Mycoscience 41: 61–78, 2000

A Multigene phylogeny of the *Gibberella fujikuroi* species complex: Detection of additional phylogenetically distinct species

Kerry O'Donnell[1], Helgard I. Nirenberg[2], Takayuki Aoki[3] and Elizabeth Cigelnik[1]

[1] Microbial Properties Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture, Peoria, Illinois 61604, U.S.A.
[2] Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Mikrobiologie, Konigin-Luise-Straße 19, D-14195 Berlin, Germany
[3] National Institute of Agrobiological Resources, Department of Genetic Resources I, Ministry of Agriculture, Forestry and Fisheries, Kannondai, Tsukuba, Ibaraki 305–8602, Japan

Accepted for publication 17 December 1999

Phylogenetic relationships within the *Gibberella fujikuroi* species complex were extended to newly discovered strains using nucleotide characters obtained by sequencing polymerase chain reaction (PCR) amplified DNA from 4 loci used in a previous study [nuclear large subunit 28S rDNA, nuclear ribosomal internal transcribed spacer (ITS) region, mitochondrial small subunit (mtSSU) ribosomal DNA, and β-tubulin] together with two newly sampled protein-encoding nuclear genes, translation elongation factor EF-1α and calmodulin. Sequences from the ribosomal ITS region were analyzed separately and found to contain of two highly divergent, nonorthologous ITS2 types. Phylogenetic analysis of the individual and combined datasets identified 10 new phylogenetically distinct species distributed among the following three areas: 2 within Asia and 4 within both Africa and South America. Hypotheses of the monophyly of *Fusarium subglutinans* and its two formae speciales, f. sp. *pini* and f. sp. *ananas*, were strongly rejected by a likelihood analysis. Maximum parsimony results further indicate that the protein-encoding nuclear genes provide considerably more phylogenetic signal that the ribosomal genes sequenced. Relative apparent synapomorphy analysis was used to detect long-branch attraction taxa and to obtain a statistical measure of phylogenetic signal in the individual and combined datasets.

Key Words——biogeography; calmodulin; DNA sequence; elongation factor EF-1α; evolution.

The *Gibberella fujikuroi* complex is a species-rich monophyletic lineage with anamorphs in *Fusarium* (O'Donnell and Cigelnik, 1997). Many of the fusaria within this complex produce toxic secondary metabolites such as fumonisins, moniliformin (Nelson et al., 1992) and beauvericin (Moretti et al., 1996), and agronomically-important plant diseases such as the gibberellin phytohormone-induced "bakanae" of *Oryza sativa* (Sun and Snyder, 1981; Cerdá-Olmedo et al., 1994). Species limits within this complex have been the subject of much debate over the past thirty years (Booth, 1971; Nirenberg, 1976; Gerlach and Nirenberg, 1982; Nelson et al., 1983). In recent years species concepts within this complex have been studied intensively using morphology (Nirenberg and O'Donnell, 1998; Nirenberg et al., 1998), mating experiments (Leslie, 1995), and phylogenetic analyses of DNA sequence data (O'Donnell and Cigelnik, 1997; O'Donnell et al., 1998a). The molecular systematic studies have revealed perfect concordance between phylogenetic species resolved as exclusive populations or lineages within the gene trees and mating populations or biological species, indicating that these two species concepts identify the same fundamental taxonomic units (O'Donnell et al., 1998a). However, because teleomorphs are only known for one-fifth of the species reported in the present study, and many species are morphologically simple, development of a robust phylogenetic species concept for these fusaria based on discrete DNA sequence data is critical to investigate fundamental aspects of their biology such as host range, mycotoxin potential and geographic distribution. Towards this end, we have studied the phylogenetic utility of sequences that encode translation elongation factor (EF-1α) (O'Donnell et al., 1998b) and calmodulin (Carbone et al., 1998), together with 4 genes employed in prior molecular systematic studies of the *G. fujikuroi* species complex (O'Donnell and Cigelnik, 1997; O'Donnell et al., 1998a), to address the following questions: (1) What is the relative contribution of phylogenetic signal of each gene? (2) Are gene trees inferred from each locus concordant? (3) Do any of the newly studied strains represent phylogenetically distinct species, and if so, what is their biogeographic history? and (4) Does the formae speciales concept as applied to *F. subglutinans* f. sp. *pini* (Correll et al., 1992) and *F. subglutinans* f. sp *ananas* (Ventura, 1998) accurately reflect a monophylet-

ic grouping with *F. subglutinans*?

Materials and Methods

Strains studied. The 14 strains that represent 10 newly discovered phylogenetically distinct species within the *Gibberella fujikuroi* species complex listed in Table 1 are stored in liquid nitrogen vapors at −175°C or by lyophilization in the ARS Culture Collection (NRRL), NCAUR, Peoria, IL. Data for the other 38 strains included in this study are reported in O'Donnell et al. (1998a) and Nirenberg and O'Donnell (1998).

Molecular methods. Total genomic DNA was extracted from mycelium grown in yeast-malt broth using a CTAB (hexadecyltrimethylammonium bromide; Sigma Chemical Co., St. Louis) miniprep described by Gardes and Bruns (1993). The mitochondrial small subunit rDNA and ribosomal internal transcribed spacer (ITS) region were amplified by the polymerase chain reaction (PCR) using conditions and primers described in White et al. (1990), the β-tubulin gene as in O'Donnell and Cigelnik (1997), and EF-1α after O'Donnell et al. (1998b). A portion of the calmodulin gene was amplified with the PCR primer pair CL1 5′-GA(GA)T(AT)CAAGGAGGCCTTCTC and CL2A 5′-TTTTTGCATCATGAGTTGGAC. Two internal primers were used to sequence this amplicon, CL11 5′-ACCATGATGGCGCGCAAG and CL22 5′-TCCTTCATCTTGCGCGCC. Following PCR, amplicons were purified with GeneClean II (Bio101, La Jolla, CA) and sequenced either with the Applied Biosystems Taq DyeDeoxy Terminator or BigDye cycle sequencing kit in a Perkin Elmer 9600 thermal cycler as described in O'Donnell et al. (1998a). All sequencing reactions were purified by gel filtration through columns containing Sephadex G-50 (Pharmacia; Piscataway, NJ) equilibrated in double-distilled H_2O and were run on an Applied Biosystems 377 DNA sequencer.

Phylogenetic analysis. DNA sequences from each of the 6 loci sequenced (Table 3) were aligned visually with the TSE DOS text editor program (SemWare, Marietta, GA) and have been deposited in GenBank under the following accession numbers: AF158288-AF158366 and AF158614-AF158627. Phylogenetic analyses were performed with PAUP*4.0b1 (Swofford, 1998) on the

Table 1. New phylogenetically distinct species within the *Gibberella fujikuroi* complex.

Clade/taxon[a]	NRRL#[b]	Equivalent#[c]	Geographical Origin	Host/Substrate	Received as
African					
Fusarium sp.	25615	BBA 63165	Nigeria	*Oryza sativa* seed	*F.* cf. *verticillioides*
Fusarium sp.	26061	BBA 70127	Madagascar	*Striga hermonthica*	*F.* cf *dlaminii*
	26152	BBA 70170	Niger		
Fusarium sp.	26064	BBA 70142	Tanzania	*Sorghum bicolor* seed	*F.* cf. *pseudonygamai*
Fusarium sp.	26793	BBA 65862= CBS 454.97	Sudan	*Striga hermonthica*	*F. nygamai*
Asian					
Fusarium sp.	26427	CBS 480.96	Papau-New Guinea	tropical rain forest soil	*F. subglutinans*
Fusarium sp.[d]	26794	MAFF 237530= BBA 70371	Japan	*Cymbidium* sp.	*Fusarium* sp.
	28852	MAFF 237529			
American					
Fusarium sp.?[e]	25622	MRC 1077	South Africa	*Zea mays*	*F. subglutinans*
Fusarium sp.[e]	25623	MRC 2802	South Africa	mango	*F. subglutinans*
Fusarium sp.[e]	26756	MRC 6747	South Africa	ornamental grass	*F. subglutinans*
	26757	MRC 6748		ornamental reed	
Fusarium sp.	29123	ERSP 199A	Ft. Pierce, FL	*Bidens pilosa*	*Fusarium* sp.
	29124	ERSP 199B			

a Clades of the *Gibberella fujikuroi* complex as reported in O'Donnell et al. (1998a).

b NRRL=ARS Culture Collection, NCAUR, Peoria, IL.

c BBA=Biologische Bundesanstalt für Land-und Forstwirtschaft, Berlin, Germany, CBS=Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; MAFF=Ministry of Agriculture, Forestry and Fisheries, NIAR, Tsukuba, Japan; MRC=Medical Research Council, Tygerberg, South Africa; ERSP=Erin Rosskopf, Agricultural Research Service, Ft. Pierce, FL.

d Collected and deposited in MAFF by Kazunori Ichikawa, Yamanashi Agriculture Research Center, Kita-koma, Yamanashi 407-0105 Japan.

e These three species were received identified as *F. subglutinans* (Viljoen et al., 1997). NRRL 25622 is listed as *Fusarium* sp.? because this species was resolved as phylogenetically distinct from *F. subglutinans* in the molecular phylogeny inferred from the combined DNA sequence data presented in the present study, and in a histone H3 gene tree (Steenkamp et al., 1999); however, this strain was reported by Steenkamp et al. (1999) to be sexually compatible with a *F. subglutinans* MP-E mating type tester strain.

Table 2. Tree statistics.

Locus	# Characters (bp)	Parsimony tree length	# Trees	CI/RI[a]	TiTv[b]	Aut/syn[c]	# Nodes ≥50% bootstrap	# Nodes ≥70% bootstrap	# Nodes ≥90% bootstrap	Ave. # steps/ character
ITS rDNA[d]	525	48	96	0.90/0.88	0.955	8/31	—	—	—	—
28S rDNA	535	28	18	0.86/0.92	4.620	13/12	2	1	1	0.052
mtSSU rDNA	701	138	>66,000	0.67/0.90	2.121	18/54	15	6	4	0.197
β-tubulin	1254	429	776	0.75/0.91	3.087	115/171	36	27	22	0.342
calmodulin	688	219	1116	0.82/0.93	3.124	67/94	27	20	13	0.318
EF-1α	671	542	2240	0.61/0.77	2.820	100/149	31	22	13	0.809
combined data (excluding ITS)	3849	1449	135	0.65/0.84	2.743	313/480	40	31	25	0.377

a CI=consistency index, RI=retention index.
b Ti=Transition/Tv=transversion ratios were estimated using the likelihood option in PAUP*4.01b.
c Aut=autapomorphies, syn=synapomorphies.
d Seventeen contiguous ITS characters were excluded as ambiguous (O'Donnell and Cigelink, 1997). Only 24 of the 52 ITS sequences were analyzed because they comprise two sets of paralogs or xenologs. Because the sequences are nonorthologous, we rooted the ITS gene tree by the midpoint method in PAUP*4.0b1 (Swofford, 1998).

individual and combined datasets, using the heuristic search option with 1,000 random addition sequences with MULPARS on and tree bisection-reconnection branch swapping. Phylogenetically informative indels were coded as a single event (i.e., fifth state). Individual and combined datasets were also analyzed with maximum likehood (ML) using the Hasegawa-Kishino-Yano (HKY) model, in which the transition/transversion ratio was estimated and with the neighbor-joining (NJ) uncorrected "p" option in PAUP*4.01b. Because the ITS sequences represent highly divergent paralogs or xenologs (O'Donnell and Cigelnik, 1997), we rooted this gene tree by the midpoint method in PAUP*4.01b. All of the other gene trees were rooted by the outgroup method using sequences of a putative sister group, the *Fusarium oxysporum* complex (O'Donnell et al., 1998b). Stability of clades was assessed by decay indices calculated with TreeRot (Sorenson, 1996) and by 1,000 parsimony bootstrap replications implemented with PAUP*4.0b1 (Hillis and Bull, 1993). Various constrained and unconstrained topologies (Table 3) were compared by the Kishino-Hasegawa likelihood test using PAUP*4.0b1. If alternate topologies were >1.96 SD less likely than the most-parsimonious tree (MPT), then they were rejected with 95% confidence. Concordance of the 5 datasets sequenced, excluding the ITS rDNA, was assessed by the nonparametric Wilcoxon Signed-Ranks (WS-R) Templeton test and Kishino-Hasegawa's modification of the Templeton test implemented in PAUP*4.0b1, using the MPT and a 70% majority rule bootstrap consensus tree as constraints in separate analyses (Kellogg et al., 1996). Based on the results of the Templeton test (Templeton, 1983; Table 4), we evaluated the concordance of the two most homogeneous datasets with the partition-homogeneity test (PHT) implemented in PAUP*4.0b1, using simple addition-sequence, tree-bisection-reconnnection, with 1,000 random repartitions with MAXTREES set to 500 (O'Donnell et al., 1998b). Unrooted RASA analyses (Relative Apparent Synapomorphy Analysis version 2.3.7; Lyons-Weiler, 1999) were used to investigate potential long-branch attraction problems and phylogenetic signal in the various individual

Table 3. Likelihood analysis of constrained and unconstrained trees from combined dataset.

Tree[a]	Tree length (steps)	# of trees[b]	Log liklihood (L) test result			
			ln L	Difference[c]	SD[d]	P[e]
MPT (Fig. 9A)	400	2	−7873.69398	(Best)		
F. subglutinans-Fusarium sp. 25622 monophyletic	404 (+4)	1	−7900.38927	−26.69529	21.65355	0.2177
F. subglutinans-F. guttiforme monophyletic (Fig. 9B)	420 (+20)	2	−7967.67268	−93.97870	25.84878	0.0003
F. subglutinans-F. circinatum monophyletic (Fig. 9C)	423 (+23)	4	−7974.97088	−101.27690	27.36212	0.0002
F. subglutinans-F. guttiforme-F. circinatum monophyletic (Fig. 9D)	428 (+28)	2	−8012.59880	−138.90482	34.86463	0.0001

a Monophyly constrains enforced with PAUP*4.0b1 (Swofford, 1998).
b Only the best tree from each constraint was included in this test.
c Difference in ln likelihood (L) between best tree and suboptimal tree.
d SD of log likelihood.
e Probability of obtaining a more extreme T-value, using the two-tailed test, with the null hypothesis being that there is no difference between the two trees. All values are significant at $P = < 0.05$.

Table 4. Summary of Templeton (WS-R) and Kishino-Hasegawa test results[a] for *Gibberella fujikuroi* DNA sequence datasets.

Comparison[b]		Templeton (WS-R) test		Kishino-Hasegawa test	
Data	Tree[c]	N	*P*[d]	Length difference	*P*[e]
β-tubulin	mtSSU rDNA	27/7	0.0003/0.0082	19/7	0.0002/0.0081
β-tubulin	calmodulin	15/1	0.0201/0.3173	9/1	0.0210/0.3175
β-tubulin	EF-1α	54/24	<0.0001/<0.0001	68/22	<0.0001/<0.0001
β-tubulin	28S rDNA	0	—	0	1.0000/1.0000
β-tubulin	combined (-β-tubulin)	12/11	0.0005/0.0009	12/11	0.0005/0.0009
mtSSU rDNA	β-tubulin	23/12	0.0012/0.1176	23/9	0.0019/0.1060
mtSSU rDNA	calmodulin	19/14	0.0007/0.0456	24/12	0.0019/0.0454
mtSSU rDNA	EF-1α	27/14	<0.0001/0.0350	57/12	<0.0001/0.0395
mtSSU rDNA	28S rDNA	0	—	0	1.0000/1.0000
mtSSU rDNA	combined (-mtSSU rDNA)	23/16	0.0008/0.0041	26/21	0.0011/0.0046
mtSSU rDNA	Asia monophyletic	10	0.4054	3	0.4058
calmodulin	β-tubulin	11/7	0.0881/0.5271	7/2	0.0896/0.5275
calmodulin	mtSSU rDNA	18/4	<0.0001/0.0455	18/4	<0.0001/0.0454
calmodulin	EF-1α	25/7	<0.0001/0.0114	45/8	<0.0001/0.0113
calmodulin	28S rDNA	0	—	0	1.0000/1.0000
calmodulin	combined (-calmodulin)	15/9	0.0047/0.0956	12/5	0.0046/0.0956
calmodulin	Africa monophyletic	11	0.0009	11	0.0009
EF-1α	β-tubulin	37/21	<0.0001/<0.0001	30/9	<0.0001/<0.0001
EF-1α	mtSSU rDNA	27/26	0.0001/0.0004	25/20	0.0001/0.0006
EF-1α	calmodulin	21/24	0.0143/0.0343	12/11	0.0142/0.0342
EF-1α	28S rDNA	0	—	0	1.0000/1.0000
EF-1α	combined (-EF-1α)	35/36	0.0007/0.0015	25/23	0.0003/0.0015
EF-1α	Africa monophyletic	21	0.0277	12	0.0284
28S rDNA	β-tubulin	5	0.0412/0.0384	10/8	0.0411/0.0324
28S rDNA	mtSSU rDNA	6/4	0.1025/0.3173	4/2	0.1025/0.3178
28S rDNA	calmodulin	6	0.0836/0.0836	8/6	0.0881/0.0833
28S rDNA	EF-1α	6/5	0.0260/0.0339	12/6	0.0338/0.0338
28S rDNA	combined (-28S rDNA)	5	0.0412/0.0384	10/8	0.0411/0.0324
combined (-β-tubulin)	β-tubulin	46/34	0.0032/0.0396	20/12	0.0032/0.0396
combined (-mtSSU rDNA)	mtSSU rDNA	52/22	<0.0001/0.0006	40/16	<0.0001/0.0006
combined (-calmodulin)	calmodulin	30/11	0.0090/0.1317	15/5	0.0090/0.1317
combined (-EF-1α)	EF-1α	98/38	<0.0001/<0.0001	126/34	<0.0001/<0.0001
combined (-28S rDNA)	28S rDNA	0	—	0	1.0000/1.0000

a Implemented in PAUP 4.0b1 (Swofford, 1998).

b In each comparison an unconstrained MPT inferred from the data in the Data column was constrained onto the tree in the Tree column. The Templeton and Kishino-Hasegawa test statistics represent two separate sets of comparisons: one for the complete dataset of 52 taxa (indicated by the number to the left in each column), and one for a reduced dataset of 47 taxa (indicated by the number to the right in each column). In the reduced dataset three African strains (*F. dlaminii*, *Fusarium* sp. NRRL 26061 and 26152) and 2 Asian strains (*Fusarium* sp. NRRL 26794 and 28852) were excluded based on their variable position within the gene trees.

c All trees are 70% majority rule bootstrap consensus except for the three monophyly constraints.

d Approximate probablity, using a two-tailed test, of getting a more extreme test statistic under the null hypothesis of no difference between the two trees. For *P* values with $N \geq 15$, we found moderate but consistent accuracy; for *P* values with $N < 15$, we found some error, but never of value greater than 4% (Bain and Engelhardt, 1992).

e Net gain of steps of the constrained tree in the Tree column compared with the unconstrained MPT inferred from the data in the Data column.

and combined datasets (Table 5), using the analytical null hypothesis option. Invariant characters were excluded in all analyses; however, separate analyses were run in which apparent autapomorphies were either included or excluded.

MacClade (Maddison and Maddison, 1992) was used to examine evolution of the major ITS2 type and biogeographic structure within the *G. fujikuroi* complex.

Table 5. Unrooted relative apparent synapomorphy analysis (RASA[a]) on various datasets.

Dataset[b]	Including apparent autapomorphies		Excluding apparent autapomorphies	
	tRASA[c]	Taxa with a significant Ftv[d]	tRASA	Taxa with a significant Ftv
28S rDNA (565/236/173/63)	−8.3818	none	2.951097	none
mSSU rDNA (701/85/14/71)	25.70826	*Fusarium* sp. 26794 (15.33474)	22.16162	*Fusarium* sp. 26794 (14.63356)
		Fusarium sp. 28852 (15.33474)		*Fusarium* sp. 28852 (14.63356)
		Fusarium inflexum (14.62679)		*Fusarium inflexum* (12.53192)
		Fusarium oxysporum (12.17986)		*Fusarium oxysporum* (10.4972)
β-tubulin (1254/405/121/284)	59.12709	*Fusarium* sp. 26061 (8.725021)	58.31345	*Fusarium* sp. 26061 (8.306092)
		Fusarium sp. 26152 (9.701594)		*Fusarium* sp. 26152 (8.306092)
		F. inflexum (10.00433)		*F. inflexum* (12.0329)
		Fusarium napiforme (7.99417)		
EF-1α (671/276/95/181)	47.86938	*F. pseudoanthophilum* (8.843337)	47.59653	*F. pseudoanthophilum* (8.474301)
		F. verticillioides (11.89439)		*F. verticillioides* (10.85759)
		F. pseudonygamai (12.75548)		*F. pseudonygamai* (10.20029)
		F. thapsinum (12.20436)		*F. thapsinum* (10.33169)
		F. nygamai (7.598962)		*F. nygamai* (7.684067)
		F. acutatum (8.233829)		*F. acutatum* (11.16473)
		F. sacchari (11.97514)		*F. sacchari* (9.120505)
		F. fujikuroi (13.75982)		*F. fujikuroi* (9.424773)
		F. proliferatum (7.601569)		*F. proliferatum* (7.52675)
		F. begoniae (7.51088)		*F. begoniae* (7.756498)
		Fusarium sp. 26794 (7.47285)		*Fusarium* sp. 26794 (7.604307)
		Fusarium sp. 25346 (7.415811)		*Fusarium* sp. 25346 (7.929488)
		Fusarium sp. 26152 (7.910438)		*Fusarium* sp. 26152 (7.24696)
		Fusarium sp. 26756 (7.741902)		*Fusarium* sp. 26756 (7.957061)
		Fusarium sp. 26757 (7.752123)		*Fusarium* sp. 26757 (7.957061)
		F. oxysporum (7.448487)		*F. oxysporum* (7.94805)
		Fusarium sp. 25807 (11.76222)		*F. inflexum* (7.97699)
				F. brevicatenulatum (7.882109)
				F. denticulatum (7.471587)
				F. lactis (7.348853)
				F. bulbicola (7.809971)
				F. circinatum (7.317918)
				F. guttiforme (7.577434)
				F. globosum (7.379686)
				F. anthophilum (7.3141029)
				Fusarium sp. 25204 (7.563562)
				Fusarium sp. 25615 (9.859931)
				Fusarium sp. 25622 (7.386989)
				Fusarium sp. 25615 (9.859931)
				Fusarium sp. 26793 (7.277243)
				Fusarium sp. 29123 (7.245568)
				Fusarium sp. 29124 (7.245568)
calmodulin (688/570/413/517)	−2.811153	none	9.142787	*Fusarium* sp. 25221 (95.06038)
combined data (3849/1600/830/770)	9.204197	*Fusarium* sp. 25221 (32.01379)	44.33425	*Fusarium* sp. 25221 (32.52776)
				F. oxysporum (7.607265)
				F. inflexum (9.102866)

a Conducted with RASA 2.3.7 (Lyons-Weiler, 1999) using the analytical null hypothesis option.

b Numbers in parentheses represent: (total number of characters/informative characters including apparent autapomorphies/apparent autapomorphies/informative characters excluding apparent autapomorphies)

c tRASA=test statistics is a measure of the difference between the observed null slopes. Except for the 2 negative values, all tRASA scores are significant at $P<0.005$ (Bain and Engelhardt, 1992).

d Ftv=taxon variance ratio where the critical value is 7.23228 when alpha=0.05.

Thirty-one of the ingroup species were given biogeographic assignments based on their exclusive distribution within a give region, or based on the fact that one or more strains of the species that appear to have restricted geographic distributions and host ranges, was collected within the same region as the clade within which they were nested in the molecular phylogeny (O'Donnell et al., 1998a). Of the 10 putative phylogenetically distinct species reported on in the present study, only 3 species (*Fusarium* spp. 25622, 25623 and 26756–26767) were given equivocal biogeographic assignments (Kluge, 1988). However, the geographic origin of all 3 species was resolved as the American clade by MacClade (Maddison and Maddison, 1992).

Results

Ingroup strains analyzed within the *Gibberella fujikuroi* species complex consisted of the 36 terminals reported in O'Donnell et al. (1998a), 14 strains recently accessioned in the ARS Culture Collection [NRRL] (Table 1), together with two outgroups within a putative sister group, the *Fusarium oxysporum* complex. Sequences of the nuclear large subunit 28S rDNA lack sufficient informative variation for phylogenetic reconstruction (Table 2; gene tree not shown) as reflected in the negative and low tRASA scores for this locus (Table 5). Gene trees inferred from the ribosomal DNA internal transcribed spacer (ITS) region resolve two highly divergent unresolved clusters (Fig. 1) corresponding to type I and type II nonorthologous ITS2 sequences described previously (Waalwijk et al., 1996; O'Donnell and Cigelnik, 1997; O'Donnell et al., 1998a). The newly discovered phylogenetically distinct species within a gene tree constructed from a subset of the ITS sequence data are equally divided between type I and type I ITS2 sequences. Sequences of the ITS region were not analyzed further because they are nonorthologous and uninformative for parsimony.

Maximum parsimony phylogenetic analysis of the mitochondrial small subunit (mtSSU) rDNA dataset yielded >66,000 MPTs in which 6 of the 10 new species were resolved as phylogenetically distinct (Fig. 2). Bootstrapping and decay analysis provided support for the monophyly of the *G. fujikuroi* complex (bootstrap=100, decay=6) and the American (bootstrap=83%, decay=2) and Asian clades (bootstrap=90%, decay=4). Two Asian strains (NRRL 26794=MAFF 237530 and NRRL 28852=MAFF 237529) ex *Cymbidium* sp. from Japan, which represent a morphologically distinct species by analysis of the original MAFF strains, formed a basal sister-group to the remaining ingroup taxa. However, a constraint forcing these strains to form a monophyletic group with the Asian clade, when subjected to the Kishino-Hasegawa test in PAUP*4.0b1, was only 3 steps longer and not significantly worse than the MPT (P=0.7591 from a two-tailed test of getting a more extreme T-value under the null hypothesis of no difference between the two trees). Maximum likelihood (ML; -Ln likelihood=1767.30949, estimated ti/tv=0.99) using

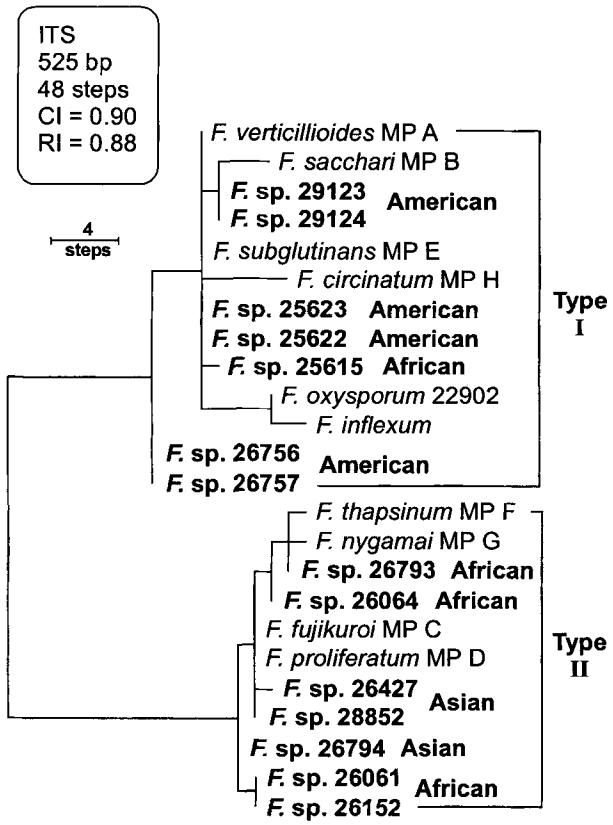


Fig. 1. One of 96 MPTs for the ITS sequences, excluding an ambiguously aligned region of 17 bp. The two divergent clusters, indicated by Type I and II, represent paralogous or xenologous ITS2 sequences. Strains in bold indicate the 10 newly discovered species. MP=mating population or biological species in the *Gibberella fujikuroi* complex.

the HKY model and neighbor joining (NJ) uncorrected "p" yielded trees nearly identical in topology to the MP trees in which the two Asian strains appeared on a long branch at the base of the trees (data not shown).

Next we analyzed exon and intron sequences of the following three protein-encoding nuclear genes phylogenetically: β-tubulin (Fig. 3), EF-1α (Fig. 4) and calmodulin (Fig. 5). The strongest support for the *G. fujikuroi* complex and an ((American (Asian, African)) sister group relationship of these biogeographically structured clades was provided by the β-tubulin gene sequences (Table 2) followed by the calmodulin dataset. Even though the EF-1α sequences possessed the highest average number of steps, synapomorphies and autapomorphies per character (Table 2, based on maximum parsimony analysis), it contained the most homoplasy (CI=0.60), and only the American clade received strong bootstrap support within the EF-1α gene tree (Fig. 4). The two most basal African taxa within the β-tubulin gene tree, *F. dlaminii* and *Fusarium* sp. (NRRL 26061 and 26152), formed a basal sister group to the remaining ingroup taxa within the EF-1α (Fig. 4) and calmodulin (Fig. 5) gene trees. Although a constraint forcing these strains to form a monophyletic group with the African clade in the

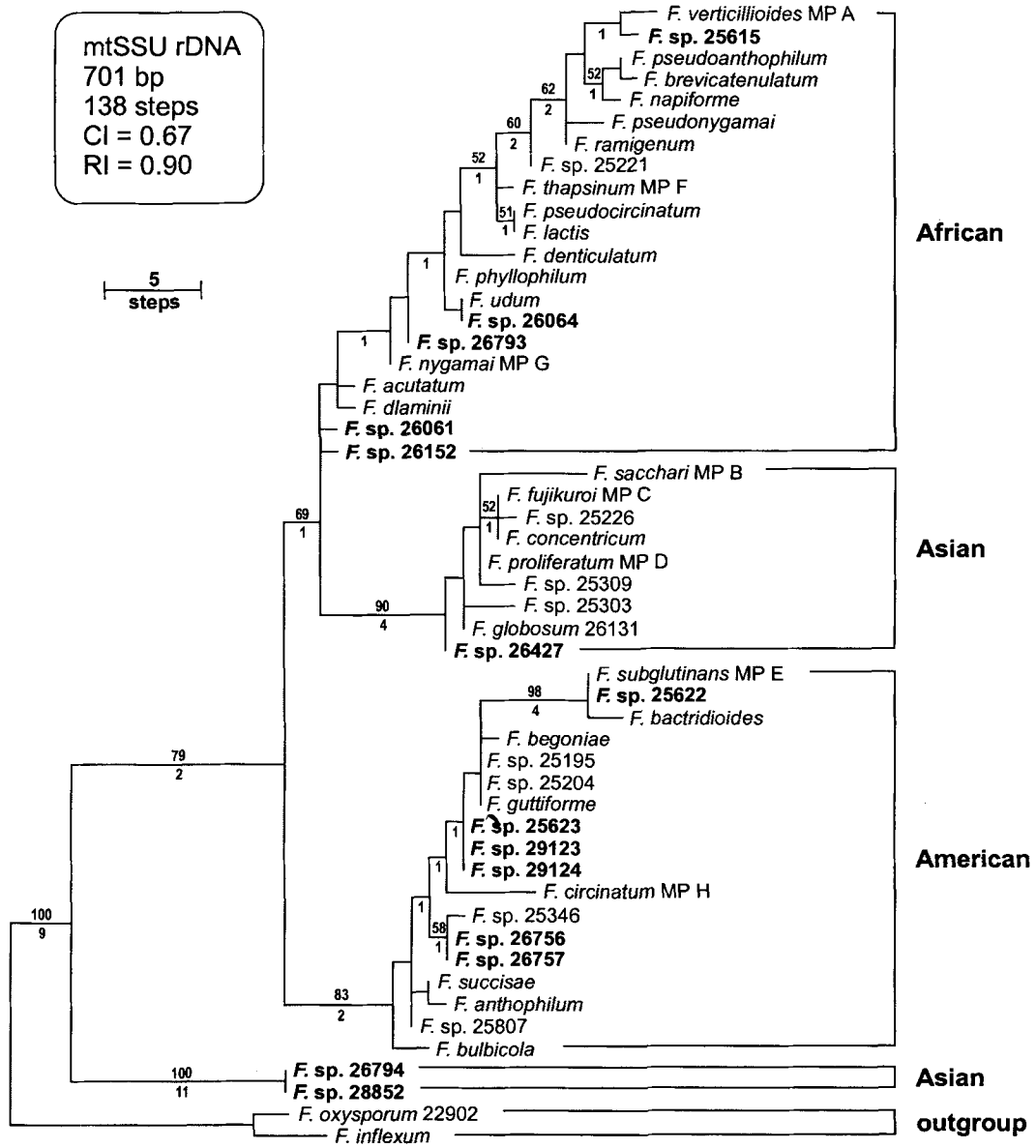


Fig. 2. One of >66,000 MPTs from the mtSSU rDNA data rooted by the outgroup method. Bootstrap intervals ≥50% (above internodes) and decay indices (below internodes) are indicated. Eight biological species in the *G. fujikuroi* complex (MP A–H) are indicated. *F. udum* produces a *G. intrica* teleomorph (Rai and Upadhyay, 1982) but has not been accessioned in the alphabetic MP series. The 10 new species reported in this study are in bold. A constraint, using PAUP*4.0b1 (Swofford, 1998), that forced *Fusarium* sp. 26794 and 28852 to form a monophyletic group with the Asian clade was 3 steps longer but not significantly worse than the MPT.

EF-1α gene tree was only 6 steps longer and not significantly worse than the MPT (P=0.2209 of getting a more extreme T-value under the null hypothesis of no difference between the two trees), a similar constraint using the calmodulin data was 11 steps longer and significantly worse than the MPT (P=0.0012) when subjected to the Kishino-Hasegawa test in PAUP*4.0b1. ML analysis of the EF-1α dataset resolved a monophyletic African clade (-Ln likelihood=3807.68337, estimated ti/tv ratio=2.77); however, ML analysis of the calmodulin data recovered a tree nearly identical in topology to the MP tree (-Ln likelihood=2322.51386, estimated ti/tv ratio=3.12) (data not shown). Including the 10 new undescribed fusaria, forty-four phylogenetically distinct species were resolved within the *G. fujikuroi* complex within the β-tubulin and EF-1α gene trees and in trees inferred from the combined sequence data (Fig. 6). Except for two African species that shared the same calmodulin haplotype [*F. udum* and *Fusarium* sp. NRRL 26064], the 42 other ingroup taxa were resolved by sequence data from this locus. Africa is the most phylogenetically diverse area studied with 20 species, followed by America (16 species) and Asia (8 species). The African and American clades each contain 4 of the

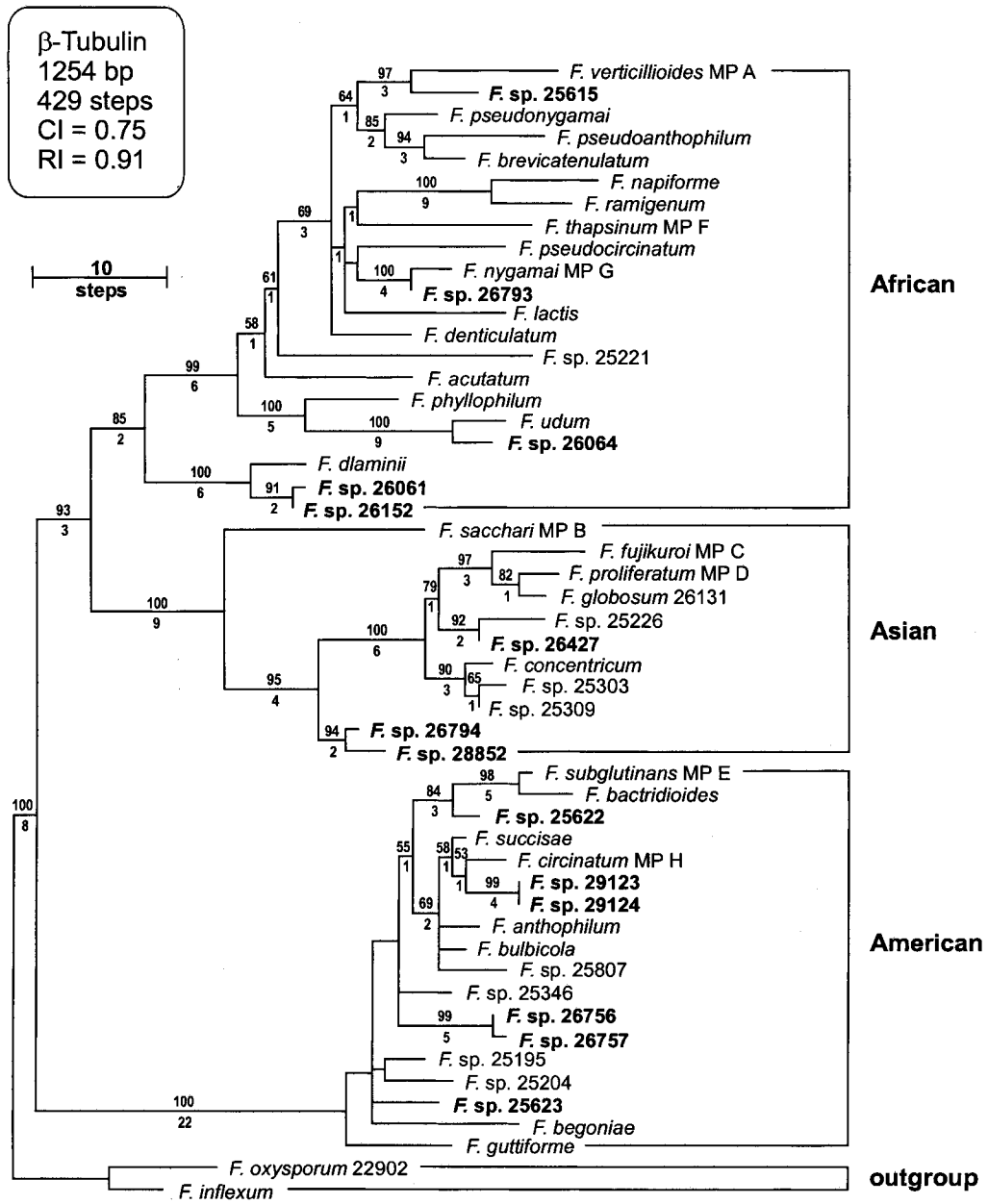


Fig. 3. One of 776 MPTs inferred from β-tubulin gene exons and introns. Bootstrap intervals ≥50% (above internodes) and decay indices (below internodes) are indicated. Monophyly of the *G. fujikuroi* complex and its three biogeographically structured clades are strongly supported by bootstrapping. New species are in bold. MP=biological species.

new species while 2 new species are nested within the Asian clade. Results of the present study indicate that 2 taxa reported as phylogenetically distinct in O'Donnell et al. (1998a), *Fusarium* sp. NRRL 25303=MAFF 237649 ex *Orzya sativa* root in Tsukuba and NRRL 25309=MAFF 237650 ex *Triticum aestivum* seed from Ibusuki, Kagoshima, Japan, may be *F. concentricum* (Fig. 6). Thorough morphological reexamination of the original MAFF strains revealed that they fit the description of this taxon (Nirenberg and O'Donnell, 1998). Of the 5 datasets included in the combined analysis, the 3 protein-encoding genes proved to be considerably more phylogenetically informative for parsimony than either the nuclear large subunit 28S rDNA or mtSSU rDNA (Fig. 7; Table 2). Results of the Templeton and Kishino-Hasegawa tests (Table 4) indicate that β-tubulin and calmodulin are the two most concordant datasets. Comparisons that included the 28S rDNA yielded high *P* values; however, this is because this locus is uninformative for parsimony. The EF-1α and β-tubulin partitions, in contrast, were identified as the most incongruent ($P = < 0.0001$) followed by the EF-1α and mtSSU rDNA datasets. Separate tests were run on the complete dataset comprising 52 taxa and on a reduced dataset after 5 problematical taxa (i.e.,

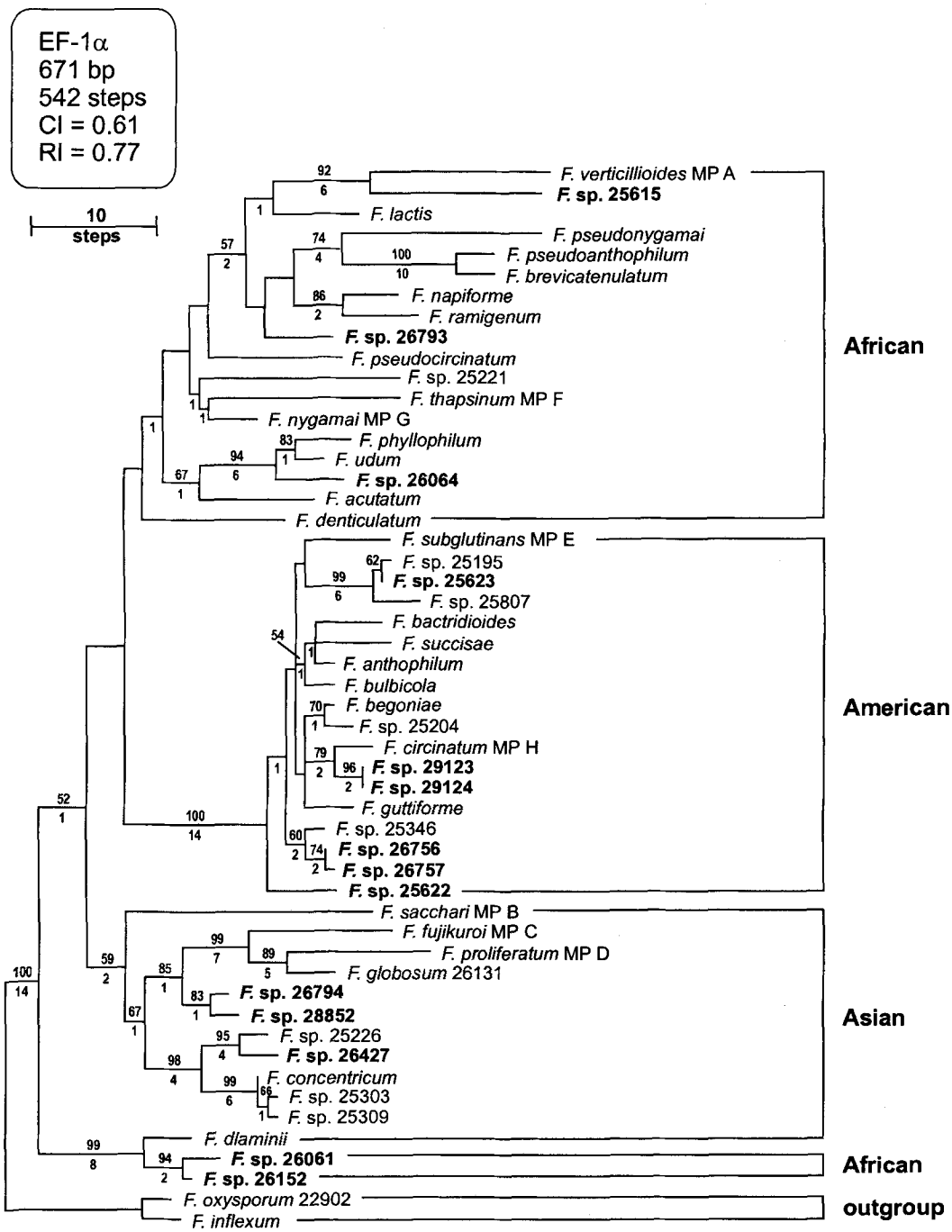


Fig. 4. One of 2240 MPTs inferred from the translation elongation factor EF-1α sequence data. Bootstrap replication frequencies $\geq$50% (above internodes) and decay indices (below internodes) are indicated. A constraint, using PAUP*4.0b1, that forced *F. dlaminii-Fusarium* sp. 26061 and 26152 to form a monophyletic group with the African clade was 6 steps longer and not significantly worse than the MPT.

F. dlaminii, *Fusarium* sp. 26061/26152 and *Fusarium* sp. 26794/28852) were pruned because their placement in the gene trees varied (Table 4). When compared with the complete dataset, *P* values from the reduced dataset of 47 taxa showed a significant increase in over one-half of the tests involving comparisons of the individual partitions. Nevertheless, the Templeton test revealed highly significant incongruence between some pairs of genes in the reduced dataset (Table 4). For this reason, we only ran the PHT on the two most concordant partitions identified by the Templeton test, β-tubulin and calmodulin. No additional PHTs were conducted because *P* values for the complete (P=0.005) and reduced datasets (P=0.014) were so low.

Because the biogeographically structured clades identified in O'Donnell et al. (1998a) were not resolved

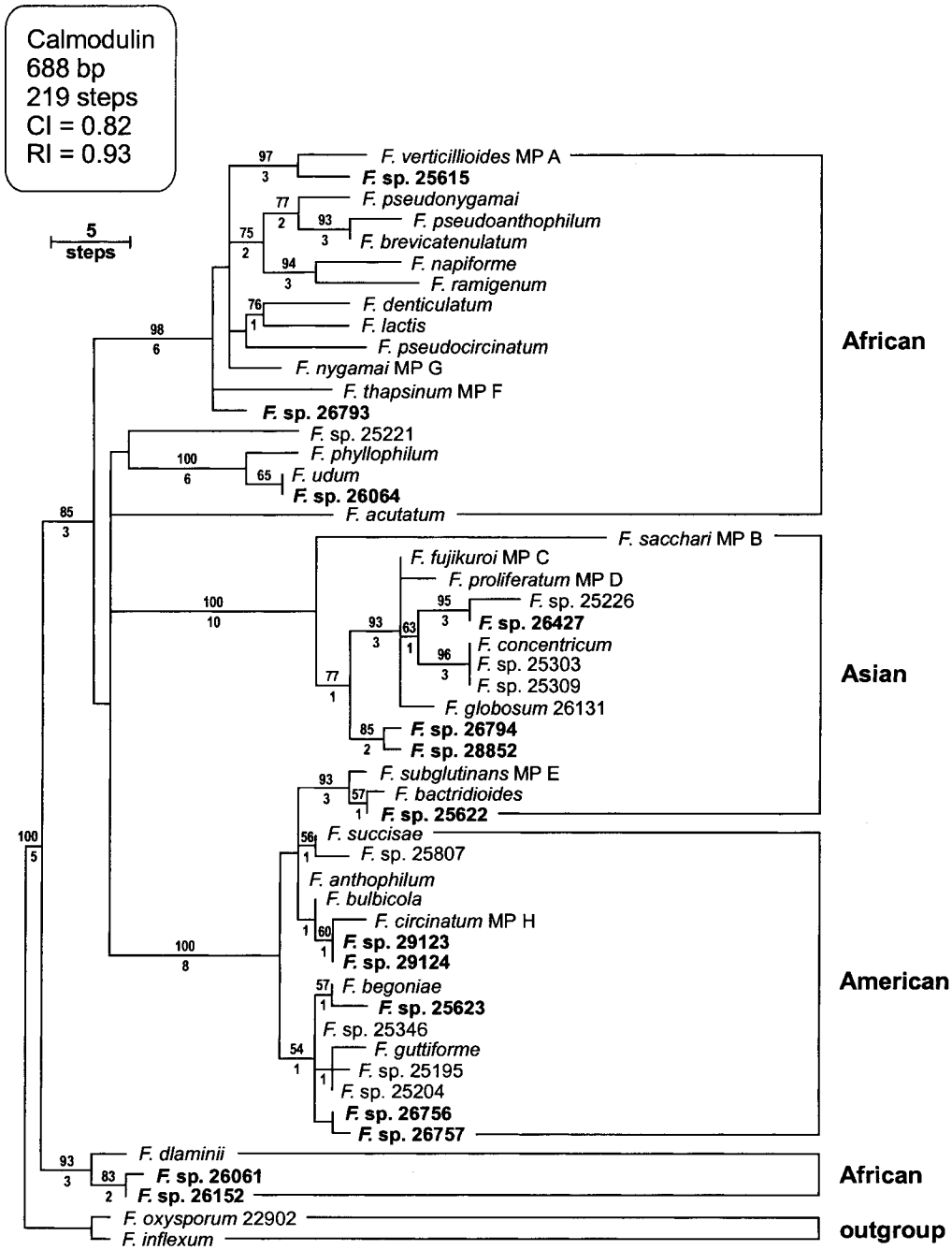


Fig. 5. One of 1116 MPTs inferred from the calmodulin sequence data. Strains in bold represent the 10 new species. Bootstrap intervals $\geq 50\%$ (above internodes) and decay indices (below internodes) are indicated. A constraint, using PAUP*4.0b1, that forced *F. dlaminii-Fusarium* sp. 26011 and 26152 to form a monophyletic group with the African clade was 11 steps longer and significantly worse than the MPT.

as monophyletic in the mtSSU rDNA (Fig. 2), EF-1α (Fig. 4) and calmodulin (Fig. 5) gene trees, we conducted unrooted RASA analyses (Lyons-Weiler, 1999) to determine whether these topological differences were due to long-branch attraction problems (Lyons-Weiler and Hoelzer, 1997), and to obtain an independent measure of phylogenetic signal from the loci sequenced (Lyons-Weiler et al., 1996). RASA analysis detected significant variance ratios ($P<0.05$) for taxa whose placement in the parsimony mtSSU rDNA (Fig. 2) and EF-1α (Fig. 4) gene trees appears to be distorted by long branches (Table 5). However, RASA analysis of the 28S rDNA data did not detect any long-branch taxa, but the low tRASA scores for this locus indicate that it is depauperate in phylogenetic signal. Interestingly, *Fusarium* sp. 25221 was identified as a long-branch taxon in the

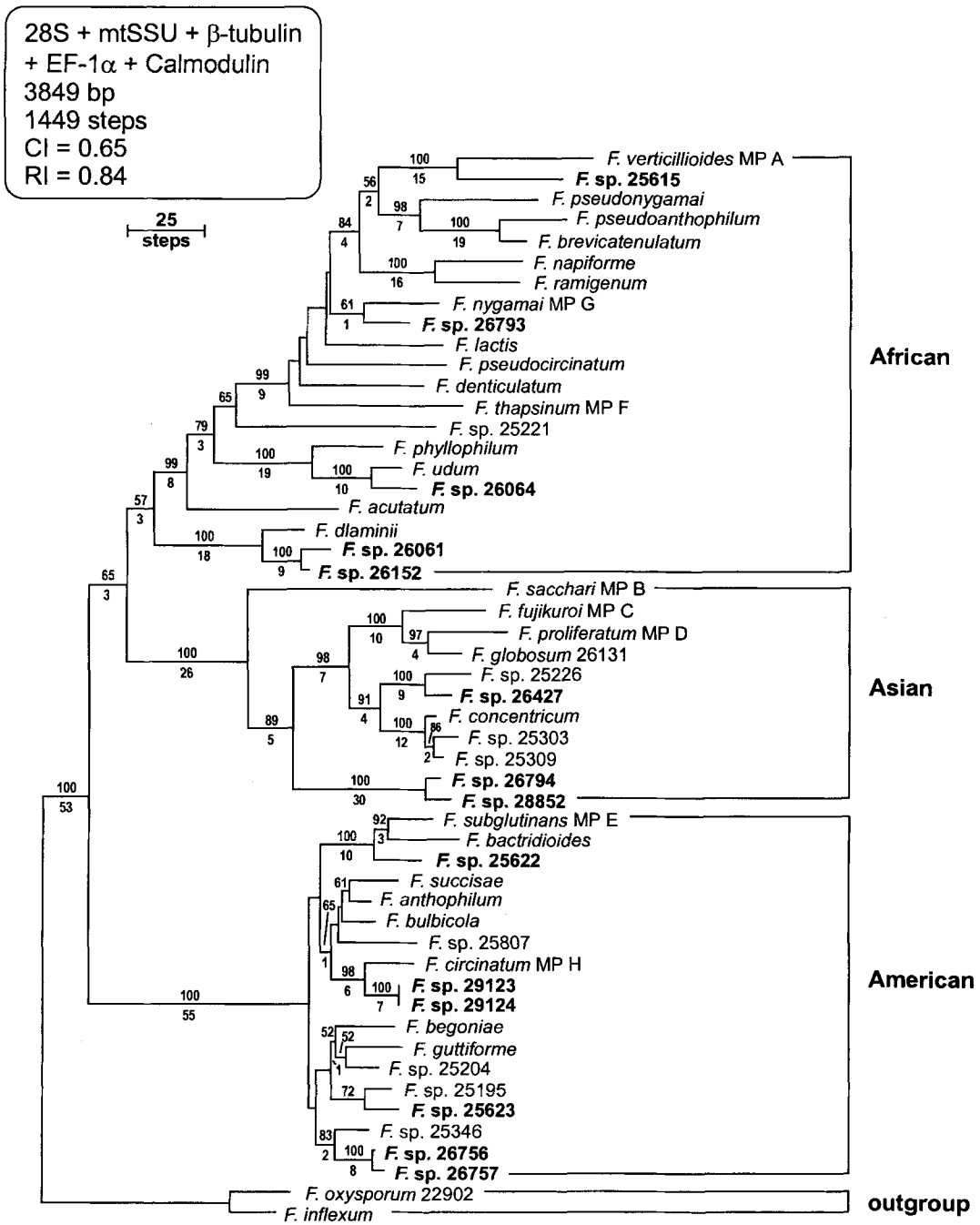


Fig. 6. One of 135 MPTs inferred from the combined 28S rDNA, mtSSU rDNA, β-tubulin, translation elongation factor EF-1α and calmodulin gene sequences rooted by the outgroup method. Bootstrap intervals (above internodes) and decay indices (below internodes) are indicated. The 10 new species identified in this study are in bold. MP=biological species in the *G. fujikuroi* complex.

calmodulin dataset only when apparent autapomorphies were excluded from the analysis (Table 5). Results of the RASA analysis indicate that phylogenetic signal is most evolved in β-tubulin, followed by EF-1α and the mtSSU rDNA (Table 5). In some cases, significantly different tRASA and taxon variance ratios were obtained depending on whether apparent autapomorphies were included in the analysis (Table 5). For example, the 28S rDNA, calmodulin and combined datasets received significantly higher tRASA scores when autapomorphies were excluded from the analysis. Overall, more long-branch taxa were detected by excluding autapomorphies from the RASA analyses (Table 5).

To examine evolution of the major ITS2 types, we mapped them on a strict consensus of the 135 MPTs inferred from the combined dataset (Fig. 8). Results of

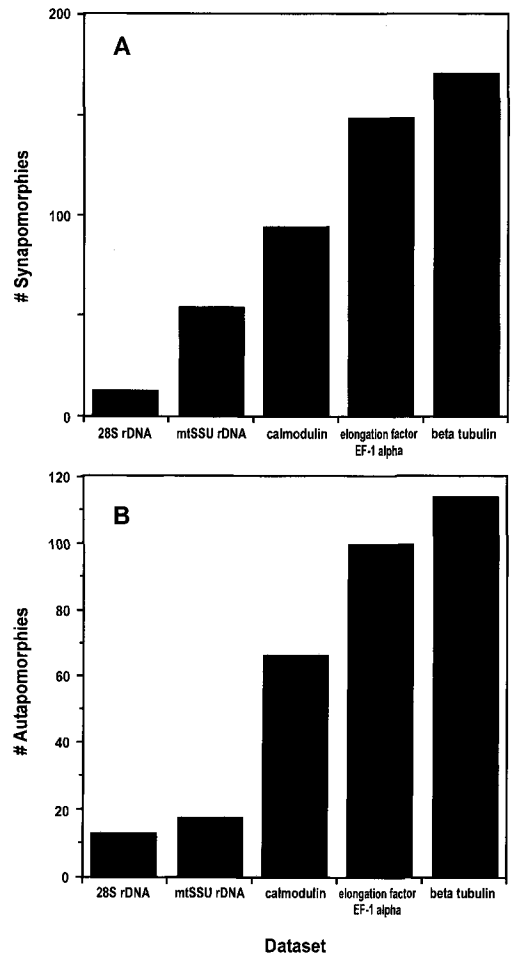


Fig. 7. Distribution of (A) synapomorphies and (B) autapomorphies in the 5 datasets analyzed phylogenetically with PAUP*4.0b1 for the 52 strains studied.

this optimization reveal that the major ITS2 types exhibit a homoplastic pattern in that there have been a minimum of three reversals between a type I and a type II sequence during the evolution of this species complex. Next, we constructed constraints to test whether two taxa described as formae speciales of *F. subglutinans*, *F. guttiforme* as *F. subglutinans* f. sp. *ananas* (Ventura, 1998; also reported as *F. moniliforme* var. *subglutinans*, Bolkan et al., 1979) and *F. circinatum* as *F. subglutinans* f. sp. *pini* (Correll et al., 1992; Britz et al., 1999), form a monophyletic group with *F. subglutinans*. Lastly, a constraint was constructed to the test whether *F. subglutinans* and *Fusarium* sp. 25622 form a monophyletic group. For the likelihood analysis we restricted the ingroup to the American clade because these taxa are nested within this clade. When compared with the MPT (Fig. 9a), topological constraints forcing the monophyly of *F. subglutinans* and *F. guttiforme* [syn.=*F. subglutinans* f. sp. *ananas*] (Fig. 9b), *F. subglutinans* and *F. circinatum* [syn.=*F. subglutinans* f. sp. *pini*] (Fig. 9c), and *F. subglutinans*, *F. guttiforme* and *F. circinatum* (Fig. 9c) were all significantly worse than the unconstrained MPT when subjected to the Kishino-Hasegawa likelihood test ($P<0.003$) (Table 3). However, the *F. subglutians-Fusarium* sp. 25622 monophyly constraint was only 4 steps longer and not sigtnificantly worse than the MPT ($P=0.2177$).

Discussion

The primary objective of this study was to extend phylogenetic analyses within the *Gibberella fujikuroi* species complex from those reported in O'Donnell and Cigelnik (1997) and O'Donnell et al. (1998a) and to test the phylogenetic utility of exons and introns sequences from two protein-encoding nuclear genes, EF-1α and calmodulin. Based on their ability to resolve phylogenetically distinct species via maximum parsimony, both genes appear to the suitable for species-level systematics within this complex. However, results of the RASA analysis indicate that phylogenetic estimates using calmodulin may be problematical, especially when apparent autapomorphies are included in the analysis (Table 5). Sequences from the ITS region were not analyzed in the combined dataset because they are composed of two divergent, nonorthologous ITS2 types as reported previously (Waalwijk et al., 1996; O'Donnell and Cigelnik, 1997; O'Donnell et al., 1998a). Although all of the strains included in this study were found to possess two sets of orthologous ITS2 sequences designated type I and type II (Waalwijk et al., 1996), gene trees inferred from these datasets are unresolved (data not shown; O'Donnell et al., 1998a, see Fig. 4). Even though the major ITS2 types exhibit a homoplastic pattern of evolution when mapped onto the species lineages, results of the present study demonstrate that, based on knowledge of the major ITS2 type of sister taxa of the new species (O'Donnell et al., 1998a; see Fig. 8), the major ITS2 type of each of the 10 new fusaria could have been accurately predicted. Given that the number of reversals between a type I and type II major ITS2 sequence within the *G. fujikuroi* complex did not increase beyond the minimum of 3 reported in O'Donnell et al. (1998a), with the addition of the 10 undescribed species reported here, suggests that it is unlikely that additional reversals in the major ITS2 type will be found as new species within this complex are diagnosed. The discovery of nonorthologous ITS and/or ribosomal intergenic (IGS) spacer sequences in the same individual in *Fusarium* (Appel and Gordon, 1996), other fungi (Sanders et al., 1995), animals (Vogler and DeSalle, 1994; Zijlstra et al., 1995), and plants (Baldwin et al., 1995; Buckler and Holtsford, 1996; Wendel et al., 1995) emphasizes the importance of assessing gene-gene concordance (Lutzoni, 1997; Geiser et al., 1998; Carbone et al., 1999), especially since sequences from the nuclear rDNA region have been and continue to be used extensively in species-level molecular systematic studies.

One of the most important discoveries of this study is that sequences from β-tubulin and translation elonga-

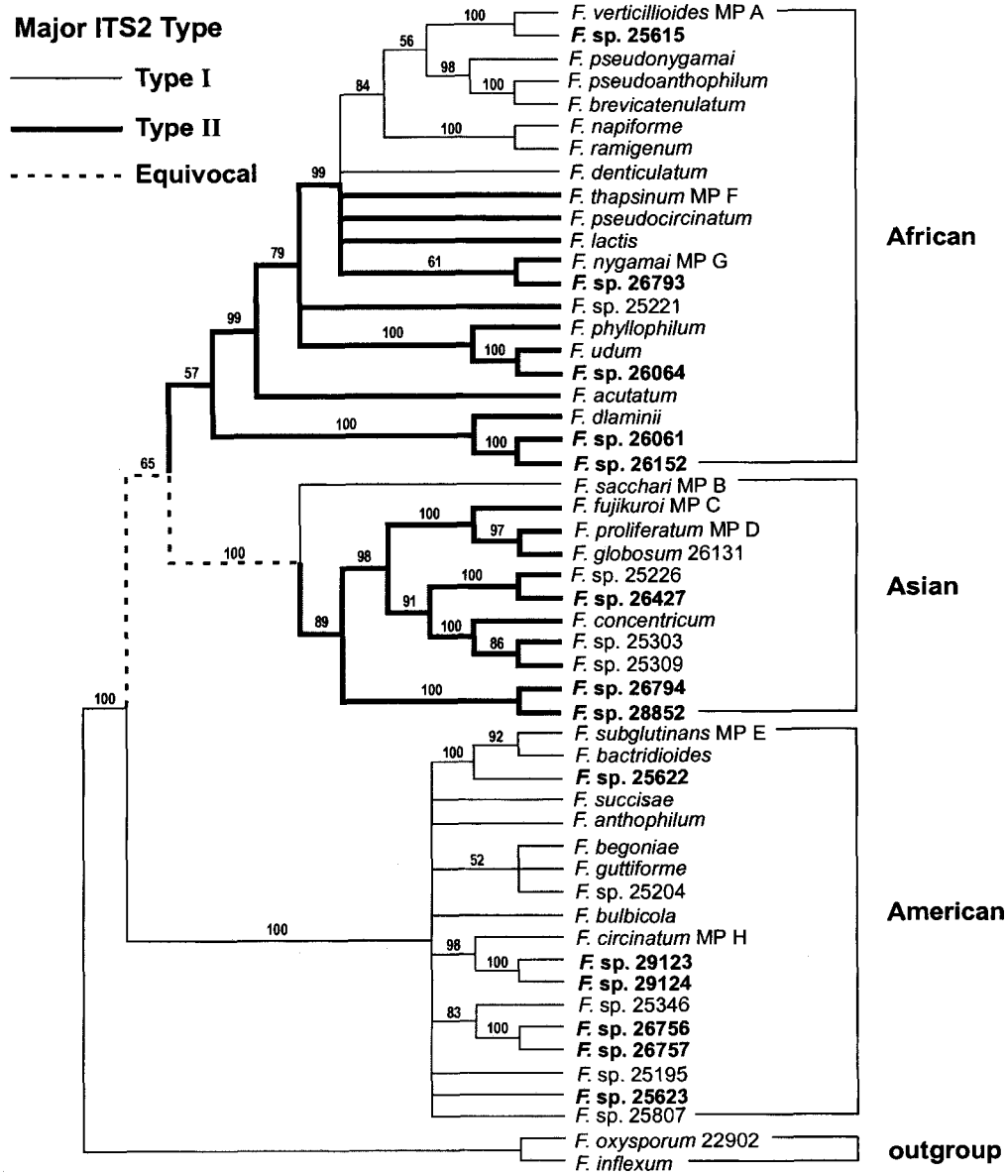
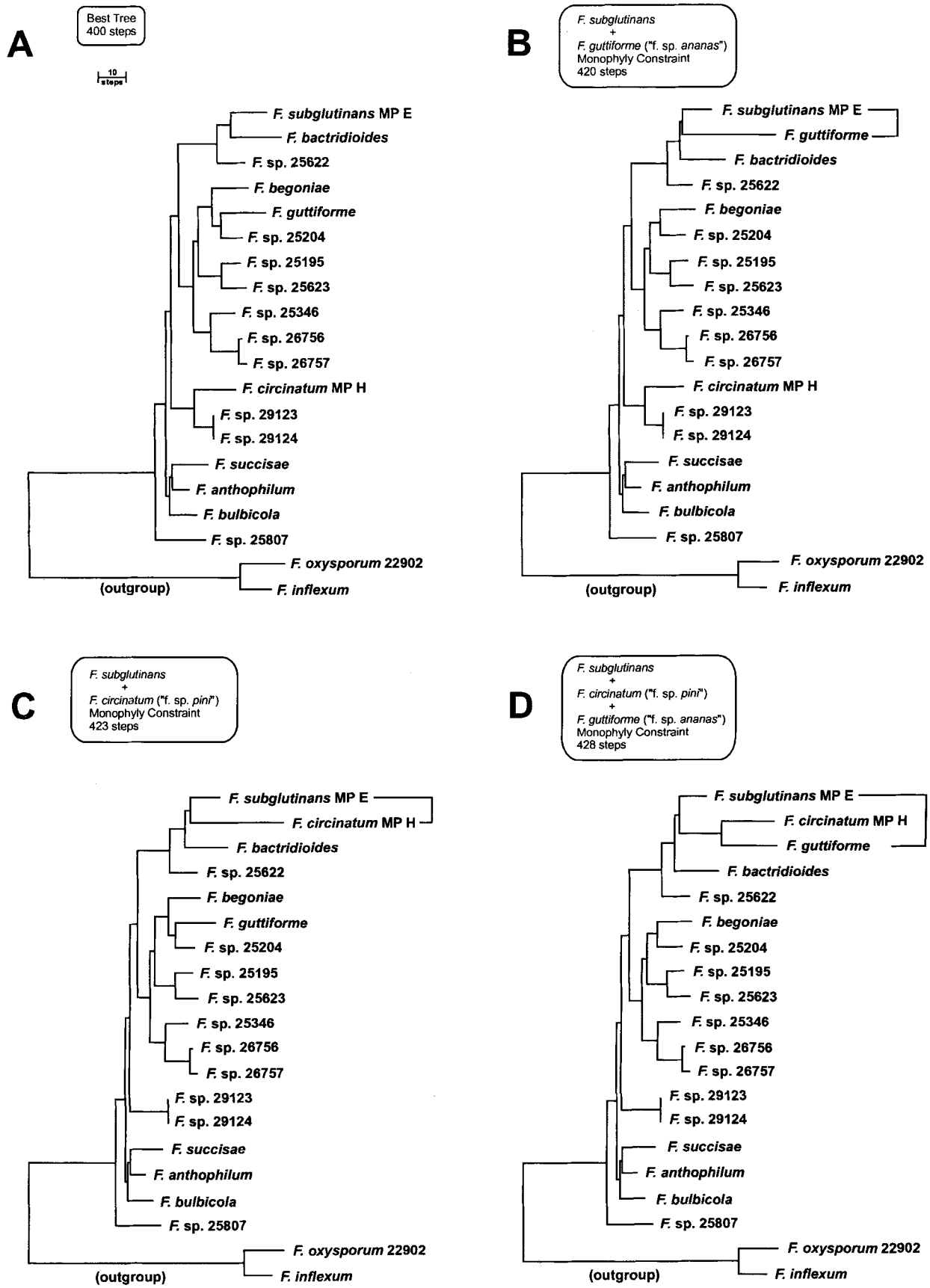


Fig. 8. Strict consensus cladogram of the 135 MPTs inferred from the combined 28S rDNA, mtSSU rDNA, β-tubulin, translation elongation factor EF-1α and calmodulin gene sequences upon which is mapped the major ITS2 type of each species using MacClade (Maddison and Maddison, 1992). The 10 new species are in bold. Bootstrap intervals $\geq 50\%$ are indicated.

tion factor EF-1α, unlike those from the nuclear 28S rDNA and mtSSU rDNA, were able to resolve all 44 species within the *G. fujikuroi* complex, and those of calmodulin were able to resolve all but a pair of species. The results we obtained with β-tubulin are consistent with those of Schardl et al. (1994), Geiser et al. (1998) and Seifert and Louis-Seize (1999) whose studies have demonstrated the utility of sequences from this locus for investigating species boundaries within *Epichloë*, *Aspergillus* and *Penicillium*, respectively. The 3 protein-encoding nuclear genes contributed 86.2% of the synapomorphies and 90.1% of the autapomorphies in the combined dataset (Table 2). In contrast, the highly conserved nuclear 28S rDNA lacks informative variation for phylogenetic reconstruction within this complex (Table 2 and 5) and 8 pairs of species shared identical mtSSU rDNA haplotypes. The weak mtSSU rDNA gene tree topology is consistent with the relatively small contribution of synapomorphies (11.3%) and autapomorphies (5.8%) of this locus to the combined dataset, although this locus received higher tRASA scores than either calmodulin or the 28S rDNA (Table 5). When compared with the individual datasets, phylogenetic analysis of the combined 28S rDNA, mtSSU rDNA and β-tubulin data yielded higher bootstrap support for the monophyly of the African clade (88% bootstrap) and an African-Asian clade (95% bootstrap) (O'Donnell et al., 1998a). However, in the present combined analysis that included


A
Best Tree
400 steps
10 steps
F. subglutinans MP E
F. bactridioides
F. sp. 25622
F. begoniae
F. guttiforme
F. sp. 25204
F. sp. 25195
F. sp. 25623
F. sp. 25346
F. sp. 26756
F. sp. 26757
F. circinatum MP H
F. sp. 29123
F. sp. 29124
F. succisae
F. anthophilum
F. bulbicola
F. sp. 25807
F. oxysporum 22902
F. inflexum
(outgroup)
B
F. subglutinans
+
F. guttiforme ("f. sp. ananas")
Monophyly Constraint
420 steps
F. subglutinans MP E
F. guttiforme
F. bactridioides
F. sp. 25622
F. begoniae
F. sp. 25204
F. sp. 25195
F. sp. 25623
F. sp. 25346
F. sp. 26756
F. sp. 26757
F. circinatum MP H
F. sp. 29123
F. sp. 29124
F. succisae
F. anthophilum
F. bulbicola
F. sp. 25807
F. oxysporum 22902
F. inflexum
(outgroup)
C
F. subglutinans
+
F. circinatum ("f. sp. pini")
Monophyly Constraint
423 steps
F. subglutinans MP E
F. circinatum MP H
F. bactridioides
F. sp. 25622
F. begoniae
F. guttiforme
F. sp. 25204
F. sp. 25195
F. sp. 25623
F. sp. 25346
F. sp. 26756
F. sp. 26757
F. sp. 29123
F. sp. 29124
F. succisae
F. anthophilum
F. bulbicola
F. sp. 25807
F. oxysporum 22902
F. inflexum
(outgroup)
D
F. subglutinans
+
F. circinatum ("f. sp. pini")
+
F. guttiforme ("f. sp. ananas")
Monophyly Constraint
428 steps
F. subglutinans MP E
F. circinatum MP H
F. guttiforme
F. bactridioides
F. sp. 25622
F. begoniae
F. sp. 25204
F. sp. 25195
F. sp. 25623
F. sp. 25346
F. sp. 26756
F. sp. 26757
F. sp. 29123
F. sp. 29124
F. succisae
F. anthophilum
F. bulbicola
F. sp. 25807
F. oxysporum 22902
F. inflexum
(outgroup)

EF-1α and calmodulin sequence data, these clades received only 57% and 65% bootstrap support, respectively. Given that neither of these clades was supported by bootstrap analyses of the individual EF-1α and calmodulin datasets, most of the support for these clades in the present combined analysis was contributed by the β-tubulin gene. This finding is supported by the fact that β-tubulin received the highest tRASA score. By coding indels within the β-tubulin gene as phylogenetically informative in the present study, an increase in bootstrap support was observed over that reported in O'Donnell et al. (1998a) for the African (from 81 to 85%) and African-Asian clades (from 83 to 93%), even with the addition of the 10 new species.

Even though the Kishino-Hasegawa test results indicate that a topological constraint forcing *F. dlaminii* and *Fusarium* sp. NRRL 26061 and 26152 to form a monophyletic group with the African clade was significantly worse than the best calmodulin gene tree, it is doubtful that calmodulin within the *F. dlaminii* lineage is tracking a different genealogy. Instead, the negative tRASA score derived from calmodulin data that included autapomorphies, coupled with the failure of RASA to identify *F. dlaminii*, *Fusarium* sp. 25061 and 26152 as long-branch taxa, may indicate that saturated sites with the introns have eroded phylogenetic signal (Lyons-Weiler et al., 1996). Although RASA did identify long-branch attraction problem taxa within the mtSSU rDNA and EF-1α datasets (Table 5), long-branch taxa do not appear to disort either the β-tubulin or the combined dataset gene tree topologies. With the exclusion of autapomorphies, the individual and combined datasets all received significant tRASA scores ($P<0.005$, two-tailed test using student's t distribution; Bain and Engelhardt, 1992), indicating that they possess phylogenetic signal.

Whereas the Templeton test identified calmodulin and β-tubulin as the two most homogeneous partitions, the partition-homogeneity test (PHT) statistically rejected combining these partitions. These results suggest that a P value of 0.05 may be too conservative for the PHT, especially since Cunningham (1997) found that phylogenetic accuracy was decreased only when partitions were combined when PHT values were lower than 0.001. Another problem with the PHT is that it frequently detects highly significant incongruence when one or both of the data partitions has relatively high levels of homoplasy (Graham et al., 1998; Lutzoni, pers. comm.). As with the PHT, the Templeton test detected highly significant incongruence between some pairs of genes which suggests that it may be premature to identify an appropriate level of incongruence from the Templeton and PHT tests since they both appear to be too conservative (Lutzoni and Vilgalys, 1995; Cunningham, 1997). Given that the Templeton and Kishino-Hasegawa test results (Table 4) indicate that the DNA sequence datasets may be giving conflicting estimates of the *Gibberella fujikuori* species complex phylogeny, which could be due to either genealogical and/or nongenealogical discordance, we have included gene trees inferred from each of the loci sequenced in this study to provide a more complete a picture of their phylogenetic history (Wendel and Doyle, 1998) together with a phylogram and strict consensus inferred from the combined data. The strict consensus of the 135 MPTs (Fig. 8) inferred from the combined dataset is accepted as the strongest hypothesis of phylogenetic relationships within the *G. fujikuroi* species complex for the following reasons: clades that are strongly supported by bootstrapping in the individual datasets are also supported in the combined dataset, decay indices of virtually every internode are higher in the combined analysis and it is nearly identical to the β-tubulin gene tree topology. Overall the combined data strongly support the monophyly of the *G. fujikuroi* species complex (bootstrap=100%, decay=53) and the American (bootstrap=100%, decay=55) and Asian (bootstrap=100%, decay=26) clades, but provided only weak support for the African clade (bootstrap=57, decay=3). It is noteworthy that bootstrap support for the African and African-Asian clades in the combined analysis decreased by 28 percentage points compared with those obtained with the β-tubulin gene (Fig. 3).

Knowledge of sister-group relationships of 5 of the 9 biological species within the *G. fujikuroi* complex were advanced in the present study. *Fusarium verticillioides* MP-A, *F. nygamai* MP-G, *F. circinatum* MP-H, and *F. udum* all appear to share a more recent common ancestor with one of the new fusaria identified in the present study while *F. proliferatum* MP-D appears to be a sister of *F. globosum* (bootstrap=97%, decay index=4) rather than *F. fujikuroi* with which it has been shown to share an intermediate level of DNA-DNA complementarity (Ellis, 1988; O'Donnell, 1998). Because strains of biological species within the *G. fujikuroi* (O'Donnell et al., 1998a), *F. solani* (O'Donnell and Gray, 1995; O'Donnell, unpubl.) and *F. graminearum* species complexes (Aoki and O'Donnell, 1999a, b) form exclusive groups within the molecular phylogenies, it should be possible to use the phylogenetic data to design optimal mating experiments to identify additional teleomorphs among the 35 anamorphic species within the *G. fujikuroi* complex for which a sexual state is unknown. Such an approach should be especially profitable within *Fusarium* since many species are difficult to diagnose morphologically. Because their extreme morphological crypsis has contributed to competing and often discordant morphology-based taxonomic treatments (Booth, 1971; Gerlach and Nirenberg, 1982; Nelson et al., 1982; Nirenberg and O'Donnell, 1998; Wollenweber and Reinking, 1935), published data on mycotoxin potential and host range is often flawed

Fig. 9. Unconstrained and constrained trees enforced with PAUP*4.0b1 restricting the ingroup to the American clade. (A) MPT. (B) *F. subglutinans* and *F. guttiforme* (syn.=*F. subglutinans* f. sp. *ananas*) monophyly constraint. (C) *F. subglutinans* and *F. circinatum* (syn.=*F. subglutinans* f. sp. *pini*) monophyly constraint. (D) *F. subglutinans*, *F. guttiforme* and *F. circinatum* monophyly constraint. All three monophyly constraints are significantly worse than the MPT (see Table 3).

because many morphological species are polyphyletic in taxonomic practice (O'Donnell et al., 1998a; Nirenberg and O'Donnell, 1998). *F. subglutinans*, for example, has been applied to 13 of the 44 species within the *G. fujikuroi* complex, including 1 African, 4 Asian, and 8 American species (O'Donnell and Cigelnik, 1997; O'Donnell et al., 1998a; Nirenberg and O'Donnell, 1998; see Table 1). To illustrate the kind of confusion a polytypic species concept can create concerning host preference, Viljoen's et al. (1997) study of the pitch canker disease of pine caused by *F. circinatum* [syn.=*F. subglutinans* f. sp. *pini*] included strains from 8 different hosts representing at least 7 phylogenetically distinct species which they all reported as *F. subglutinans*, although *F. subglutinans* MP-E appears to be restricted to *Zea mays* (O'Donnell et al., 1998a). The phylogenetic status of *Fusarium* sp. 25622 deserves further study because it is resolved as phylogenetically distinct from *F. subglutians* in the present study and in the histone H3 gene tree presented by Steenkamp et al. (1999); however, Steenkamp et al. (1999) reported that *Fusarium* sp. 25622 and *F. subglutinans* were mating compatible.

Based on results from prior molecular phylogenetic studies, the formae speciales naming system has been challenged within the *F. oxysporum* complex because many of these special forms are poly- or paraphyletic (O'Donnell et al., 1998a; O'Donnell, unpubl.), and in the *F. solani* complex where formae speciales described by Matuo and Snyder (1973) represent phylogenetically distinct, reproductively isolated biological species (O'Donnell and Gray, 1995; O'Donnell unpubl.). Therefore, we were interested in testing whether formae speciales described for *F. subglutinans*, f. sp. *pini* and f. sp. *ananas*, form an exclusive, monophyletic group with *F. subglutinans* MP-E in the molecular phylogeny. Hypotheses of the monophyly of these taxa were strongly rejected by the Kishino-Hasegawa test implemented in PAUP*4.01b (Table 3). Results of these likelihood analyses indicate that the formae speciales designation for morphologically, phylogenetically and biologically distinct species (Nirenberg and O'Donnell, 1998) such as *F. circinatum* [teleomorph=*G. circinata*] obscures communication of critical information concerning the systematics and genetic diversity of these pathogens necessary to develop effective disease control efforts and breeding programs.

Biogeographical interpretation of the evolutionary origin of the 4 new African and 2 new Asian species is supported by their geographic origins in these two respective areas (Table 1). However, biogeography of the new putative American fusaria is complicated because 3 of the 4 species nested within this clade were isolated in South Africa and one came from Florida. Based on results of the molecular phylogeny, we theorize that 3 of the species isolated in South Africa were most likely introduced to Africa from South America on the following economically important hosts: *Fusarium* sp. NRRL 25622 on corn, *Fusarium* sp. NRRL 26756 and 26757 on an unidentified ornamental grass and reed, and *Fusarium* sp. NRRL 25623 on mango. Moreover, the latter species, *Fusarium* sp. 25623, was isolated from mango imported into South Africa from South America (M. Wingfield, pers. comm.). Further it should be noted that this American species is phylogenetically distinct from the Asian species (*Fusarium* sp. NRRL 2526) responsible for the mango malformation disease in India, Israel and Egypt (Kumar et al., 1993; S. Freeman et al., 1999; R. Ploetz pers. comm.). Based on the histone H3 phylogeny published by Steenkamp et al. (1999), the mango pathogen from Asia is also present in South Africa and the United States. Although Steenkamp et al. (1999) refer to the Asian species as *F. subglutinans*, it represents a phylogenetically unrelated, unnamed species designated as *Fusarium* sp. NRRL 25226 in O'Donnell et al. (1998a). Biogeographical interpretation of the undescribed American species (*Fusarium* sp. 29123 and 29124) isolated from *Bidens pilosa* in Florida is consistent with a range expansion of this noxious weed from the neotropics.

The complex historical biogeographic pattern of the *G. fujikuroi* complex inferred from the phylogenetic evidence appears to reflect vicariant events associated with the breakup of Gondwanaland, at least one transoceanic jump dispersal (O'Donnell et al., 1998a), and the relatively recent alteration in the distribution of these fusaria associated with movement of agronomically important plants (Simpson and Ogorzaly, 1995). Sampling of species in continents that were formally part of Gondwana is fragmentary as no species have been discovered within this complex that are endemic to Australia or New Zealand. For this reason in part, we theorize that sampling biogeographically rich regions such as Asia, the least speciose clade of the *G. fujikuroi* complex, should reveal considerable genetic diversity that will advance our understanding of the host range, geographic distribution and phylogenetic relationships of these agronomically important mycotoxigenic phytopathogens. Comparative morphological analyses are in progress directed at formally describing the phylogenetically distinct species discovered in the present study. To facilitate future studies of these fungi within a phylogenetic context, the aligned database of the *G. fujikuroi* complex has been deposited in TreeBase on the World Wide Web: http://www.herbaria.harvard.edu/treebase. Because this dataset and the one described by Bruns et al. (1998) for the ectomycorrhizal basidiomycetes are electronically portable, anyone should be able use these publicly accessible molecular systematic tools to identify and diagnose species within these groups using molecular phylogenetics.

Acknowledgements——Thanks are due Kazunori Ichikawa (Yamanashi Agriculture Research Center, Kita-koma, Yamanashi, Japan), Erin Rosskopf (Agricultural Research Service, Ft. Pierce, FL, USA) and the culture collections cited in Table 1 for generously providing strains, David Hibbett (Clark University, Worcester, MA, USA) for advice on running the Templeton test in PAUP*4.01b, David Geiser (Pennsylvania State University, University Park, PA, USA) for recommending RASA for the de-

tection of long-branch attraction taxa and phylogenetic signal within DNA sequence datasets, François Lutzoni (Field Museum of Natural History, Chicago, IL, USA) for discussing the partition-homogeneity test, Larry Tjarks for oligonucleotides, and Steve Prather for the illustrations. Names are necessary for report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

Literature cited

Aoki, T. and O'Donnell, K. 1999. Morphological and molecular characterization of *Fusarium pseudograminearum* sp. nov., formerly recognized as the Group 1 population of *F. graminearum*. Mycologia **91**: 597–609.

Aoki, T. and O'Donnell, K. 1999b. Morphological characterization of *Gibberella coronicola* sp. nov., obtained through mating experiments of *Fusarium pseudograminearum*. Mycoscience **40**: 443–453.

Appel, D. J. and Gordon, T. R. 1996. Relationships among pathogenic and nonpathogenic isolates of *Fusarium oxysporum* based on the partial sequence of the intergenic spacer region of the ribosomal DNA. Mol. Pl.-Microbe Interact. **9**: 125–138.

Bain, L. J. and Engelhardt, M. 1992. Introduction to probability and mathematical statistics. Duxbury Press, Belmont, California.

Baldwin, B. G., Sanderson, M. J., Porter, J. M., Wojciechowski, M. F., Campbell, C. S. and Donoghue, M. J. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann. Missouri Bot. Gard. **82**: 247–277.

Bolkan, H. A., Dianese, J. C. and Cupertino, F. P. 1979. Pineapple flowers as principal infection sites for *Fusarium moniliforme* var. *subglutinans*. Pl. Dis. Reptr. **63**: 655–657.

Booth, C. 1971. The genus *Fusarium*. CMI, Kew, Surrey.

Britz, H., Coutinho, T. A., Wingfield, M. J., Marasas, W. F. O., Gordon, T. R. and Leslie, J. F. 1999. *Fusarium subglutinans* f. sp. *pini* represents a distinct mating population in the *Gibberella fujikuroi* species complex. Appl. Environm. Microbiol. **65**: 1198–1201.

Bruns, T. D., Szaro, T. M., Gardes, M., Cullings, K. W., Pan, J. J., Taylor, D. L., Horton, T. R., Kretzer, A., Garbelotto, M. and Li, Y. 1998. A sequence database for the identification of ectomycorrhizal basidiomycetes by phylogenetic analysis. Mol. Ecol. **7**: 257–272.

Buckler, E. S. and Holtsford, T. P. 1996. *Zea* systematics: Ribosomal ITS evidence. Mol. Biol. Evol. **13**: 612–622.

Carbone, I., Anderson, J. B. and Kohn, L. M. 1999. Patterns of descent in clonal lineages and their multilocus fingerprints are resolved with combined gene genealogies. Evolution **53**: 11–21.

Cerdá-Olmedo, E., Fernández-Martín, R. and Ávalos, J. 1994. Genetics and gibberellin production in *Gibberella fujikuroi*. Antonie van Leeuwenhoek Int. J. Gen. Mol. Microbiol. **65**: 217–225.

Correll, J. C., Gordon, T. R. and McCain, A. H. 1992. Genetic diversity in California and Florida populations of the pitch canker fungus *Fusarium subglutinans* f. sp. *pini*. Phytopathology **82**: 415–420.

Cunningham, C. W. 1997. Can three incongruence tests predict when data should be combined? Mol. Biol. Evol. **14**: 733–740.

Ellis, J. J. 1988. Lesser known species in section *Liseola* of *Fusarium* and near relatives in the *Fusarium oxysporum* group. Mycologia **80**: 734–738.

Freeman, S., Maimon, M. and Pinkas, Y. 1999. Use of GUS transformants of *Fusarium subglutinans* for determining etiology of mango malformation disease. Phytopathology **89**: 456–461.

Graham, S. W., Kohn, J. R., Morton, B. R., Eckenwalder, J. E. and Barrett, S. C. H. 1998. Phylogenetic congruence and discordance among one morphological and three molecular data sets from Pontederiaceae. Syst. Biol. **47**: 545–567.

Gardes, M. and Bruns, T. D. 1993. ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Mol. Ecol. **2**: 112–118.

Geiser, D. M., Pitt, J. I. and Taylor, J. W. 1998. Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. Proc. Natl. Acad. Sci. USA **95**: 388–393.

Gerlach, W. and Nirenberg, H. 1982. The genus *Fusarium* – A pictorial atlas. Mitt. Biol. Bundesanst. Land- u. Forstwirtsch. Berlin-Dahlem **209**: 1–406.

Hillis, D. M. and Bull, J. J. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst. Biol. **42**: 182–192.

Kellogg, E. A., Appels, R. and Mason-Gamer, R. J. 1996. When genes tell different stories: the diploid genera of Triticeae (Gramineae). Syst. Bot. **21**: 321–347.

Kluge, A. G. 1988. Parsimony in vicariance biogeography: a quantitative method and a greater antillean example. Syst. Zool. **37**: 315–328.

Kumar, J., Singh, U. S. and Beniwal, S. P. S. 1993. Mango malformation: one hundred years of research. Annu. Rev. Phytopathol. **31**: 217–232.

Leslie, J. F. 1995. *Gibberella fujikuroi*: available populations and variable traits. Can. J. Bot. **73**: S282–S291.

Lutzoni, F. 1997. Phylogeny of lichen- and non-lichen-forming omphalinoid mushrooms and the utility of testing for combinability among multiple data sets. Syst. Biol. **46**: 373–406.

Lutzoni, F. and Vilgalys, R. 1995. Integration of morphological and molecular data sets in estimating fungal phylogenies. Can. J. Bot. **73**: S649–S659.

Lyons-Weiler, J. 1999. RASA 2.3 software and documentation for the MAC. http:test1.bio.psu.edu/LW/rasatext/html.

Lyons-Weiler, J. and Hoelzer, G. A. 1997. Escaping from the Felsenstein zone by detecting long branches in phylogenetic data. Mol. Phylo. Evol. **8**: 375–384.

Lyons-Weiler, J., Hoelzer, G. A. and Tausch, R. J. 1996. Relative apparent synapomorphy analysis (RASA) I: The statistical measurement of phylogenetic signal. Mol. Biol. Evol. **13**: 749–757.

Maddison, W. P. and Maddison, D. R. 1992. MacClade: Analysis of phylogeny and character evolution, Version 3. Sinauer Associates, Sunderland, MA.

Matuo, T. and Snyder, W. C. 1973. Use of morphology and mating populations in the identification of formae speciales in *Fusarium solani*. Phytopathology **63**: 562–565.

Moretti, A. A., Logrieco, A., Bottalico, A., Ritieni, A., Fogliano, V. and Randazzo, G. 1996. Diversity in beauvericin and fusaproliferin production by different populations of *Gibberella fujikuroi* (*Fusarium* section *Liseola*). Sydowia **48**: 44–56.

Nelson, P. E., Plattner, R. D., Shackelford, D. D. and Desjardins A. E. 1992. Fumonisin B_1 production by *Fusarium* species

other than *F. moniliforme* in Section *Liseola* and by some related species. Appl. Environm. Microbiol. **58**: 984–989.

Nelson, P. E., Toussoun, T. A. and Marasas, W. F. O. 1983. *Fusarium* Species: An illustrated manual for identification. Pennsylvania State University, University Park, PA.

Nirenberg, H. 1976. Untersuchungen über die morphologische und biologische Differenzierung in der *Fusarium*-Sektion *Liseola*. Mitt. Biol. Bundesanst. Land- u. Forstwirtsch. Berlin-Dahlem **169**: 1–117.

Nirenberg, H. and O'Donnell, K. 1998. New *Fusarium* species and combinations within the *Gibberella fujikuroi* species complex. Mycologia **90**: 434–458.

Nirenberg, H., O'Donnell, K., Kroschel, J., Andrianaivo, A. P., Frank, J. M. and Mubatanhema, W. 1998. Two new species of *Fusarium*: *Fusarium brevicatenulatum* from the noxious weed *Striga asiatica* in Madagascar and *Fusarium pseudoanthophilum* from *Zea mays* in Zimbabwe. Mycologia **90**: 459–464.

O'Donnell, K. 1998. Molecular genetic approaches for measuring microbial diversity: Examples from filamentous fungi. In: Diversity and use of agricultural microorganisms: The fifth international workshop on genetic resources, (ed. by National Institute of Agrobiological Resources), pp. 7–24. National Institute of Agrobiological Resources, Tsukuba, Japan.

O'Donnell, K. and Cigelnik, E. 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol. Phylo. Evol. **7**: 103–116.

O'Donnell, K., Cigelnik, E. and Nirenberg, H. I. 1998a. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia **90**: 465–493.

O'Donnell, K. and Gray, L. E. 1995. Phylogenetic relationships of the soybean sudden death syndrome pathogen *Fusarium solani* f. sp. *phaseoli* inferred from rDNA sequence data and PCR primers for its identification. Mol. Pl. Microbe Interact. **8**: 709–716.

O'Donnell, K., Kistler, H. C., Cigelnik, E. and Ploetz, R. C. 1998b. Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. Proc. Natl. Acad. Sci. USA **95**: 2044–2049.

Rai, B. and Upadhyay, R. S. 1982. *Gibberella indica*: the perfect state of *Fusarium udum*. Mycologia **74**: 343–346.

Sanders, I. R., Alt, M., Groppe, K., Boller, T. and Wiemken, A. 1995. Identification of ribosomal DNA polymorphisms among and within spores of the Glomales: application to studies on the genetic diversity of arbuscular mycorrhizal fungal communities. New Phytol. **130**: 419–427.

Schardl, C. L., Leuchtmann, A., Tsai, H.-F., Collett, M. A., Watt, D. M. and Scott, D. B. 1994. Origin of a fungal symbiont of perennial ryegrass by interspecific hybridization of a mutualist with the ryegrass choke pathogen, *Epichloë typhina*. Genetics **136**: 1307–1317.

Seifert, K. A. and Louie-Seize, G. 1999. Phylogeny and species concepts in the *Penicillium aurantiogriseum* complex as inferred from partial beta-tubulin gene DNA sequences. Proceedings 3rd International Workshop on *Penicillium* and *Aspergillus*, Baarn, The Netherlands.

Simpson, B. B. and Ogorzaly, M. C. 1995. Economic botany: Plants in our world. McGraw-Hill, New York.

Sorenson, M. D. 1996. TreeRot. Computer program and documentation. University of Michigan, Ann Arbor, MI.

Steenkamp, E. T., Wingfield, B. D., Coutinho, T. A., Wingfield, M. J. and Marasas, W. F. O. 1999. Differentiation of *Fusarium subglutians* f. sp. *pini* by histone gene sequence data. Appl. Environm. Microbiol. **65**: 3401–3406.

Sun, S.-K. and Snyder, W. C. 1981. The bakanae disease of the rice plant. In: *Fusarium*: Diseases, biology, and taxonomy, (ed. by Nelson, P. E., Toussoun, T. A. and Cook, R. J.), pp. 104–113. Pennsylvania State University, University Park, PA.

Swofford, D. L. 1998. PAUP*4.0: Phylogenetic analysis using parsimony. Sinauer Associates, Sunderland, MA.

Templeton, A. 1983. Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. Evolution **37**: 221–244.

Ventura, J. A. 1998. Resistance of *Fusarium subglutinans* f. sp. *ananas* to benomyl. 8th Intern. *Fusarium* Workshop, Egham, England, August 17–20.

Viljoen, A., Marasas, W. F. O, Wingfield, M. J. and Viljoen, C. D. 1997. Characterization of *Fusarium subglutinans* f. sp. *pini* causing root disease of *Pinus patula* seedlings in South Africa. Mycol. Res. **101**: 437–445.

Volger, A. P. and DeSalle, R. 1994. Evolution and phylogenetic information content of the ITS-1 region in the tiger beetle *Cicindela dorsalis*. Mol. Biol. Evol. **11**: 393–405.

Waalwijk, C., De Koning, J. R. A., Baayen, R. P. and Gams, W. 1996. Discordant groupings of *Fusarium* spp. from sections *Elegans*, *Liseola* and *Dlaminia* based on ribosomal ITS1 and ITS2 sequences. Mycologia **88**: 316–328.

Wendel, J. F. and Doyle, J. J. 1998. Phylogenetic incongruence: Window into genome history and molecular evolution. In: Molecular systematics of plants, (ed. by Soltis, P. S., Soltis, D. E. and Doyle, J. J.), pp. 265–296. Chapman and Hall, New York.

Wendel, J. F., Schnabel, A. and Seelanan, T. 1995. Bidirectional interlocus concerted evolution following allopolyploid speciation in cotton (*Gossypium*). Proc. Natl. Acad. Sci. USA **92**: 280–284.

White, T. J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR protocols: A guide to methods and application, (ed. by Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J.), pp. 315–322. Academic Press, New York.

Wollenweber, H. W. and Reinking, O. A. 1935. Die Fusarien, ihre Beschreibung, Schadwirkung und Bekämpfung. Paul Parey, Berlin.

Zijlstra, C., Lever, A. E. M., Uenk, B. J. and Van Silfhout, C. H. 1995. Differences between ITS regions of isolates of root-knot nematodes *Meloidogyne hapla* and *M. chitwoodi*. Phytopathology **85**: 1231–1237.