Phylogeny of Some *Fusarium* Species, as Determined by Large-Subunit rRNA Sequence Comparison[1]

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Fifty-two strains from eight species of *Fusarium* were analyzed by rapid rRNA sequencing. Two highly variable stretches (138 and 214 nucleotides) of the 5′ end of the 28S-like rRNA molecule were sequenced. Such stretches permit evaluation of the divergence between closely related species and even between varieties within a species. The phylogenetic tree computed from the number of nucleotide differences shows seven *Fusarium* species to be more closely related to one another than the eighth species, *F. nivale,* is to them. On the basis of these data, we discuss both the phylogenetic value of taxonomical criteria and the impact of our findings on the demarcation of the genus *Fusarium.* We conclude that this method is suitable for establishing a precise phylogeny between closely related species within a genus.

Introduction

Fusarium is one of the most heterogeneous and difficult to classify fungal genera. Species of *Fusarium* are ubiquitous or limited to more or less specialized habitats, as saprophytes or parasites (Booth 1984). Many of them are of practical significance as food contaminants in industry and as pathogens in agriculture, where, for example, in the species *F. oxysporum,* >100 *formae speciales* (morphologically similar strains characterized by their adaptation to different hosts) and races can be identified (Armstrong and Armstrong 1981). Another difficulty stems from the various degrees of morphological and cultural variation seen, within a species, for such characters as pigmentation, growth rate, and potential perithecium differentiation. Sexuality has been described in only half of the taxa (Booth 1981), and even then is not a common occurrence. As a consequence of the large variability of asexual morphology on which traditional taxonomy has relied, the number of defined taxa varies over a wide range: nine species for Snyder and Hansen (1945), 44 species and seven varieties for Booth (1971), 65 species and 55 varieties for Wollenweber and Reinking (1935), and >70 species and ≥55 varieties for Gerlach and Nirenberg (1982, pp. 4–16). The uncertainty in *Fusarium* classification is further complicated by a double nomenclature: one for the asexual state (anamorph) and one for the sexual state (teleomorph). Species in which only the anamorph state is known are classified as fungi imperfecti. Until now this uncertain and ambiguous taxonomy did not allow construction of a consistent phylogeny.

Classification criteria derived from various biochemical techniques have been tried. Soluble protein electrophoretic patterns (Glyn and Reid 1969), zymograms (Scala et al. 1981), and restriction-fragment-length polymorphism (Kistler et al. 1987; Manicom et al. 1987) have improved strain identification. Serological similarities

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(Iannelli et al. 1982; Rataj-Guranowska 1984) and DNA reassociation (Szécsi and Dobrovolsky 1985*a,* 1985*b*) have been tentatively used for evaluating phylogenetic relationships.

Ribosomal RNAs (rRNAs) provide a powerful taxonomic indicator, because they are highly conserved and are universally found in living cells. The 5S rRNA was first used for this purpose (reviewed by Hori and Osawa 1987). However, the 5S rRNA is so short and so conserved that it cannot be used for studying closely related species; for such species one has to look at larger rRNA molecules: 16S (Salim and Maden 1981; Woese et al. 1985) and 28S (Qu et al. 1983). The development of a technique for rapid and easy sequencing of large stretches of 18S or 28S rRNA opened the way for systematic exploitation of the remarkable properties of these molecules as phylogenetic indicators (Qu et al. 1988).

The present study aims at evaluating the rRNA sequencing methodology as a tool for rapid identification and classification of strains within the same genus, using *Fusarium* as a model. We show that this method is efficient for these purposes and may provide a phylogenetic tree. In addition, a precise knowledge of genetic distances between strains may help in biological manipulations, such as protoplast fusion.

Material and Methods

Source of Fungal Strains

The 53 strains used in this study (table 1) originated from various culture collections or individual investigators who are responsible for their identification. None of the strains is a type. However, it is worth mentioning that strain CBS 203,31 was identified by Wollenweber (1931, pp. 269–276) as *Fusarium javanicum* var. *theobromae* in 1931 and was synonymized by him to *F. javanicum* var. *javanicum* in 1935. For each strain, microconidia were isolated, and single-spore strains were maintained, in our collection, on potato dextrose agar slants at 12°C. A 2-ml inoculum from a 2-d-old preculture fragmented in a Measuring and Scientific Equipment, Ltd. homogenizer (catalog no. 7700; Measuring and Scientific Equipment Ltd., London) was grown in a Roux flask for 2 d at 23°C in 150 ml liquid medium (Daboussi-Bareyre 1980) supplemented with 0.1% yeast extract. The mycelium from four flasks was harvested, washed with sterile water, lyophilized, and stored at −20°C.

RNA Template Isolation

A miniscale extraction procedure was developed. Lyophilized mycelium (120 mg) mixed with an equal weight of sand was placed in a mortar with liquid nitrogen and was ground to a fine powder. The powdered material was transferred to a 2-ml Eppendorf tube and was soaked in 1 ml extraction buffer [50 mM hydroxymethylaminomethan (Tris) pH 7.4, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), and 5% sodium dodecyl sulfate (SDS)]. Nucleic acids were purified by three 1.0-ml phenol:chloroform (1:1) extractions. Centrifugations were performed in a Beckman microfuge. Contaminating double-strand DNA was eliminated by precipitation in 3 M LiCl (Maccecchini et al. 1979). The RNA material sufficient to perform ≤40 sequencing reactions was stored at −20°C.

RNA Sequencing by Using Synthetic Primers

The sequencing protocol involves a base-specific, dideoxynucleotide-terminated chain elongation, modified for the use of reverse transcriptase and RNA template (Hamlyn et al. 1978; Qu et al. 1983). Three oligonucleotide primers (P1, P2, and P3;

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Table 1
List of Sequenced Strains, Arranged by Sequence Class

Species	Subspecies[a]	Isolate	Source	Supplier[b]	Class
Fusarium oxysporum . . .	f.sp. *cubense*	FO cub	Banana tree	1	A
	f.sp. *raphani*	FO 437	Radish	2	
	f.sp. *cyclaminis*	FO 393	Cyclamen	2	
	f.sp. *melonis* race 0	FOM 15	Melon	3	
	f.sp. *melonis* race 0	FOM 25	Melon	3	
	f.sp. *melonis* race 1–2	FOM 7	Melon	3	
	f.sp. *lycopersici* race 2	FOL 15	Tomato	3	
	f.sp. *lini*	FOLn 3	Flax	3	
	. . .	FO 47	Soil	3	
	. . .	FO 1235	Crawfish	1	
	var. *bulbeginum*[c]	FO bul	Vanilla	2	
	var. *redolens*	FO red	. . .	2	B
F. moniliforme	. . .	*F. moniliforme* 1	Sorghum	2	C
	. . .	*F. moniliforme* 2	Maize	2	
	. . .	*F. moniliforme* 3	Asparagus	2	D
Gibberella fujikuroï	. . .	GK	. . .	2	
F. moniliforme	var. *subglutinans*	*F. subglutinans* 1	Maize	2	E
	var. *subglutinans*	*F. subglutinans* 2	Vanilla	2	
	var. *subglutinans*	*F. subglutinans* 3	. . .	2	
F. graminearum	. . .	FG 1	Maize	2	F
	. . .	FG 2	Maize	2	
	. . .	FG 3	Vanilla	2	
	. . .	CBS 389/62	Wheat	4	
G. zeae	. . .	GZ 1103	Maize	2	
	. . .	GZ 1	Maize	2	
F. culmorum	. . .	FC 15			G
	. . .	FC X29	Wheat	2	
	. . .	FC 1	Wheat	2	
	. . .	FC 2	Millet	1	
F. decemcellulare	. . .	FD 1	Cacao tree	2	H
	. . .	FD 2	Cacao tree	2	
	. . .	FD 3	. . .	2	I
	. . .	FD 4	Acacia	2	J
F. solani	var. *coeruleum*	51.1500	Carnation	5	K
	var. *coeruleum*	51.215	Maybug	5	
	. . .	F. sol 1	Pea	1	
	. . .	F. sol D158	. . .	6	
	f.sp. *pisi*	F. sol 2	Pea	7	
	var. *martii*[c]	M 808/1	. . .	6	L
Nectria haematococca . . .	var. *cucurbitae* race 1 +	R1CU4A	. . .	8	M
	var. *cucurbitae* race 1 +	R1CUBrazil	. . .	8	
	var. *cucurbitae* race 2	R2CUS1	. . .	8	
	. . .	CBS 181/29	Potato	4	
	var. *minus*[c]	M 47/378	Papaya	5	N
	. . .	*F. solani* 3	Soil	3	
	. . .	*F. solani* 4	Millet	1	
F. javanicum[c]	var. *javanicum*[c]	CBS 203/31	Coffee tree	4	O
	var. *radicicola*[c]	M 49/592	. . .	5	P
N. haematococca	. . .	CBS 225/58	Soil	4	Q
F. nivale	. . .	*F. nivale* 1	Wheat	2	R
	. . .	*F. nivale* 2	Wheat	1	
	. . .	*F. nivale* 4	Brome	1	
Neurospora crassa	. . .	N 4317	. . .	1	

NOTE.—Classes of identical sequences are as depicted in fig. 2.

[a] f.sp. = *formae speciales* (see text); var. = variety (i.e., defined on the basis of morphological traits).

[b] 1 = Authors' own collection; 2 = Drs. Roger Cassini and Renée Cassini, Centre National de la Recherche Agronomique, Versailles; 3 = Dr. J. Louvet, Institut National de la Recherche Agronomique, Dijon; 4 = Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands; 5 = Museum National d'Histoire Naturelle, Paris; 6 = Eidgenosische Technische Hochschule, Zurich; 7 = Dr. P. Van Etten, Department of Plant Pathology, Cornell University, Ithaca, N.Y.; 8 = Snyder and Hansen (1945).

[c] Strains received with a name departing from Booth's (1971) taxonomical system.

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see fig. 1) complementary to the evolutionarily conserved portion of 28S rRNA segments and positioned either just by the side of domains known as variable (P1 and P3) or inside an evolutionarily conserved stretch (P2) were used as primers and were labeled at their 5′ end with [gamma-^{P}32] ATP and polynucleotide kinase. Two gels, 8% and 6% acrylamide, were generally run on each set of sequencing reactions, allowing the determination of 250–280 nucleotide stretches (Qu et al. 1983). Cloned reverse transcriptase was purchased from Bethesda Research Laboratories, and deoxynucleotides and dideoxynucleotides were from Boehringer. Oligonucleotide primers were synthesized using the phosphoramidite protocol on an Applied Biosystems DNA synthesizer (model 380 A). With these techniques, one nucleotide sequence can be obtained from lyophilized mycelium in 3 d.

Analysis of Data

The sequences were aligned manually. Divergence (or distance) between two sequences was estimated either as the number of nucleotide positions containing different symbols or as Kimura's (1980) K_{nuc} index. Dendrograms were constructed from the distance data by the Fitch and Margoliash (1967) least-squares clustering procedures using the FITCH and KITSCH programs of Felsenstein's PHYLIP package (version 2.9). We also looked for the most-parsimonious trees implied by the sequences. For that purpose, we used the DNAPENNY program from the same package. To root

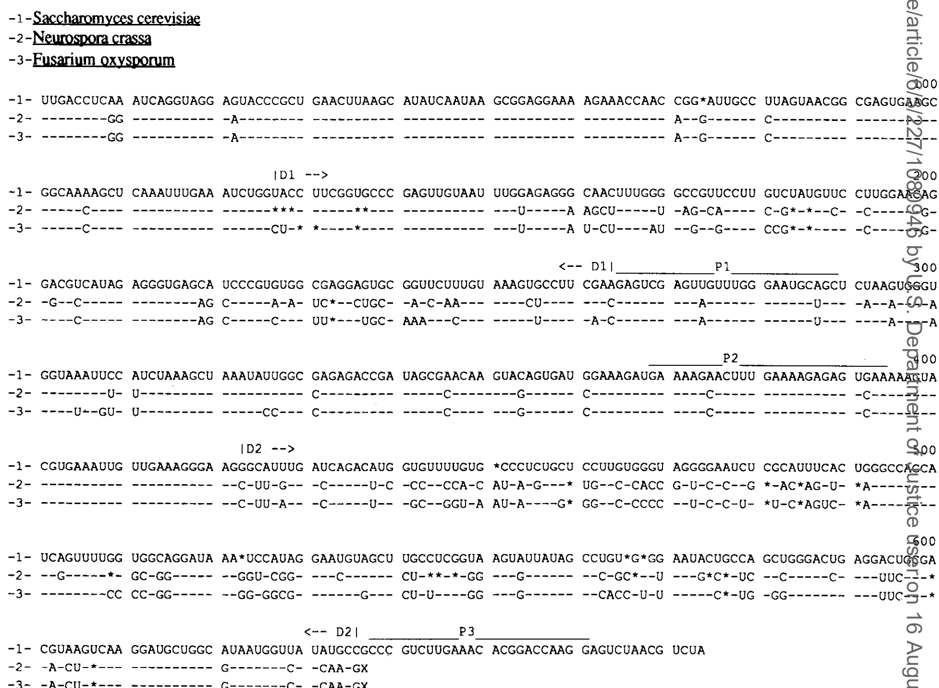

FIG. 1.—Comparison of three fungi large-subunit rRNAs. −1− = RNA sequence of *Saccharomyces cerevisiae* from Georgiev et al. (1981); −2− and −3− = RNA sequences deduced from sequencing cDNA from *Neurospora crassa* and *Fusarium oxysporum* (FOM 15). Positions are numbered from the 5′ end of the molecule. Identical bases are denoted by a dash (—); undetermined bases are denoted by an ×; and deletions are denoted by an asterisk (∗). Domains D1 and D2 are flagged, and oligonucleotide primers (P1, P2, and P3) are overlined.

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the phenetic tree when needed, we added *Neurospora crassa* and *Saccharomyces cerevisiae* as outgroup species.

Results

The common features of structural organization shared by 28S-like molecules in all species should provide a versatile and sensitive phylogenic indicator since large portions of the sequence, well-conserved throughout evolution, are interspersed with much more rapidly evolving domains (Clark et al. 1984; Hassouna et al. 1984). Referring to data and figures published by Clark et al. (1984) and Hassouna et al. (1984) (in the absence of published complete secondary structure for fungi large rRNAs), we selected the 5′-terminal end of the 28S-like RNA molecule which contains both slowly and rapidly evolving domains (the latter termed D1 and D2). Also Lane et al. (1985) reported that phylogenetic trees constructed by using limited regions of 16S RNA molecules have the same topologies as those obtained from the complete molecules. Therefore the region we selected, ~640 nucleotides long, should provide enough data and reduce the risk of spurious fluctuations in the statistics of nucleotide substitutions. Indeed, this part of the molecule has been found useful for partial phylogeny determination of unicellular eukaryotes (Baroin et al. 1988).

In a first set of experiments, the 5′-terminal end of the 28S-like rRNA from one *Fusarium oxysporum* strain (FOM 15 in table 1) and one *Neurospora crassa* strain were sequenced and aligned with the corresponding *Saccharomyces cerevisiae* sequence (fig. 1). The length variations (deletions or insertions) of this stretch are limited to a few bases along the 640 nucleotides. This alignment clearly confirms that the stretches 1–126 and 265–422 are evolutionarily conserved (<20 nucleotide subtitutions) and that variability is almost restricted to stretches 127–264 (D1) and 423–636 (D2). Therefore, for the 51 additional *Fusarium* strains analyzed, sequencing was limited to the 352 nucleotides of these D1 and D2 regions. These sequences can be sorted into 18 classes, each with identical sequences (fig. 2). When comparing the *Fusarium* sequence classes with taxonomical units (table 1), we observed two situations: (1) One class combines all the strains from one species. This is the case for classes G, F, and R corresponding to *F. culmorum, F. graminearum,* and *F. nivale,* of which species there are four, five, and three strains, respectively. (2) A single species is split into several classes. For instance, in *F. oxysporum,* 11 strains belong to class A and only one (*F. oxysporum* var. *redolens*) to class B. This contrasts with the *F. solani* species, in which the 16 strains are divided into seven classes (K–Q). *Fusarium moniliforme* and *F. decemcellulare* are both split into three different classes—respectively, classes C–E and H–J.

One approach was to compare the class sequences pairwise and construct a distance matrix (table 2). From this matrix, we took sequences A–R, plus *N. crassa* and *S. cerevisiae* as outgroups, and ran the FITCH program, which gave a phenetic tree sketched in the figure 3 inset. The same program, run on a K_{nuc} index distance matrix to get a better estimate of evolutionary distances, resulted in a relative increase of large distances compared with shorter ones, without modification of the branching pattern of the phenetic tree. Except for the *F. nivale* class, which branched as far away from all other *Fusarium* as did *N. crassa,* the remaining *Fusarium* classes are grouped into three clusters (I–III), branching from a common stem, with 10% maximal divergence between them. There can only be one teleomorph state, if any, in each cluster: *Gibberella* (I), *Calonectria* (II), and *Nectria* (III). These three clusters were further analyzed by adjusting branch lengths so that every total length from each tip to the

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				|D1		150			180
Δ	Reference	GGCAACAGCU	CAAAUUUGAA	AUCUGGCUC*	*UCGG*GCCC	GAGUUGUAAU	UUGUAGAGGA	UGCUUUUGGU	GAGGUG[illegible]CUU
A	F. oxysporum	----------	----------	----------	----------	----------	----------	-A------A-	-C--------
B	F. oxysp. var. redolens	----------	----------	----------	----------	----------	----------	--------A-	-C--------
C	F. moniliforme 1	----------	----------	----------	----------	----------	----------	-A------A-	-C--------
D	F. moniliforme 3	----------	----------	----------	----------	----------	----------	-A------A-	-C--------
E	F. monili. subglutinans	----------	----------	----------	----------	----------	----------	-A------A-	-C--------
F	F. graminearum	----------	----------	--------U-	----------	----------	----------	--A-----A-	-C--------
G	F. culmorum	----------	----------	--------U-	----------	----------	----------	--A-----A-	-C--------
H	F. decemcellulare 1	----------	----------	----------	----------	----------	----------	----------	-C--------
I	F. decemcellulare 3	----------	----------	----------	----------	----------	----------	----------	-C--------
J	F. decemcellulare 4	----------	----------	----------	----------	----------	----------	----------	-C--------
K	F. sol. var. coeruleum	----------	----------	----------	----------	----------	----------	----------	----------
L	F. sol. var. martii	----------	----------	----------	----------	----------	----------	----------	----------
M	F. sol. f. sp. cucurbitae	----------	----------	----------	----------	----------	----------	----------	----------
N	F. sol. var. minus	----------	----------	----------	----------	----------	----------	----------	----------
O	F. sol. var. javanicum	----------	----------	----------	----------	----------	----------	----------	----------
P	F. sol. var. radicicola	----------	----------	----------	----------	----------	----------	----------	----------
Q	N. haematococca	----------	----------	----------	----------	----------	----------	----------	----------
R	F. nivale	----------	----------	-------CU-	-X-U---U--	----------	----------	--A-------	U----A----
2	N. crassa	----------	----------	--------*-	---*------	----------	----------	A---------	----CA----
1	S. cerevisiae	-----A----	----------	------UA-C	U----U----	----------	---G-----G	CAAC-----G	-CC--U----

		200					250		<-D1|
Δ	*CCGA*GUUC	CCUGGAACGG	GACGCCAUAG	AGGGUGAGAG	CCCCGUCUGG	UU*GGAUGCC	GAUCCUCUGU	AAAGCUCCUU	CGAC
A	----------	----------	----------	----------	----------	----------	A-AU------	----U-----	-A--
B	----------	----------	----------	----------	----------	----------	A-AU------	----------	-A--
C	----------	----------	----------	----------	----------	----------	A-AU------	----U-----	----
D	----------	----------	----------	----------	----------	----------	A-AU------	----U-----	----
E	----------	----------	----------	----------	----------	----------	A-AU------	----U-----	----
F	----------	----------	----------	----------	----------	----------	A-AU------	---U------	----
G	----------	----------	----------	----------	----------	----------	A-AU------	---U------	----
H	--------G-	----------	----------	----------	----------	----------	A---------	----------	----
I	--------G-	----------	----------	----------	----------	----------	A---------	----------	----
J	--------G-	----------	----------	----------	----------	----------	A---------	----------	----
K	----------	----------	----------	----------	----------	------C---	--A-------	----------	----
L	----------	----------	----------	----------	----------	------C---	--A-------	----------	----
M	----------	----------	----------	----------	----------	------C---	--A-------	----------	----
N	----------	----------	----------	----------	----------	------CA--	----------	----------	----
O	----------	----------	----------	----------	----------	------CA--	----------	----------	----
P	----------	----------	----------	----------	----------	------CA--	----------	----------	----
Q	----------	----------	----------	----------	----------	------CA--	--G-------	----------	----
R	----------	-U------A-	------U---	----------	------AC--	----------	--G-------	---U------	----
2	--U-----C-	----------	-G--------	----------	------A-A-	-C--------	-----AA---	----------	----
1	GU-U-U----	-U------A-	----U-----	--------CA	U-----G---	CGA---GUG-	-G-U--U---	-----UG---	---A

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		410		D2		450					
A	Reference	CGUGAAAUUG	UUGAAAGGGA	AGCGUUUAUG	ACCAGACUUG	GGCUUGGUUG	AUCAUCUGG*	GGUUCUCCCC	GGUGCACUCU	*UCC*AGCCC	*A[illegible]CCA
A	F. oxysporum	----------	----------	----------	----------	---------A	----------	----------	A-------U-	-------U--	------
B	F. oxy. var. redolens	----------	----------	----------	----------	---------A	----------	----------	A-------U-	-------UU-	------
C	F. moniliforme 1	----------	----------	----------	----------	---------A	----------	----------	A-------U-	-------U--	------
D	F. moniliforme 3	----------	----------	----------	----------	---------A	----------	----------	A-------U-	-------U--	------
E	F. monili. subglutinans	----------	----------	----------	----------	---------A	----------	----------	A-------U-	-------U--	------
F	F. graminearum	----------	----------	----------	----------	---------A	----------	------U---	A-------U-	-------U--	------
G	F. culmorum	----------	----------	----------	----------	---------A	----------	-------U--	A-------U-	----------	------
H	F. decemcellulare 1	----------	----------	----------	----------	---------A	------CA--	---------U	--------U-	-------U--	------
I	F. decemcellulare 3	----------	----------	----------	----------	---------A	------CA--	---------U	--------U-	-------U--	------
J	F. decemcellulare 4	----------	----------	----------	----------	---------A	------CA--	---------U	--------U-	-------U--	------
K	F. sol. var. coeruleum	----------	----------	----C--G--	----------	----------	------**--	----------	----------	-----*----	------
L	F. sol. var. martii	----------	----------	----C--G--	----------	----------	------**--	----------	----------	-----*----	------
M	F. sol. f. sp. cucurbitae	----------	----------	----C--G--	----------	----------	------**--	----------	----------	-----*----	------
N	F. sol. var. minus	----------	----------	----C--G--	----------	----------	------**--	----------	----------	-----*--U-	------
O	F. sol. var. javanicum	----------	----------	----C--G--	----------	----------	------**--	----------	----------	-----*--U-	------
P	F. sol. var. radicicola	----------	----------	----C--G--	----------	----------	------**--	----------	----------	-----*----	------
Q	N. haematococca	----------	----------	----C--G--	----------	----------	------**--	----------	----------	-----*--U-	------
R	F. nivale	----------	----------	--GA------	-------C-U	U----*-CG-	------*---	U------A--	--------U-	-G-UUG-UUG	------
2	N. crassa	----------	----------	-------G--	---------C	-C---CCA-C	-----G--C-	U------A--	---------G	-GA-----U-	------
1	S. cerevisiae	----------	----------	--G-CA-U--	-U-----A--	-UG--UUG--	*C-C----CU	CC--G-GGGU	A-G-G-A---	CG-AUUU-A-	UG----

				550					600			
1	CCGGGGGAUA	AAGGCUUCGG	GAAUGUGGCU	CUCUUCGGGG	AGUGUUAUAG	CCCGUUGCGU	AAUACC*CUG	GGGGGGACUG	AGGUUCGCG*	CAUCUG*CAA	GGAUGCUGGC	G[illegible]AAU
A	----------	-----GG---	----------	----------	----------	---ACC-U--	----------	----------	----------	----------	----------	-----
B	----------	-----GG---	----------	----------	----------	----CC-U--	----------	----------	----------	----------	----------	-----
C	----------	---A------	----------	----------	----------	-------U--	----*-----	-C--------	----------	----------	----------	-----
D	----------	---A------	----------	----------	----------	-------U--	----*-----	----------	----------	----------	----------	-----
E	----------	---A------	----------	----------	----------	-------U--	----------	----------	----------	----------	----------	-----
F	----------	----------	----------	-C-C------	----------	-------U--	----------	-U--------	----------	-U--------	----------	-----
G	----------	---A------	----------	-C-C------	----------	-------U--	----------	-U--------	----------	-U--------	----------	-----
H	U---------	----------	----------	----C-----	----------	----------	----------	---C------	----A-----	-U--------	----------	-----
I	U---------	----------	----------	----------	----------	-------U--	----------	---C------	----A-----	-U--------	----------	-----
J	U---------	----------	----------	-CUC------	----------	----------	----------	---C------	----A-----	-U--------	----------	-----
K	-U------C-	-----A----	---C------	----C-----	----------	----------	-------*--	C--C------	----------	---UC-----	----------	-----
L	-U--------	-----A----	---C------	----C-----	----------	----------	----------	C--C------	----------	---UC-----	----------	-----
M	-U--------	----------	---C------	----C-----	----------	----------	----------	U--C------	----------	---UC-----	----------	-----
N	-U--------	----------	----------	----C-----	----------	----C-----	----------	U--C------	----------	---UC-----	----------	-----
O	-U------A-	----------	----------	----C-----	----------	----C-----	----------	U--C------	----------	---UC-----	----------	-----
P	-U------C-	----------	----------	----C-----	----------	----C-----	----------	U--C------	----------	---UC-----	----------	-----
Q	-U--------	-----AG---	----------	----C-----	----------	----C-----	----------	U--C------	----------	---UC-----	----------	-----
R	GG-C------	---A----A-	---C---A--	-C-*C-*---	----------	--U-C--U--	-------GCC	-CU-------	---AC-----	-G--G---U-	----------	A----
2	G---------	-----UCCG-	---C--A---	----**-*--	----------	----GC**--	---G*----C	-CC-----C-	----------	----------	----------	-----
1	UG-CA-----	--*U-CAUA-	------A---	UG-C----UA	---A------	--U--*-*-G	-----UG-CA	-CU-------	---ACU---A	-G-AA-U---	----------	A----

FIG. 2.—Nucleotide variations among sequence classes within domains D1 (upper sequences) and D2 (lower sequences). The reference sequen
taking the most frequent base for each position; it is valid only for the set of sequences aligned with it and does not represent an ancestor sequence.
fig. 1.

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Table 2
Distance Matrix between the 18 Classes of Identical Sequences, plus Reference Sequence (Δ) and Two External Species

CLASS[a]	Δ	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	2	1
Δ		5.4	4.8	4.8	4.3	4.0	5.7	5.7	4.3	4.8	5.4	5.7	5.1	4.8	5.1	5.4	5.1	6.0	17.0	14.5	33.5
A	19		1.1	2.8	2.3	2.0	4.8	5.4	6.8	6.2	7.4	10.2	9.7	9.7	9.9	10.2	9.9	9.7	19.9	17.6	34.4
B	17	4		3.4	2.8	2.6	4.3	4.8	6.2	5.7	6.8	9.7	9.1	9.1	8.8	9.1	9.4	8.5	18.7	16.5	34.7
C	17	10	12		0.6	0.9	3.4	3.4	6.0	5.4	6.5	9.9	9.4	9.1	9.9	10.2	9.9	10.5	18.7	16.8	32.2
D	15	8	10	2		0.3	3.7	3.7	5.7	5.1	6.2	9.4	8.8	8.5	9.4	9.7	9.4	9.9	18.7	17.3	33.8
E	14	7	9	3	1		3.4	3.4	5.4	4.8	6.0	9.1	8.5	8.2	9.1	9.4	9.1	9.7	18.5	17.6	33.5
F	20	17	15	12	13	12		1.1	6.2	5.7	5.7	10.8	10.2	9.9	10.8	11.1	10.8	11.4	17.6	17.9	33.8
G	20	19	17	12	13	12	4		6.8	6.2	6.2	10.8	10.2	9.9	10.8	11.1	10.8	11.4	17.3	17.6	33.5
H	15	24	22	21	20	19	22	24		0.6	1.1	8.2	7.7	7.4	7.7	8.0	7.7	8.5	19.0	17.6	35.2
I	17	22	20	19	18	17	20	22	2		1.1	8.8	8.2	8.0	8.2	8.5	8.2	9.1	19.0	17.6	34.9
J	19	26	24	23	22	21	20	22	4	4		9.4	8.8	8.5	8.8	9.1	8.8	9.7	19.3	18.2	34.9
K	20	36	34	35	33	32	38	38	29	31	33		0.6	1.1	2.6	2.6	2.0	2.6	19.6	18.7	36.1
L	18	34	32	33	31	30	36	36	27	29	31	2		0.6	2.0	2.3	2.0	2.0	19.3	18.2	35.5
M	17	34	32	32	30	29	35	35	26	28	30	4	2		1.4	1.7	1.4	2.0	19.0	18.2	35.5
N	18	35	31	35	33	32	38	38	27	29	31	9	7	5		0.3	0.6	0.9	19.0	18.2	35.2
O	19	36	32	36	34	33	39	39	28	30	32	9	8	6	1		0.6	1.1	19.3	18.5	35.5
P	18	35	33	35	33	32	38	38	27	29	31	7	7	5	2	2		1.4	19.6	18.7	35.5
Q	21	34	30	37	35	34	40	40	30	32	34	9	7	7	3	4	5		19.3	18.5	35.5
R	60	70	66	66	66	65	62	61	67	67	68	69	68	67	67	68	69	68		22.4	35.2
2	51	62	58	59	61	62	63	62	62	62	64	66	64	64	64	65	66	65	79		37.8
1	118	121	122	117	119	118	119	118	124	123	123	127	125	125	124	125	125	125	124	133	

NOTE.—Distances are expressed in % of differing bases (upper matrix) within the D1 + D2 regions (352 nucleotides) and in absolute value (lower matrix). Any difference (transition, tranversion, or each nucleotide deletion) counts as one, without correction for multiple changes at one given site.

[a] For each class, one strain is given as an example. Δ Is constructed by taking the most frequent base for each position. A = *Fusarium oxysporum;* B = *F. oxysporum* var. *redolens;* C = *F. moniliforme* 1; D = *F. moniliforme* 3; E = *F. moniliforme* var. *subglutinans;* F = *F. graminearum;* G = *F. culmorum;* H = *F. decemcellulare* 1; I = *F. decemcellulare* 3; J = *F. decemcellulare* 4; K = *F. solani* var. *coeruleum;* L = *F. solani* var. *martii;* M = *Nectria haematococca* var. *cucurb;* N = *F. solani* var. *minus;* O = *F. javanicum* var. *javanicum;* P = *F. javanicum* var. *radicicola;* Q = *N. haematococca;* R = *F. nivale;* 2 = *N. crassa;* 1 = *Saccharomyees cerevisiae.*

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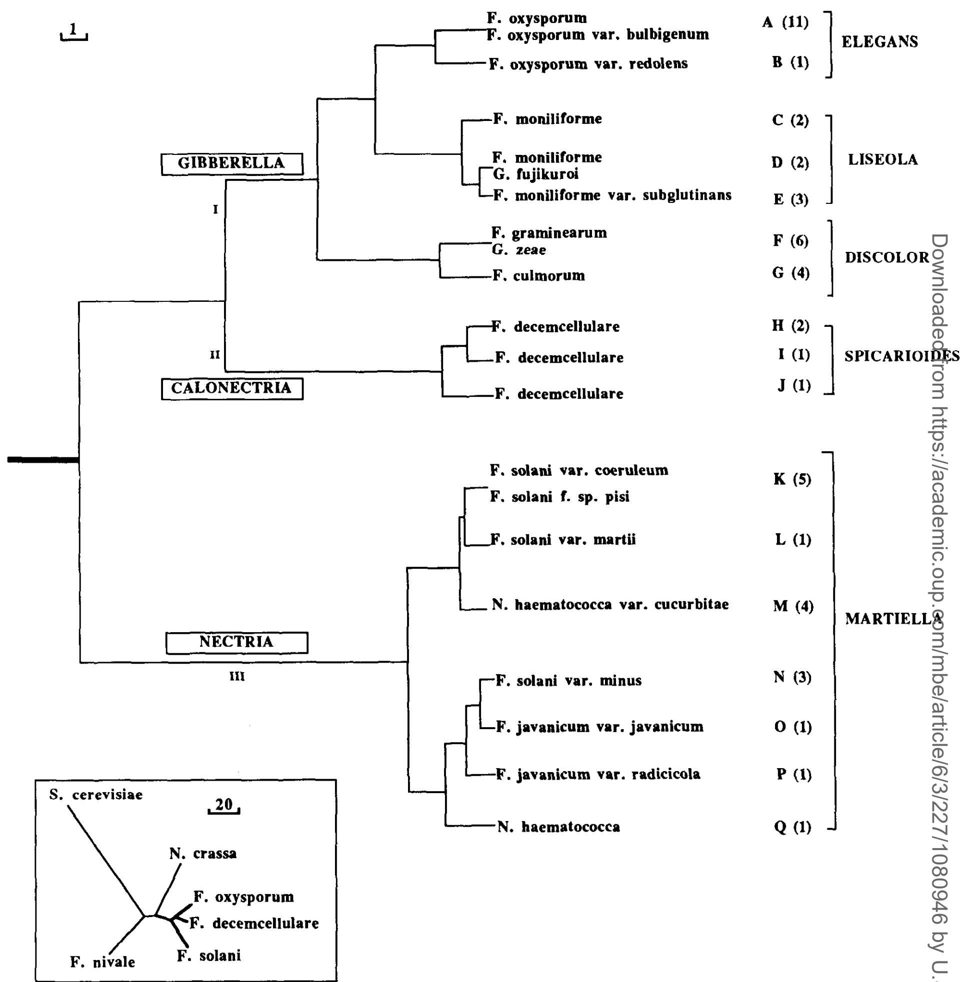

FIG. 3.—*Fusarium* phylogenetic trees constructed from table 2 data (lower matrix). Branch lengths are computed by the KITSH program [Fitch and Margoliash (1967) method, assuming a constant evolutionary clock in all branches]; scale bar = one nucleotide difference; average % SD = 12.9. Sequence classes (capital letters), number of analyzed strains, and taxonomical sections are listed at right. *Inset:* General sketch of the unrooted tree computed, from the same data, by the FITCH program (unconstrained branch lengths). Scale bar = 20 nucleotide differences; average % SD = 6.4. One typical sequence class from each *Fusarium* cluster is cited: *F. oxysporum* for *Gibberella*, *F. decemcellulare* for *Calonectria*, and *F. solani* for *Nectria*.

common root is the same. This was done by the KITSCH program, and several trees were obtained, with identical average percent standard deviations. These trees varied locally in their branch lengths, depending on the input order in which distances were entered into the computer program, but the grouping of sequence classes remained the same. One of these trees, that most similar to the FITCH tree, is shown in figure 3.

Another complementary approach was to construct parsimony trees from the *Fusarium* sequence classes (excluding *F. nivale*). However, parsimony calculations, in this version of PHYLIP, are practically limited to the simultaneous treatment of ~10 sequences. Thus, the DNAPENNY program was run on each cluster's sequences

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plus one outgroup sequence, and a hypothetical ancestral sequence was estimated for each node of the resulting tree. Then, step by step, we could reconstruct a possible most parsimonious *Fusarium* tree from these common ancestor sequences for each cluster and could infer a possible general ancestor for the whole *Fusarium* tree. This method gives clues about possible multiple substitutions at each site; for 56 variable sites within the reconstructed *Fusarium* tree (17 sequences), we found an average 1.4 substitutions/variable site (range 1–3; four sites showed three substitutions).

Discussion

The simultaneous computation of nucleotide differences within the D1 and D2 domains allowed us to evaluate the phylogenetic distances between very closely related species as well as between less related species. But for distantly related species such as *Saccharomyces cerevisiae, Neurospora crassa,* and *Fusarium oxysporum,* multiple substitutions may have occurred at one given site, and the number of nucleotide differences over domains D1 and D2 is an underestimate of the total divergence. This is the case for the species depicted in the insert of figure 3. We found that the variability of our sequences is high enough to distinguish 18 sequence classes among seven *Fusarium* species chosen to be representative of the diversity of this genus. These sequence classes can be arranged into a phenetic tree (fig. 3). This tree appears to be well structured, with a first radiation of two main branches (defined as clusters I plus II and III), each one with further branching. If we regard rRNA molecules as good phylogenetic indicators, we may accept this tree as a phylogenetic tree. A very similar branching pattern was observed in the parsimony trees. This similarity is a direct consequence of the low average substitution per variable site (1.4) and reflects the closeness of the analyzed strains. However, we shall rely mostly on the phenetic tree for the following discussion.

What is the contribution of this molecular analysis to the knowledge of the genetic diversity in the genus *Fusarium*? On the basis of sequence analysis, *F. nivale* is about equally divergent (17.3%–19.9%) from all other *Fusarium,* which cluster into a group of 11.4% maximal internal divergence. The divergence shown by *F. nivale* could represent either a higher evolutionary rate in this species ("fast evolutionary clock") or an earlier branching in the Pyrenomycetes. The first interpretation is easy to check by comparison with a distantly related species, such as *S. cerevisiae.* We found that the latter is as distant from *F. nivale* as from every other *Fusarium* (33%–36%). Hence the distance data do not support this fast-evolutionary-clock hypothesis (McCarroll et al. 1983). Thus, we suggest that *F. nivale* diverged from the mainstream leading to the *Fusarium* before the branching of some other Pyrenomycetes such as *N. crassa.* This result gives grounds for the taxonomical meandering of *F. nivale* during the past 40 years. Indeed, *F. nivale* has long been known as a morphologically anomalous *Fusarium* on the basis of its conidiogenous cells proliferating, the shape of its conidia, and its light requirement for sporulation (Parkinson et al. 1981). Our results would support its removal from the anamorph genus *Fusarium* and its reassignment to *Gerlachia* (Gams and Müller 1980), itself recently reassigned to the genus *Microdochium* (Samuels and Hallet 1983) corresponding to the teleomorph genus *Monographella* (Müller 1977) (fig. 4). The macroconidium shape of *F. nivale* is now considered as a convergence with macroconidia of other *Fusarium* for two reasons: first, the macroconidium fine structure of *F. nivale* is different (Parkinson 1980); second, convergence, implying two distinct occurrences of a similar character, is simpler to imagine (it is more economical from a cladistic point of view) than is an ancestral character which

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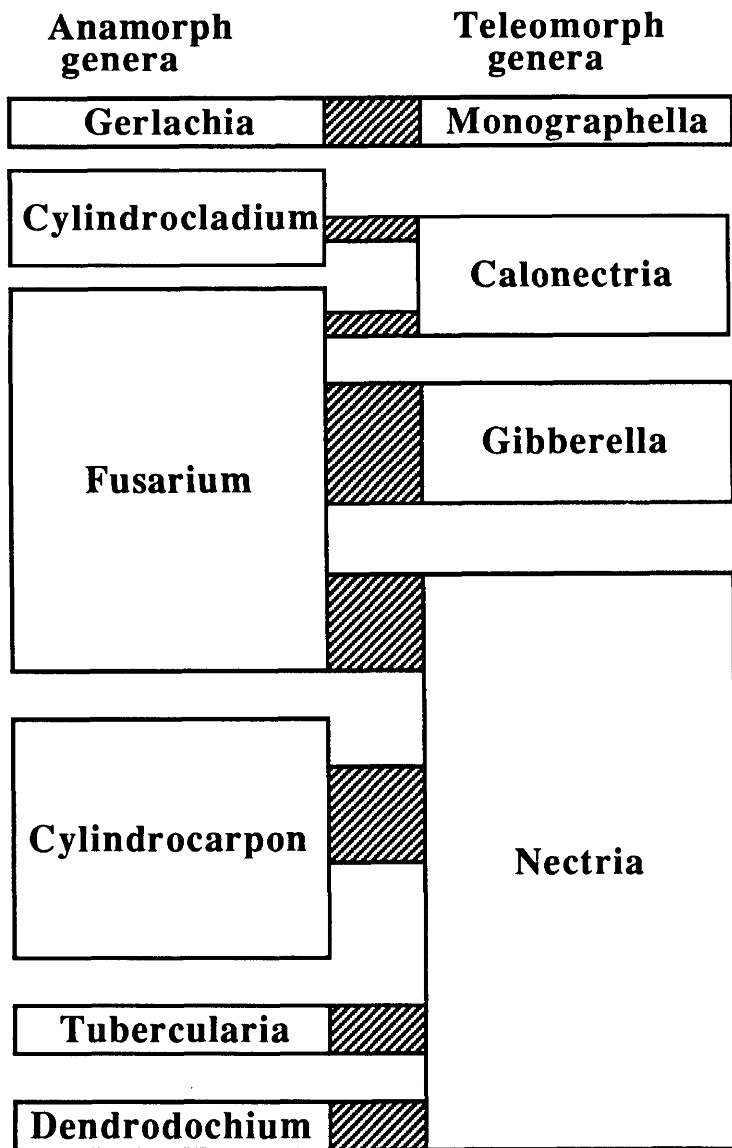

FIG. 4.—Correspondences between anamorph (*left*) and telemorph (*right*) genera related to *Fusarium*, from the data of Subramanian (1983, pp. 52–94). The surfaces of hatched connections and genus blocks are roughly proportional to the number of species.

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would have disappeared many times in all Pyrenomycetes branches but two (see fig. 3, inset). Therefore, from now on in this discussion we shall no longer consider *F. nivale* to belong to the genus *Fusarium*. The remaining species of *Fusarium* form a rather homogeneous group of sequences (≈11% maximal divergence) when compared with external species such as *N. crassa, S. cerevisiae,* or *F. nivale*. This is compatible with a monophyletic origin for the *Fusarium* species analyzed. However, sequencing other strains either from anamorph genera related to *Fusarium* (e.g., *Cylindrocarpon* or *Cylindrocladium*) or from species described as doubtful *Fusarium* (some species

in the sections *Epispheria* and *Arachnites*) might clarify the branching point and the extent to which the whole genus is within the Pyrenomycetes.

As shown in figure 4, the partition of the single anamorph genus *Fusarium* into three distinct subgroups corresponding to three teleomorph genera (*Gibberella, Calonectria,* and *Nectria*) stresses the ambiguity of the genus concept. The genera *Calonectria* and *Nectria* comprise several species, the asexual state of which might be rather distantly related to that of *Fusarium* (e.g., *Tubercularia* and *Dendrodochium*). The generic name *Fusarium,* defined on the basis of the asexual state, clearly combines closely related species, with exceptions such as *F. nivale.* Therefore, referring to these species by a generic name based on their sexual state, results in a somewhat unnecessary fragmentation of an otherwise cohesive genus. In our opinion, the perithecial morphology, combined with conidial characteristics, should be a classification criterion limited to a subgeneric level within the genus *Fusarium.*

As early as 1913, Wollenweber defined several sections, on the sole basis of anamorph characters. The correspondence between sectional and perithecial groups is not obvious, as one section may include different perithecial morphologies. Our dendrogram reveals, first, an unequivocal separation of section *Martiella* from section *Spicarioides* and from the other three sections analyzed (*Elegans, Liseola,* and *Discolor*) and, second, three clearly distinct clusters corresponding exactly to three different teleomorphs: strains with *Nectria* perithecia are grouped in cluster III, strains described as having *Calonectria* teleomorphs are in cluster II, and three strains with *Gibberella* teleomorphs are all found in cluster I. The subgeneric group composed of these three last strains indeed combines organisms with great affinity (≤3.7% internal divergence). This validates the fact that morphotaxonomists consider *Gibberella* as a rather uniform and distinctive genus (Samuels and Rossman 1979). The species *F. oxysporum* and *F. culmorum,* without known sexual state, can be exactly positioned by our analysis: they fit easily in cluster I, with just a slight increase of its internal variability (5.4%). Thus, we have no doubt about their membership in the teleomorph *Gibberella.* This result is not consistent with the conclusion of Szécsi and Dobrovolsky (1985*b*), who found, on the basis of DNA thermal denaturation analyses, that *F. oxysporum* was far away from other *Fusarium* species; but it is in agreement with the commonly accepted taxonomic position of *F. oxysporum* (Booth 1971). Hence, despite the limited number of strains analyzed, which belong to five different taxonomical sections, we again find a good coincidence between groupings based on sequence data and previous taxonomical assignments at the subgeneric level (section). It turns out that both anamorph and teleomorph criteria are useful for a common classification of *Fusarium.* It is suggested that these two criteria are ordered in order to get groupings with a phylogenetical value: anamorphic criteria should define the genus, sections, and species, while teleomorphic criteria may be used to refine the definition of species—as well as that of subgenera (i.e., between genus and section levels).

The good agreement between rRNA sequence data and morphotaxonomy at the subgeneric level prompted us to apply our molecular tool to a lower taxonomical level: species, varieties, *formae speciales,* and races. There is a large variation from one species to another in terms of the number of varieties: species with some variable characters comprise several varieties, while homogeneous species do not. Accordingly, we tried to determine how our smallest sequence differences matched the previously identified distinct taxa (table 1). In fact, we observe that there is no one-to-one correspondence between a sequence class and a previously defined taxon. The sequence classes may correspond to two different taxonomic levels: a species level (classes F,

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G, and R) and an infraspecific level, such as varieties (classes A–E and I–Q). For instance, the sequence data for *F. solani* scatters the 16 strains into seven sequence classes, with divergence levels ≤2.6%. We can relate this result to the wide polymorphism of *F. solani* macroconidia, a polymorphism that was used by Wollenweber and Reinking (1935, pp. 1–15) for the partition of this group into three species and nine varieties: *F. coeruleum, F. javanicum* (four varieties), and *F. solani* (five varieties). On the other hand, we do not find any single difference in the sequence stretches from the four *F. culmorum* strains analyzed here. In this latter case, the species *F. culmorum* shows a rather uniform asexual morphology. Finally, within the species *F. oxysporum*, only *F. oxysporum* var. *redolens* can be clearly distinguished from all other *F. oxysporum* by the shape and proportion of its macroconidia; when the sequences are considered, all the *F. oxysporum* are identical, except for *F. oxysporum* var. *redolens*. Hence, we observe that, within a morphologically homogeneous group, a single sequence class is found. From these facts, we conclude that at a lower (infraspecific) level, a majority of varieties can be sorted out by sequence data; but so far we are unable to go further down with this molecular tool in distinguishing between *formae speciales* and a fortiori between races. This confirms that *formae speciales* are so genetically similar that presently available molecular techniques are not suitable for identification purposes at that level.

Then, can sequence analyses contribute to a better demarcation of *Fusarium* species? Which divergence between two sequences is compatible with a membership in the same species? The two investigated strains *F. solani* f.sp. *pisi* (also called *Nectria haematococca* f.sp. *pisi*) and *F. solani* var. *martii* interbreed and produce perithecia with *N. haematococca* morphology (Holenstein and Défago 1983). So we consider them as belonging to the same species. However, because their sequences diverge by 0.6%, we admit some intraspecific sequence variability. To estimate a divergence threshold above which we would be dealing with two species, we would need to analyze some other strains and species from the genus. Cluster III comprises one species only (*F. solani*) with a 2.6% maximum divergence. But a similar divergence is found in cluster I, which includes several species. These facts suggest either that the concepts of genus and species are not consistent between these two clusters or that species of the same genus may have different infraspecific variabilities (justifying the definition of varieties). In fact, two mutually exclusive solutions may yield a coherent classification system: either *F. solani* is maintained as a single species, in which case *F. oxysporum* and *F. moniliforme, F. culmorum* and *F. graminearum* correspond to two species only, or *F. solani* is split into several species and four species are maintained in cluster I. As we cannot decide from sequence data, we have to refer to morphology. Knowing that several classification systems consider *F. solani* as a group of species rather than as a single species (Gordon 1952), we can split *F. solani* into two subgroups: classes K–M and classes N–Q, each of them including morphologically similar strains with a divergence limited, respectively, to 1.1% and 1.4%. These divergence values are of the same order as those found within the four species analyzed from the *Gibberella* cluster. Hence, the partition of *F. solani* into two species can be inferred from sequence data. A similar partition of *F. solani* into *F. solani* and *F. javanicum* has indeed been proposed by Joffe (1986, pp. 386–440) on the basis of morphological analyses.

The good correspondence, at levels as low as species and variety, with the morphotaxonomy presented as early as 1935 by Wollenweber and Reinking (1935, pp. 1–15) confirms both the phylogenetic validity of morphological criteria (e.g., macroconidia morphology) used by mycologists and the usefulness of this molecular tool,

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never used before, to our knowledge, for establishing a phylogeny at such a low taxonomical level. These molecular methods can be used for a rapid determination and phylogenetic classification of strains, especially for atypical isolates (i.e., those lacking any diaspore). More widely, it could also be applied to the classification of filamentous fungi at higher taxonomical levels, as well as to the establishment of a common phylogenetic tree for both the Fungi Imperfecti (teleomorphic) and the Perfecti (anamorphic).

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