1α data set. In both cases, high consistency (CI) and retention (RI) indices were obtained (Figures 1 and 2). One of six most parsimonious trees was also obtained from the combined data sets (Figure 3); in this case the CI and RI indices were lower than indices obtained for the individual data sets. All the phylogenetic analyses, i.e. the individual and combined data sets (Figures 1–3) as well as those performed using the Jukes and Cantor model (data not

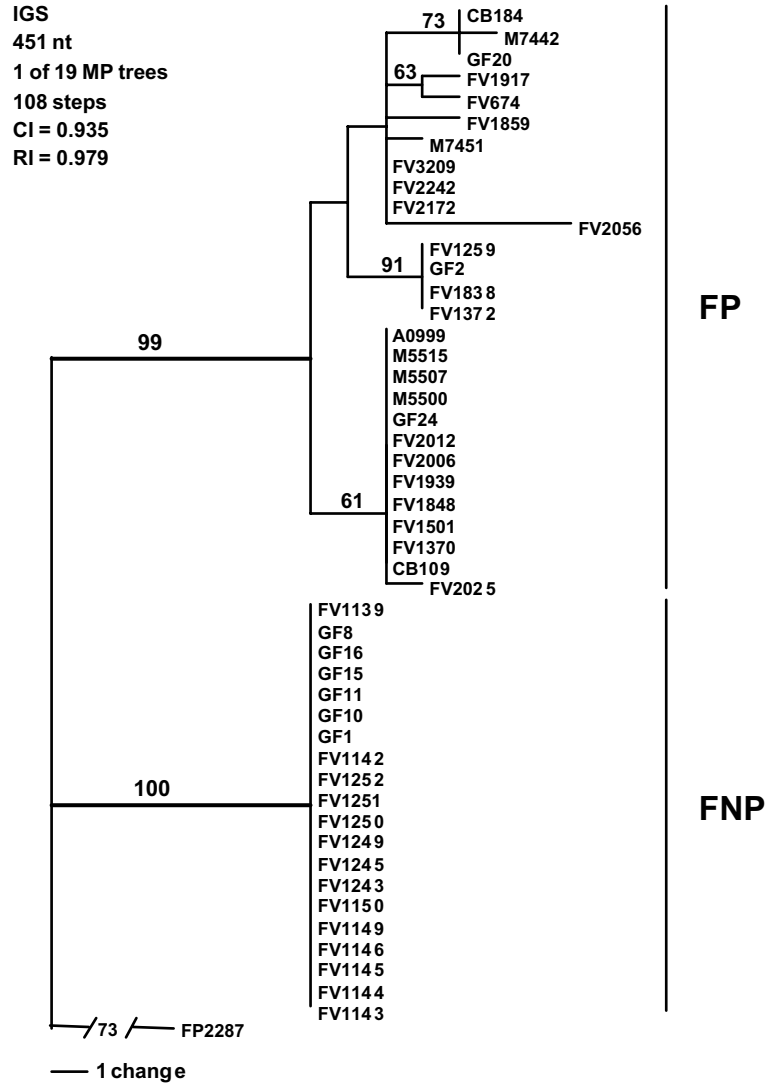

Figure 1. One of the most parsimonious trees inferred from the partial IGS sequence data set with assigned bootstrap values.

shown), revealed two distinct groups of isolates which corresponded to groups FP and FNP. The statistics Fst was calculated to estimate the degree of genetic separation of both populations, FP and FNP. The Fst obtained was 0.55911 and 0.52497 for IGS and EF-1α, respectively.

The FNP group was homogenous: the IGS and EF-1α sequences were identical. On the contrary, variability was high among strains from the FP group. Nucleotide diversity per site within this group was $\pi = 0.00812 \pm 0.00084$ and $\pi = 0.00279 \pm 0.00052$, for IGS and EF-1$\alpha$, respectively. The clusters detected within the FP group with the IGS data set were not supported by the genealogy obtained with the EF-1α data set. The clusters observed did not show a link to geographical origin or host. When PHT was performed on this group using a combination of both sequences, three replicas out of 1000 showed a summed length equal to the number of steps (17) ($p = 0.003$), a result that suggested the existence of incongruence. Additionally, the Templeton–Wilcoxon test (Templeton, 1983) and the Kishino–Hasegawa test (Kishino and Hasegawa, 1989) were

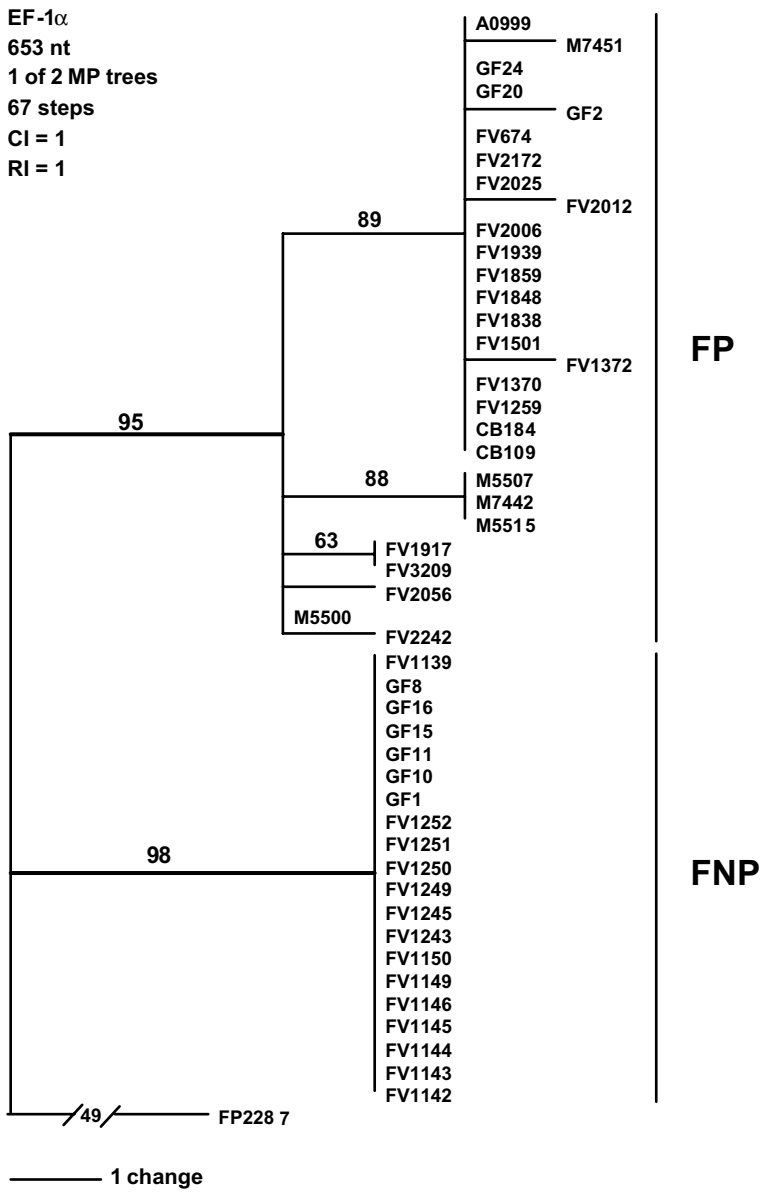

Figure 2. One of the most parsimonious trees inferred from the partial EF-1α gene sequence data set with assigned bootstrap values

performed to test the null hypothesis of congruence between both sequences within the FP group. In both cases the null hypothesis was rejected (data not shown).

Discussion

The analysis of a representative sample of strains of *Fusarium verticillioides* (Table 1) using two partial genomic sequences from the IGS region and the EF-1α gene revealed the existence of variability and detected a cluster of strains which could be considered a distinct population within *F. verticillioides*. The existence of variability within *F. verticillioides* could be inferred from its worldwide distribution, the different diseases which are produced in maize, the diverse hosts and the differences observed in vegetative compatibility groups (Leslie, 1995; Desjardins and Plattner, 2000).

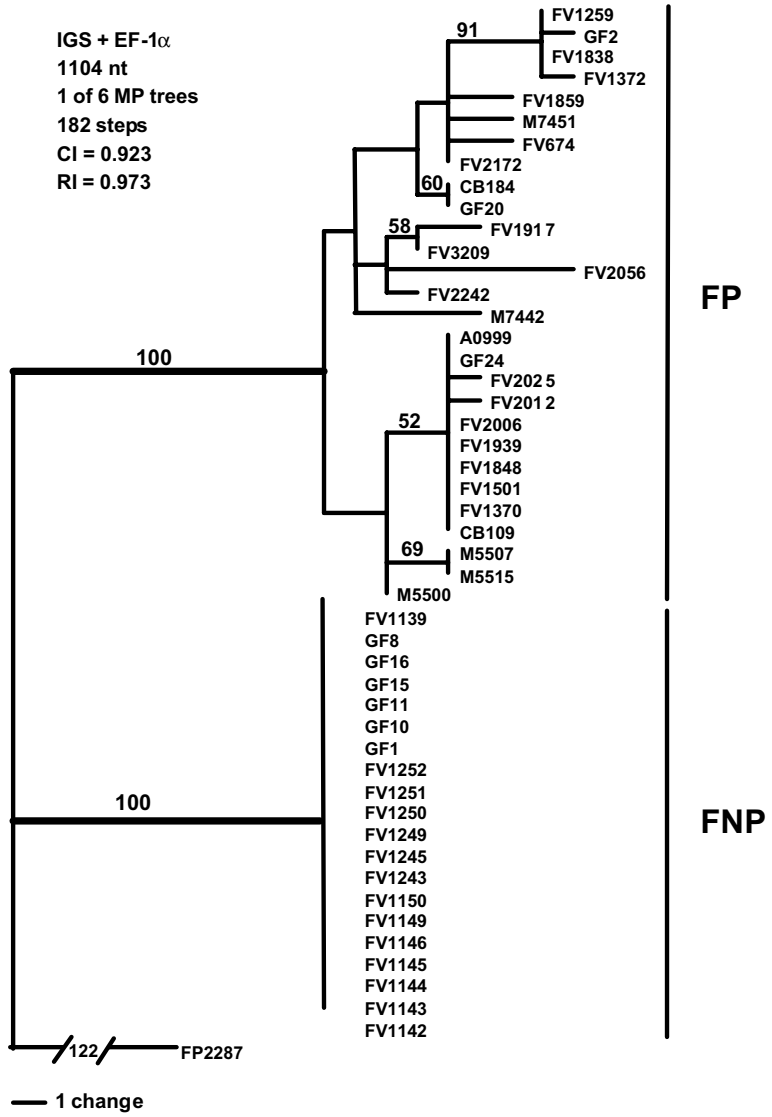

Figure 3. One of the most parsimonious trees inferred from the combination of the partial IGS and EF-1α gene sequence data sets with assigned bootstrap values.

However, previous studies using polymorphic markers (isozymes) did not detect variability (Huss and Leslie, 1996), suggesting that *F. verticillioides* could be a homogenous biological population (Leslie and Klein, 1996). In our analysis, both DNA sequences, particularly the IGS region, revealed variability within *F. verticillioides* showing a high number of parsimony-informative sites and nucleotide diversity in comparison with other sequences such as β-tubulin or histones (Steenkamp et al., 2002).

Phylogenetic analysis using IGS and EF-1α sequences revealed the existence of two distinct clusters of strains within *F. verticillioides* (Figures 1–3). The first group contained a major population with a wide geographical distribution, wide host preferences and the ability to produce fumonisins (Table 1) (FP group). The second group included strains associated with banana, which were located in Central America and the Canary Islands and which were unable to produce fumonisins (FNP group) (Table 1). No variability was found for

both IGS and EF-1α within this group. The different origin of those isolates and the existence of strains belonging to both mating types, MAT-1 and MAT-2, suggest that this cluster contained different individuals. Reproductive isolation of these two populations is apparently taking place, since the crosses of individuals belonging to both populations performed in the laboratory showed some features which are different to those observed in normal crosses within each group (Moretti et al., this issue). Moreover, when the presence of some of the genes involved in fumonisin synthesis (Seo et al., 2001), namely *fum*5 and *fum*8, was investigated in some of the isolates of the FNP group by Southern blot analysis, no hybridization was detected. This suggests that they had lost part of the fumonisin biosynthetic cluster (data not shown). The three isolates from Nepal which produced low levels of fumonisins amplified partial regions of *fum*5 and *fum*8 genes, while none of the FNP group of strains showed positive amplification of these genes.

Variability within the FP group was high and some clusters with significant bootstraps were detected using both data sets, IGS and EF-1α. None of the clusters was related to geographic origin or host. However, the clustering observed was not congruent according to the results of the test PHT performed using a combination of both sequences in group FP. The two additional tests used, the non-parametric Templeton–Wilcoxon test and the Kishino–Hasegawa test, confirmed the existence of incongruence within FP group. Occurrence of recombination among the groups could be an explanation for these inconsistencies (Geiser et al., 1998). Indeed, sexual reproduction is common in *F. verticillioides* (Leslie and Klein, 1996). In contrast, the low variability within the FNP group and the association of this group with one host would also be indicative of clonality.

This situation would fit the hypothesis by Taylor et al. (1999) which suggests that asexual populations could have arisen from a more variable sexual population. Evidence obtained from other *Fusarium* related species, such as *F. subglutinans sensu stricto* (*G. fujikuroi* mating population E) (Steenkamp et al., 2002), or the more distantly related *F. oxysporum* (Appel and Gordon, 1996; O'Donnell et al., 1998b) or *F. graminearum* (O'Donnell et al., 2000a) indicates that in these species there are several isolated lineages which support a clonal or asexual mode of reproduction; no variable population with sexual reproduction was detected. However, it is possible that the loss of sexual reproduction occurred in a previous step, during the events leading to the formation of these species. Studies on the rest of the main species included in the *G. fujikuroi* species complex will be necessary to gain insight into the evolutionary trends in relation to reproductive strategies and to evaluate the incidence of factors such as toxin production, global trade and agricultural practices and their effects on the evolution of *Gibberella fujikuroi* complex.

Acknowledgements

This work was supported by an EU project: Early Detection of Toxigenic *Fusarium* Species and Ochratoxigenic Fungi in Plant Products (QLK1-CT-1998-001380); V Frame Programme (Quality of Life and Management of Living Resources) and by the Spanish MCyT (AGL2001/2974/C05/05). S. Mirete and M. Jurado were supported by EU and MCyT predoctoral grants, respectively. We wish to thank Gema Rodríguez for skilful technical assistance.

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