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Cryptic speciation in *Fusarium subglutinans*

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Abstract: *Fusarium* isolates that form part of the *Gibberella fujikuroi* species complex have been classified using either a morphological, biological, or phylogenetic species concept. Problems with the taxonomy of *Fusarium* species in this complex are mostly experienced when the morphological and biological species concepts are applied. The most consistent identifications are obtained with the phylogenetic species concept. Results from recent studies have presented an example of discordance between the biological and phylogenetic species concepts, where a group of *F. subglutinans sensu stricto* isolates, i.e., isolates belonging to mating population E of the *G. fujikuroi* complex, could be sub-divided into more than one phylogenetic lineage. The aim of this study was to determine whether this sub-division represented species divergence or intraspecific diversity in *F. subglutinans*. For this purpose, we included 29 *F. subglutinans* isolates belonging to the E-mating population that were collected from either maize or teosinte, from a wide geographic range. DNA sequence data for six nuclear regions in each of these isolates were obtained and used in phylogenetic concordance analyses. These analyses revealed the presence of two major groups representing cryptic species in *F. subglutinans*. These cryptic species were further sub-divided into a number of smaller groups that appear to be reproductively isolated in nature. This suggests not only that the existing *F. subglutinans* populations are in the process of divergence, but also that each of the resulting lineages are undergoing separation into distinct taxa. These divergences did not appear to be linked to geographic origin, host, or phenotypic characters such as morphology.

Key Words: *Fusarium subglutinans*, maize, reproductive isolation, teosinte

INTRODUCTION

Gibberella fujikuroi (Sawada) Wollenw. is a species complex that encompasses many *Fusarium* species (Nirenberg and O'Donnell 1998, O'Donnell and Cigelnik 1997, O'Donnell et al 1998a, 2000a, Steenkamp et al 1999, 2000a, 2001). In the global environment, species in this complex are important, because of their association with diseases of agronomically important plants (Correll et al 1991, Leslie 1995, Leslie et al 1990, Sun and Snyder 1981, Varma et al 1974, Ventura et al 1993). These fungi also affect human and animal health, since many species produce toxic secondary metabolites such as moniliformin, beauvericin, fumonisin, fusaproliferin, and fusaric acid (Leslie et al 1992, Logrieco et al 1993, Marasas et al 1983, Sewram et al 1999a, 1999b, Shephard et al 1999, Vesonder et al 1995).

The taxonomy of *Fusarium* species in the *G. fujikuroi* complex has been subject to much controversy (Leslie 1995). This is mainly due to a lack of consensus among researchers on how to define a morphological species for the fungi in this complex (Gerlach and Nirenberg 1982, Nirenberg and O'Donnell 1998, Snyder and Hansen 1945). In an attempt to resolve this problem, a biological species concept (Dobzhansky 1937, Mayr 1940) was introduced, whereby eight biological species have been identified (Britz et al 1999, Hsieh et al 1977, Klittich and Leslie 1992, Kuhlman 1982, Leslie 1991, 1995, 1996). These biological species were designated as mating populations A to H, where mating population E, for example, represents *F. subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas *sensu stricto* (Britz et al 1999, Hsieh et al 1977, Klittich and Leslie 1992, Kuhlman

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1982, Leslie 1991, 1995, 1996). The eight biological species, however, exclude the more than 80% of species in this complex with no apparent sexual reproductive cycle. Currently, the only method for classifying all the fungal strains in the *G. fujikuroi* species complex is through the application of the phylogenetic species concept (Cracraft 1983, Taylor et al 2000). With this method, fungi in the *G. fujikuroi* complex are classified into more than 40 different phylogenetic species (O'Donnell et al 1998a, Steenkamp et al 1999, 2000a).

In a recent study, ten additional phylogenetically distinct species in the *G. fujikuroi* complex were reported (O'Donnell et al 2000a). Among these was a *F. subglutinans* strain associated with maize in South Africa, that had previously been classified in mating population E of the *G. fujikuroi* species complex using the biological species concept (Steenkamp et al 1999). Application of the phylogenetic species concept has, however, indicated that this biological species is subdivided into more than one phylogenetic lineage (O'Donnell et al 2000a, Steenkamp et al 1999, 2001). These lineages may either reflect species divergence within *F. subglutinans* or are simply the result of intraspecific diversity. However, all of the studies dealing with the molecular classification of strains representing *F. subglutinans* were based on small sets of different isolates (O'Donnell et al 2000a, Steenkamp et al 1999, 2001) and no definite conclusions could be drawn on the taxonomic status of these fungi. Clarification of the relationships among different *F. subglutinans* isolates would undoubtedly shed light on how the phylogenetic species concept might influence our interpretation of the biological species concept in the *G. fujikuroi* species complex. The aim of this study was, therefore, to address the phylogenetic status of members of *F. subglutinans* by (i) including strains isolated from maize and its wild teosinte relatives from a wide geographic range; (ii) obtaining DNA sequence data from six nuclear regions for these strains; and (iii) using phylogenetic concordance analysis (Avise and Ball 1990, Taylor et al 2000) to determine whether these isolates are interbreeding in nature and, if not, to define subgroups within *F. subglutinans*.

MATERIAL AND METHODS

Fungal isolates.—Twenty-nine *F. subglutinans sensu stricto* isolates belonging to the E-mating population of the *G. fujikuroi* species complex were included in this study (TABLE I). The six South African, ten United States and six Mexican strains were isolated from maize. The remaining five Mexican strains and the two Guatemalan strains were isolated from teosinte. For outgroup purposes we also included two isolates of *F. circinatum* Nirenberg et O'Donnell that were isolated from pines.

DNA isolation, PCR amplification and sequencing.—DNA was isolated using a CTAB (*N*-cetyl-*N,N,N*-trimethyl-ammonium bromide) extraction method (Steenkamp et al 1999). A portion of three nuclear genes, histone *H3* (Glass and Donaldson 1995), calmodulin (Carbone and Kohn 1999) and β-tubulin (Glass and Donaldson 1995), were amplified from all 31 isolates. We also used an additional set of primers that amplify three unlinked nuclear regions of unknown function (H. Britz unpubl). The first primer set is HB9-a (5′-tcaataccctcgcctagaa-3′) and HB9-b (5′-gaccacagcctcgagaacat-3′), the second is HB14-a (5′-ttccaccatgagaggaaaccc-3′) and HB14-b (5′-ccattgccaatcttgatcct-3′), and the third HB26-a (5′-gacttgagtatctgcactgc-3′) and HB26-b (5′-gaatgtactactcgacgtcg-3′).

For amplification of all these loci, the PCR mixture contained 1 mM deoxynucleotide triphosphates (0.25 mM each), 2.5 mM $MgCl_2$, 0.2 μM of each primer, 0.25 ng/μL DNA, 0.05 U/μL of Super-Therm DNA polymerase [Southern Cross biotechnology (Pty.) Ltd., Cape Town, South Africa] and 1 × Super-Therm reaction buffer. PCR-cycling conditions were as follows: denaturation at 92 C for 20 s, annealing for 20 s at 55 C (calmodulin, tubulin, and histone) or 47 C (HB9, HB14, and HB26), and elongation for 20 s at 72 C. This was repeated 30 times and was preceded by an initial denaturation at 92 C for 1 min and followed by a final elongation step at 72 C for 5 min.

After PCR, the products were purified with a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and sequenced in both directions with the respective primers. Reactions were performed on an ABI PRISM™ 377 automated DNA sequencer, with an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Warrington, United Kingdom). Sequences were analyzed with Sequence Navigator version 1.0.1.™ (Perkin Elmer Applied BioSystems, Inc., Foster City, California).

Phylogenetic analyses.—The data sets obtained for each primer set were aligned manually by inserting gaps (TreeBase accession numbers S789 and M1249). Phylogenetic analyses were performed with PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b1 (Swofford 1998), with gaps treated as fifth characters in heuristic parsimony searches, and with tree-bisection-reconnection (TBR) branch swapping and MULTREES (saving of all optimal trees) effective. For bootstrap analyses 1000 replications were performed.

RESULTS

PCR amplification and sequencing.—With the primers used, we were able to amplify and sequence 480 base pairs (bp), 456 bp, and 332 bp of the calmodulin, β-tubulin, and histone *H3* genes, respectively. For the three regions of unknown function, 250 bp, 235 bp, and 236 bp were sequenced for HB9, HB14, and HB26, respectively. Of the 1989 nucleotides (nc) sequenced, 17 nc (0.9%) were polymorphic in the dif-

TABLE I. Hosts, geographic origins and sources of the *Fusarium subglutinans* and *F. circinatum* isolates used in this study

Isolate[a]	Host[b]	Geographic origin[c]	Source[d]	GenBank accession No.[e]
MRC115	*Z. mays*	Eastern Cape, South Africa	W.F.O. Marasas (Steenkamp et al 1999)	AF150843, AF374015, AF374036, AF374084, AF374116, AF374148
MRC714	*Z. mays*	Northern Province, South Africa	W.F.O. Marasas (Steenkamp et al 1999)	AF150841, AF366548, AF366520, AF374085, AF374117, AF374149
MRC756	*Z. mays*	Mpumalanga, South Africa	W.F.O. Marasas (Steenkamp et al 2001)	AF150839, AF366549, AF366527, AF374086, AF374118, AF374150
MRC837	*Z. mays*	Eastern Cape, South Africa	W.F.O. Marasas (Steenkamp et al 1999)	AF150840, AF374016, AF374037, AF374087, AF374119, AF374151
MRC1077	*Z. mays*	Eastern Cape, South Africa	W.F.O. Marasas (Steenkamp et al 1999)	AF150837, AF374017, AF374038, AF374088, AF374120, AF374152
MRC1084	*Z. mays*	Eastern Cape, South Africa	W.F.O. Marasas (Steenkamp et al 2001)	AF150838, AF366550, AF366521, AF374089, AF374121, AF374153
MRC6483, M3696, KSU990	*Z. mays*	Illinois, United States	J.F. Leslie (Yan et al 1993)	AF150845, AF366552, AF366522, AF374100, AF374132, AF374164
MRC6512, M3693, KSU2192	*Z. mays*	Illinois, United States	J.F. Leslie (Yan et al 1993)	AF 150844, AF366551, AF366525, AF374099, AF374131, AF374163
KSU434, M 6496	*Z. mays*	Kansas, United States	J.F. Leslie (Yan et al 1993)	AF374061, AF374018, AF374039, AF374090, AF374122, AF374154
KSU507, M5119	*Z. mays*	Kansas, United States	J.F. Leslie (Yan et al 1993)	AF374062, AF374019, AF374040, AF374091, AF374123, AF374155
KSU731, M5126	*Z. mays*	Kansas, United States	J.F. Leslie (Yan et al 1993)	AF374063, AF374020, AF374041, AF374092, AF374124, AF374156
KSU993, M3698	*Z. mays*	Illinois, United States	J.F. Leslie (Yan et al 1993)	AF374064, AF374021, AF374042, AF374093, AF374125, AF374157
KSU1257	*Z. mays*	Kansas, United States	J.F. Leslie (Yan et al 1993)	AF374065, AF374022, AF374043, AF374094, AF374126, AF374158
KSU1417	*Z. mays*	Kansas, United States	J.F. Leslie (Yan et al 1993)	AF374066, AF374023, AF374044, AF374095, AF374127, AF374159
KSU2921, M3763	*Z. mays*	Ohio, United States	J.F. Leslie (Yan et al 1993)	AF374068, AF374025, AF374046, AF374097, AF374129, AF374161
KSU3815, M851	N/a	N/a	J.F. Leslie (Yan et al 1993)	AF374069, AF374026, AF374047, AF374098, AF374130, AF374162
MRC7786, Fst9	*Z. diploperennis*	Jalisco, Mexico	A.E. Desjardins	AF374050, AF374008, AF374029, AF374073, AF374105, AF374137
MRC7787, Fst10	*Z. diploperennis*	Jalisco, Mexico	A.E. Desjardins (Steenkamp et al 2001)	AF374051, AF366544, AF366523, AF374074, AF374106, AF374138
MRC7790, Fst13, M7794	*Z. luxurians*	Chiquimula, Guatemala	A.E. Desjardins (Desjardins et al 2000)	AF374052, AF374009, AF374030, AF374075, AF374107, AF374139
MRC7794, Fst17, M7799	*Z. mays* ssp. *huehuetenangensis*	Huehuetenen, Guatemala	A.E. Desjardins (Desjardins et al 2000)	AF374053, AF374010, AF374031, AF374076, AF374108, AF374140

TABLE I. Continued

Isolate[a]	Host[b]	Geographic origin[c]	Source[d]	GenBank accession No.[e]
MRC7799, Fst22	*Z. mays* ssp. *mexicana*	Durango, Mexico	A.E. Desjardins	AF374054, AF374011, AF374032, AF374077, AF374109, AF374141
MRC7803, Fst26	*Z. mays* ssp. *mexicana*	Michoacan, Mexico	A.E. Desjardins (Steenkamp et al 2001)	AF374055, AF366545, AF366526, AF374078, AF374110, AF374142
MRC7817, Fst40	*Z. mays* ssp. *parviglumis*	Guerrero, Mexico	A.E. Desjardins	AF374056, AF366546, AF366524, AF374079, AF374111, AF374143
MRC7828, Fst51, M8372	*Z. mays* ssp. *mexicana*	Texcoco, Mexico	A.E. Desjardins (Desjardins et al 2000; Steenkamp et al 2001)	AF374057, AF366547, AF366529, AF374080, AF374112, AF374144
MRC7833, Fst54, M8375	*Z. mays* ssp. *mays*	Texcoco, Mexico	A.E. Desjardins	AF374058, AF374012, AF374033, AF374081, AF374113, AF374145
MRC7838, Fst58, M8377	*Z. mays* ssp. *mays*	Texcoco, Mexico	A.E. Desjardins	AF374059, AF374013, AF374034, AF374082, AF374114, AF374146
MRC7849, Fst69, M8380	*Z. mays* ssp. *mays*	Texcoco, Mexico	A.E. Desjardins (Desjardins et al 2000)	AF345060, AF374014, AF374035, AF374083, AF374115, AF374147
MRC7861, M3935	*Z. mays* ssp. *mays*	N/a, Mexico	A.E. Desjardins	AF374049, AF374007, AF374028, AF374072, AF374104, AF374136
MRC7862, M3869	*Z. mays* ssp. *mays*	N/a, Mexico	A.E. Desjardins	AF374048, AF374006, AF374027, AF374071, AF374103, AF374135
MRC7870, Fsp34	*Pinus* spp.	California, United States	T.R. Gordon (Desjardins et al 2000)	AF374070, AF366555, AF366528, AF374101, AF374133, AF374165
MRC6213	*Pinus* spp.	Mpumalanga, South Africa	A. Viljoen (Britz et al 1999)	AF150849, AF366553, AF366531, AF374102, AF374134, AF374166

[a] MRC-isolates are maintained at PROMEC, Medical Research Council, Tygerberg, South Africa; KSU-isolates at the Department of Plant Pathology, Kansas State University, Manhattan, Kansas; Fst-isolates at NCAUR, USDA, Peoria, Illinois; Fsp-isolates at the Department of Plant Pathology, University of California, Davis, California; and M-isolates at the Fusarium Research Center, Pennsylvania State University, University Park, Pennsylvania.

[b] N/a, information on the plant host from which the strain was isolated is not available.

[c] N/a, exact location of isolation of the strain is not available.

[d] References of studies where isolates were used in sexual compatibility tests are indicated in parentheses.

[e] GenBank Accession numbers for the sequences for each of six loci analyzed are listed in the following order: histone H3, β-tubulin, calmodulin, HB14, HB9 and HB26.

TABLE II. Summary of polymorphic nucleotides (nc) in the six nuclear regions sequenced among the *Fusarium subglutinans* isolates from maize and teosinte

	Polymorphisms (nc)[a]																			
	CAL		TUB			HIS						HB14				HB 26	HB9		Geno-
Isolates	33	377	144	204	285	27	99	275	286	351	426	99	230	231	233	69	101	204	type
MRC7828, MRC7833, MRC837, MRC1077, MRC1084	T	C	A	T	T	C	C	T	G	C	T	T	−	−	−	A	C	C	1-1
MRC7786	T	C	A	T	T	C	T	C	G	C	C	T	−	−	−	A	C	C	1-2
MRC7803	T	C	A	T	C	C	T	C	G	C	C	T	−	−	−	A	C	C	1-3
MRC7862, MRC7861, MRC7838, MRC7849, MRC756	T	C	G	T	T	C	C	T	G	C	T	T	−	−	−	A	C	C	1-4
MRC7787, MRC7790, MRC7794, MRC7799, MRC115, KSU1257, KSU1417, KSU3815	C	C	A	C	T	C	C	T	A	T	T	C	T	A	A	G	T	G	2-1
KSU434, KSU731	C	T	A	C	T	T	C	T	A	T	T	C	T	A	A	G	T	G	2-2
MRC7817, MRC714, MRC6512, MRC6483, KSU507, KSU993, KSU2921	C	T	A	C	T	C	C	T	A	T	T	C	T	A	A	G	T	G	2-3

[a] Numbering of polymorphic nucleotides corresponds with their nucleotide positions in the respective DNA sequences. Horizontal lines (−) indicate nucleotide deletions.

ferent *F. subglutinans* strains and none of the sites had more than two different nucleotide character states. Among these strains, between one and six polymorphic nucleotides in each of the six regions were identified (TABLE II). Upon combination of the polymorphisms for each individual, we recognized seven different genotypes within the set of 29 *F. subglutinans* isolates (TABLE II). The most frequently sampled genotype was 2-1, which was represented by eight strains associated with maize in South Africa and the United States and teosinte in Mexico and Guatemala. Seven strains displayed genotype 2-3, and were associated with maize in the United States and South Africa, as well as teosinte in Mexico. Five isolates displayed genotype 1-1 and were collected from maize in South Africa and Mexico, as well as Mexican teosinte. Genotype 1-4 was also represented by five strains, all of which were isolated from maize in South Africa and Mexico. Genotype 2-2 was represented by two strains associated with maize in the United States and both genotypes 1-2 and 1-3 were represented by single strains that were isolated from teosinte in Mexico.

TABLE III. Number of polymorphic and parsimony informative characters, as well as the actual length of trees, generated from the individual and combined sequence data sets

Locus	Informative characters	Polymorphic characters	Tree length
Calmodulin	2	2	2
Histone *H3*	6	6	6
β-tubulin	2	3	2
HB9	2	2	2
HB14	4	4	4
HB26	1	1	1
Combined	17	18	17

Phylogenetic analyses.—The number of parsimony informative characters in the six data sets ranged from one for HB26 to six for histone *H3* (TABLE III). In all the data sets, only the β-tubulin sequence harbored a parsimony uninformative character (TABLE III, FIG. 1). This variable character was present only in isolate MRC7803. Phylogenetic analyses generated unique gene genealogies for each of the individual data sets (FIG. 1). In each case, a single tree was generated and the consistency (CI) and retention (RI) indices for each were 1.00 and 1.00, respectively, indicating no homoplastic characters in any of the six individual data sets. As a result, all of the single-gene genealogies were of minimal length, i.e., equal to the number of parsimony informative sites. A single most parsimonious tree was also obtained from the combined data sets (FIG. 2). The length of this tree was

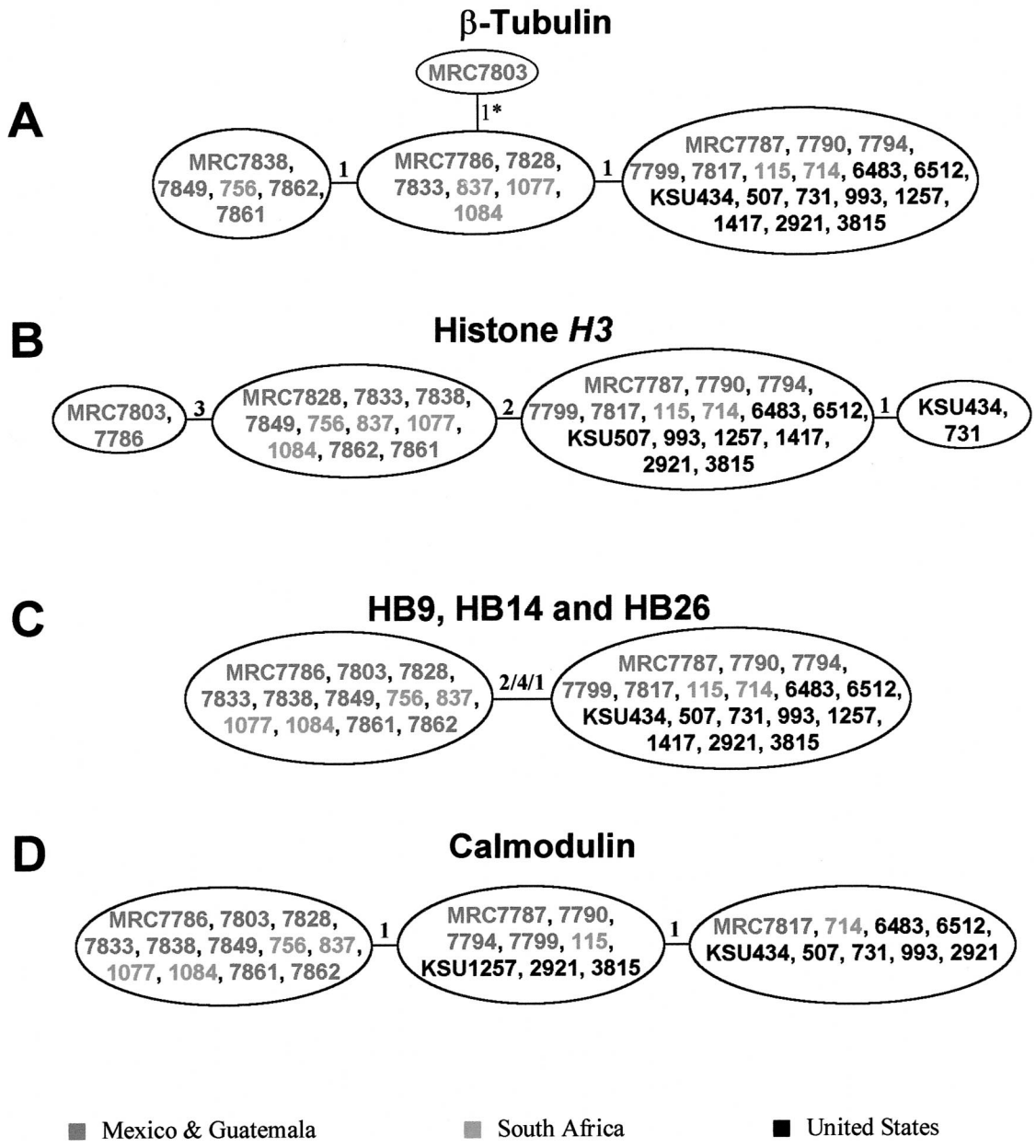

FIG. 1. Single-gene genealogies generated from sequence data sets for six different loci studied in 29 *F. subglutinans* strains associated with maize and teosinte. In each case only one most parsimonious tree was obtained. The branch associated with the single parsimony uninformative character in the β-tubulin data set is indicated with an asterisk (*). The consistency (CI) and retention (RI) indices for each were 1.00 and 1.00, respectively. The different geographic origins are indicated in color (red = South Africa; black = United States; blue = Mexico and Guatemala). A: β-tubulin gene genealogy consisting of 2 parsimony informative characters and 2 steps. B: Histone *H3* genealogy consisting of 6 parsimony informative characters and 6 steps. C: The single-gene genealogy for each of the HB9, HB14, and HB26 nuclear regions. Because the clustering for each of these regions was identical, they are represented by a single tree with lengths 2, 4, and 1, respectively. D: Calmodulin gene genealogy consisting of 2 steps.

equal to the number of parsimony informative characters (TABLE III), since homoplastic characters were also absent in the combined data set (CI = 1.00, RI = 1.00). The length of this tree was equal to the summed lengths of the individual gene trees (TABLE III), which is a distinctive feature of absolute congruence among individual gene genealogies (Dykhuizen and Green 1991, Maynard Smith and Smith 1998, Taylor et al 1999b).

Clustering within the different genealogies was very similar. For the data sets HB9, HB14, and HB26 the isolates were separated into two groups that al-

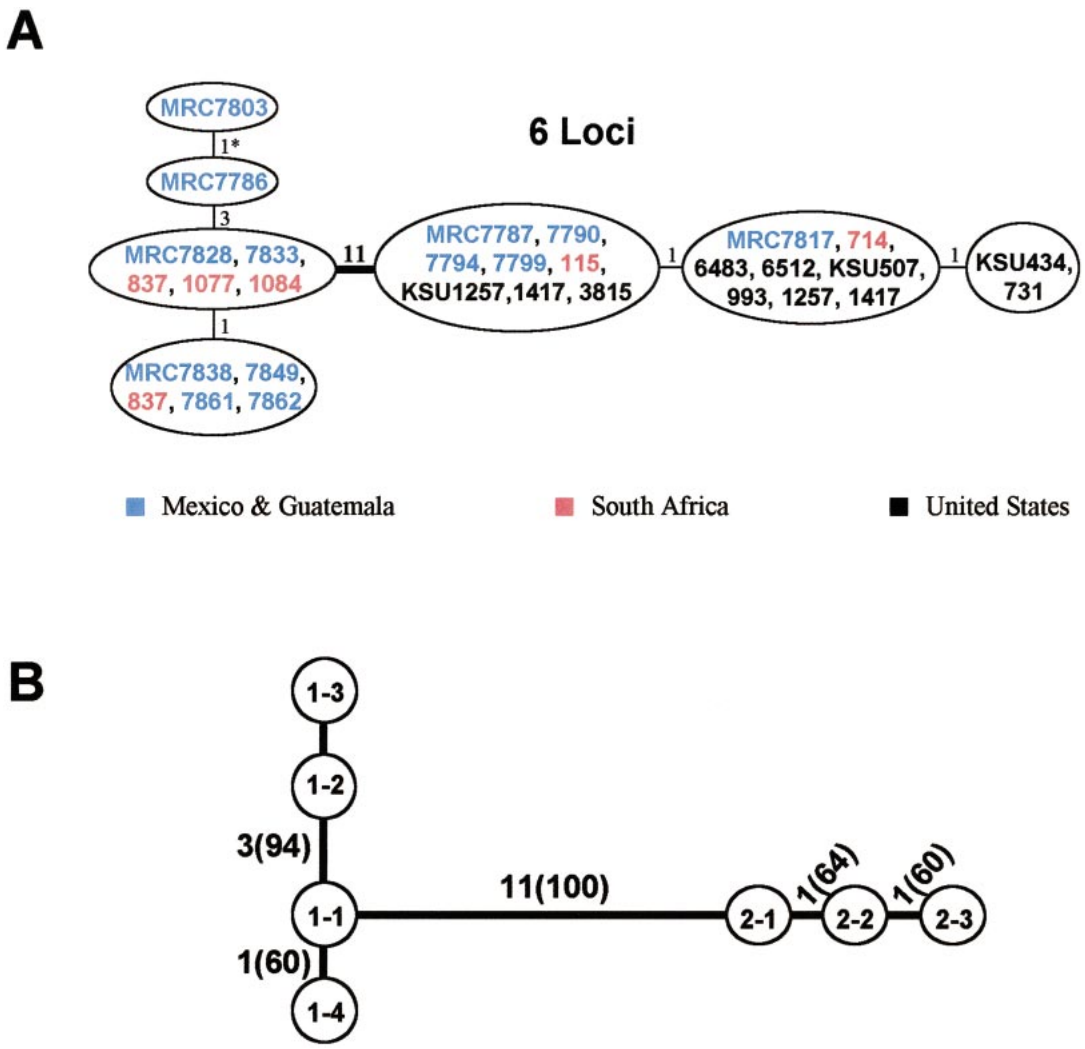

FIG. 2. A: Genealogy generated from the combined data sets (color coding same as in FIG. 1). The branch associated with the single parsimony uninformative character in the β-tubulin data set is indicated with an asterisk (*). One single most parsimonious tree with a length of 17 steps was obtained (CI = 1.00; RI = 1.00). B: The individuals included in each of the seven clusters correspond with the individuals displaying each of seven genotypes. Bootstrap values based on 1000 replications are indicated in parentheses.

ways included the same isolates (FIG. 1). The β-tubulin and calmodulin genealogies consisted of four and three clusters of isolates, respectively. Although there was some overlap, the clustering patterns for the β-tubulin and calmodulin genes were unique. The genealogy generated from the histone *H3* data generated four groups of isolates, some of which showed some resemblance to those generated for the other data sets.

Phylogenetic analysis of the combined data set from all the isolates included in this study revealed the presence of two distinct groups among the isolates associated with maize and teosinte (FIGS. 2 and 3). They were designated as Group 1 and 2 (FIG. 3). The genotypes 1-1, 1-2, 1-3 and 1-4 were present in Group 1 and the genotypes 2-1, 2-2 and 2-3 were present in Group 2 (FIGS. 2 and 3). Although this clustering pattern was not immediately detectable from the individual gene trees, the combined gene genealogy was not discordant with any of them.

DISCUSSION

In this study, we set out to use phylogenetic tools to answer what appeared to be either a population or species level question. Although this approach is not widely used in fungal taxonomy, it is not without precedence and a number of researchers have reported on the value of using phylogenetic concordance analysis (Carbone et al 1999, Geiser et al 1998, Koufopanou et al 1997, O'Donnell et al 1998b, 2000a, b, Taylor et al 1999a, b, 2000). Based on previous work

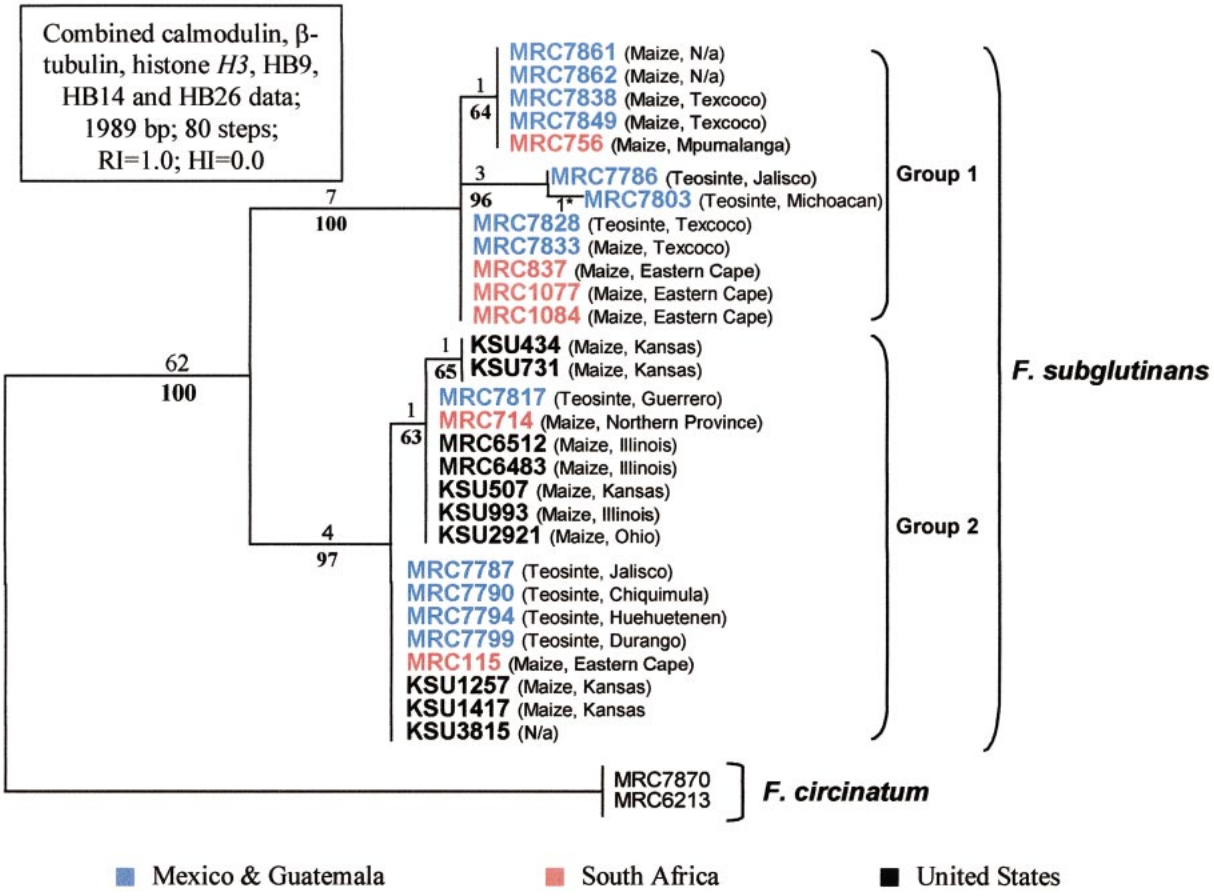

FIG. 3. A single most parsimonious phylogram generated from the combined sequence data sets. Parsimony informative and parsimony uninformative characters (indicated with an asterisk) were included in the analysis. In parentheses are indicated the different hosts followed by their specific geographic origins within South Africa, the United States, or Mexico and Guatemala. The tree is rooted to *F. circinatum*. Branch lengths are indicated above the branches and bootstrap values based on 1000 replications are indicated in bold. (N/a = not available)

(Steenkamp et al 1999, 2001), our null hypothesis was that the set of isolates associated with maize and teosinte, from a wide geographic range, would form part of the existing E-mating population of the *G. fujikuroi* species complex (i.e., *F. subglutinans sensu stricto*), and that the observed sequence variation merely reflect intraspecific diversity. However, considering the data generated in this study, we had to reject this hypothesis. The results clearly showed that *F. subglutinans* is separated into reproductively isolated populations that probably constitute separate sibling species.

The basic rationale behind the use of phylogenetic concordance analyses involves the detection of congruence or the lack thereof, among different gene trees (Avise and Ball 1990, Dykhuizen and Green 1991, Taylor et al 1999a, 1999b, 2000). Incongruence among gene trees from different loci indicates interbreeding among individuals, since sexual recombination 'reshuffles' their genomes. In an interbreeding population this 'reshuffling' results in unique evolutionary histories for the genes in a specific individual, while the genes from different individuals share numerous characteristics or polymorphisms. In reproductively isolated populations genes do not normally flow among individuals, resulting in a lack of shared polymorphisms and consequently perfectly congruent gene trees. Our analyses showed that the evolutionary histories of the six nuclear regions were perfectly congruent in each of the *F. subglutinans* individuals studied. This is because every gene tree, as well as the one generated from the combined data, was of minimum length (FIGS. 1 and 2). The *F. subglutinans* isolates from the United States, South Africa, Mexico, and Guatemala thus appear to represent reproductively isolated populations.

The tree generated from the combined data separated the 29 *F. subglutinans* isolates into seven groups (FIG. 2). These groups corresponded to the seven multilocus genotypes identified from the se-

quence comparisons (TABLE II). The evolutionary histories for each of the six nuclear regions in all the isolates from genotype 1-1, for example, are identical, indicating a lack of genome 'reshuffling' through sexual reproduction. All the isolates in genotype 1-1 can, therefore, be considered as clones of one another, since there is no evidence of interbreeding. The same is also true for genotypes 1-4, 2-1, 2-2 and 2-3. Although genotypes 1-2 and 1-3 are represented by single isolates, analysis of additional isolates will most likely reveal a similar trend. In theory, the overall lack of shared polymorphisms in the six nuclear regions among the seven groups or genotypes, separate the 29 isolates into seven clones, a notion that is probably not entirely correct (see below).

In light of similar studies on other fungi (reviewed by Taylor et al 1999a, 1999b, 2000) our results were rather unexpected. In most of these studies, phylogenetic concordance analysis revealed the presence of so-called cryptic species where individuals from one species were shown to be reproductively isolated from those in the other species. However, these tests also detected that individuals within some of these cryptic species were interbreeding in nature, because of the presence of numerous shared polymorphisms. As a result, generation of consensus trees from combined sequence data resulted in trees that were longer than the expected minimum (observed tree length > number of polymorphic characters). In contrast, the observed tree length in our study was equal to the number of polymorphic characters (TABLE III), providing no evidence for interbreeding among any of the seven *F. subglutinans* genotypes. By employing phylogenetic concordance analysis, our study is one of few, and perhaps the first to suggest that a group of fungi capable of interbreeding in the laboratory appears to be propagating exclusively asexually in nature.

The idea that isolates of *F. subglutinans* exist in clonal populations is not congruent with previous reports. Based on analyses of phenotypic characters, isolates of a specific genotype do not always display similar degrees of resistance to antimicrobial agents and demonstrate similar abilities to produce mycotoxins (Desjardins et al 2000, Marasas et al 1983, 1984, Sewram et al 1999a, 1999b, Shephard et al 1999, Yan et al 1993). For example, the two isolates in genotype 2-2 are significantly different in their resistance to hygromycin B (Yan et al 1993) and the three South African isolates of genotype 1-1 produce markedly different levels of moniliformin and fusaproliferin (Sewram et al 1999a, 1999b, Shephard et al 1999). Another trait suggesting that isolates with similar genotypes are not 'true clones' is the distribution of mating type. If all the isolates with a specific genotype are clones of one another, their mating types determined using either laboratory crosses or PCR-based methods (Steenkamp et al 2000b) should be the same. Among the *F. subglutinans* isolates studied, this was not the case, because each of the five multi-isolate genotypes was represented by both *MAT-1* and *MAT-2* isolates (Desjardins et al 2000, Steenkamp et al 2000a, Yan et al 1993). This variation within groups of isolates with the same genotype, was undoubtedly generated by earlier sexual recombination events. Even though we did not include sufficient isolates and polymorphic loci to address questions on the reproductive mode of *F. sublutinans*, our results suggest that sexual reproduction is not a common phenomenon. Among the 17 polymorphic sites analyzed, all were fixed within isolates associated with a specific genotype. It is possible that the inclusion of additional data, especially those associated with mycotoxin production, antibiotic resistance and mating, will result in the identification of non-fixed or shared polymorphism. However, the ongoing absence of sex will also bring about fixation in these characters and eventually the absence of shared polymorphisms. Our results, therefore, suggest *F. subglutinans* populations are diverging into various reproductively isolated lineages that will most probably each eventually constitute separate species.

Classifying the isolates used in this study into their smallest diagnosable units or redefining species limits in the existing *F. subglutinans sensu stricto* is problematic. Given that sexual reproduction between these isolates appears to be absent in nature, the biological species concept cannot be applied. The phylogenetic species concept assists to some extent, but as shown above, it identifies 'clones'. Determining whether these 'clones' represent separate species is an arbitrary exercise, since it is impractical to designate each clonal population as a distinct species. Likewise, to classify each as the same species would be incorrect and would not reflect the natural situation. Separating *F. subglutinans* into smaller units/taxa is further complicated by the fact that some isolates differ by no more than a single nucleotide at the six nuclear regions analyzed and display no known or diagnosable phenotypic differences. Among the isolates studied, we have, however, observed two major phylogenetic groups (groups 1 and 2) (FIG. 3), which may represent a species partition where groups 1 and 2 represent cryptic species. Genotypes 1-1, 1-2, 1-3 and 1-4 would belong to group 1 and 2-1, 2-2 and 2-3 would belong to group 2. Whether this partition will prove to be diagnosable using phenotypic characters remains to be tested.

There are no apparent links between the genotype to which the *F. subglutinans* isolates belong and geo-

graphic origins or host (FIG. 3). South African isolates were present in all genotypes except 1-2, 1-3 and 2-2, while Mexican/Guatemala isolates were present in all but genotype 2-2. The United States isolates appeared to be restricted to genotypes 2-1, 2-2 and 2-3. Isolates from maize were present in all genotypes except 1-2 and 1-3, while those from teosinte were only absent from genotype 2-2. From the limited data available, the separation of *F. subglutinans* into the seven genotypes/groups also does not appear to be linked to mycotoxin production (Desjardins et al 2000, Marasas et al 1983, 1984, Sewram et al 1999a, 1999b, Shephard et al 1999) or morphology (Desjardins et al 2000, Steenkamp et al 1999, 2001). There were also no apparent links between the Groups 1 and 2 separation and geographic origin, host or mycotoxin production (FIG. 3) (Desjardins et al 2000, Marasas et al 1983, 1984, Sewram et al 1999a, 1999b, Shephard et al 1999, Steenkamp et al 1999, 2001). Noticeably, maize in the United States is associated only with group 2, but this is probably a sampling effect. The 17 polymorphic sites, therefore, appear to be the only diagnosable features separating the seven genotypes and Groups 1 and 2. As with other fungi (Geiser et al 2000, Peng et al 1999, Taylor et al 2000), our results may lead to the future identification of morphological, physiological, pathogenic, or toxigenic characters supporting either the presence of Groups 1 and 2 or their sub-lineages.

Based on the results presented here, we have attempted to interpret the findings of previous mating studies (Desjardins et al 2000, Steenkamp et al 1999, 2001). Again, there was no relationship between the genotype represented and ability to sexually interact in the laboratory. The genotype 1-1 isolate MRC1084 from South African maize, for example, was capable of producing fertile offspring when mated with genotype 1-3 and 2-1 isolates. Many other isolates were also capable of successful sexual interaction with isolates displaying genotypes other than their own. The presence of the two proposed cryptic species helped to clarify some of the problems that were encountered in previous mating studies (Desjardins et al 2000, Steenkamp et al 1999, 2001). For example, some of the *F. subglutinans* strains that were collected from maize and teosinte in Mexico and Central America were sexually incompatible with those from the United States (Desjardins et al 2000). As revealed here all the available *F. subglutinans* isolates from the United States belong to Group 2, whereas those from Mexico and Central America belong to both Groups 1 and 2, which may partially explain their incompatibility. Our results can, however, not explain why the United States isolates were sexually incompatible even with the Mexican strains from Group 2, or why most of the isolates collected from Mexico and Central America are capable of fertile sexual interaction, even though they represent separate cryptic species. Inclusion of all 29 isolates in a mating study might eventually shed light on the connection between the ability to reproduce sexually and genotype/cryptic species, but conditions in the laboratory do not necessarily mimic those in nature. Many fungi are able to sexually interact across the species barrier (e.g., Vilgalys and Sun 1994), which was also demonstrated for strains of *F. subglutinans* and *F. circinatum* (Desjardins et al 2000, Steenkamp et al 2001).

The inconsistency between the biological and phylogenetic species concepts illustrated in this study introduces serious complications for the classification of fungi, especially those in the *G. fujikuroi* species complex, since classification relies heavily on both concepts. Problems with using the morphological species concept have been reported by many workers (O'Donnell et al 1998a, 2000a, Steenkamp et al 1999, 2001), but the current study is the first to report disparities using the biological species concept for classifying fungi in the *G. fujikuroi* species complex. Undoubtedly, species recognition using phylogenetic concordance analysis will yield the most informative results, but there are two major problems associated with the use of this approach. Firstly, even though we have considerable knowledge on the subject of phylogenetics, new ideas are constantly emerging and we are still in the learning-phase with respect to its methodologies and the interpretation of results. Secondly, we are still in the process of developing a definition for a fungal species and how to recognize this unit. Although we have attempted to address the taxonomic status and reproductive mode of isolates residing in *F. subglutinans sensu stricto*, many questions remain unanswered. These include an understanding of how an asexual species should be defined, how many nucleotide differences constitute a species divergence in *Fusarium* and others. Our study and similar cases will thus represent an interesting topic for discussion as the fields of fungal reproduction and phylogenetic taxonomy advance.

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