Genetic Diversity and Pathogenic Variability Among Isolates of *Colletotrichum* Species from Strawberry

Béatrice Denoyes-Rothan, Guy Guérin, Christophe Délye,
Barbara Smith, Dror Minz, Marcel Maymon, and Stanley Freeman

First and second authors: UREFV, INRA, BP 81, 33883, Villenave d'Ornon Cedex, France; third author: Laboratoire de Malherbologie et Agronomie, INRA, BV 1540, 21034, Dijon Cedex, France; fourth author: U.S. Department of Agriculture-Agriculture Research Services, Poplarville, MS 39470; fifth author: Institute of Soil, Water and Environmental Sciences, ARO, The Volcani Center, P.O. Box 6, Bet Dagan 50250; and sixth and seventh authors: Department of Plant Pathology, ARO, The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel.

Accepted for publication 25 September 2002.

ABSTRACT

Denoyes-Rothan, B., Guérin, G., Délye, C., Smith, B., Minz, D., Maymon, M., and Freeman, S. 2003. Genetic diversity and pathogenic variability among isolates of *Colletotrichum* species from strawberry. Phytopathology 93:219-228.

Ninety-five isolates of *Colletotrichum* including 81 isolates of *C. acutatum* (62 from strawberry) and 14 isolates of *C. gloeosporioides* (13 from strawberry) were characterized by various molecular methods and pathogenicity tests. Results based on random amplified polymorphic DNA (RAPD) polymorphism and internal transcribed spacer (ITS) 2 sequence data provided clear genetic evidence of two subgroups in *C. acutatum*. The first subgroup, characterized as CA-clonal, included only isolates from strawberry and exhibited identical RAPD patterns and nearly identical ITS2 sequence analysis. A larger genetic group, CA-variable, included isolates from various hosts and exhibited variable RAPD patterns and divergent ITS2 sequence analysis. Within the *C. acutatum* population isolated from strawberry, the CA-clonal group is prevalent in Europe (54 isolates of 62). A subset of European *C. acutatum* isolates isolated from strawberry and representing the CA-clonal and CA-variable groups was assigned to two pathogenicity groups. No correlation could be drawn between genetic and pathogenicity groups. On the basis of molecular data, it is proposed that the CA-clonal subgroup contains closely related, highly virulent *C. acutatum* isolates that may have developed host specialization to strawberry. *C. gloeosporioides* isolates from Europe, which were rarely observed were either slightly or nonpathogenic on strawberry. The absence of correlation between genetic polymorphism and geographical origin in *Colletotrichum* spp. suggests a worldwide dissemination of isolates, probably through international plant exchanges.

Additional keywords: *Glomerella cingulata*, internal transcribed spacer, phylogeny, rDNA.

Three species have been reported as causal agents of strawberry anthracnose, *Colletotrichum acutatum* J.H. Simmonds, *C. fragariae* Brooks, and *C. gloeosporioides* (Penz.) Penz. & Sacc. (teleomorph *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk) (19,24). These species cause similar symptoms on strawberry including crown rot, fruit rot, and stolon lesions (15). In Europe, *C. acutatum* is the most prevalent species causing anthracnose, whereas *C. gloeosporioides* is found only occasionally, and *C. fragariae* has not yet been observed (5).

Colletotrichum spp. are currently identified using morphotaxonomic criteria such as morphological characters, i.e., conidial shape and size, appressorium morphology and size, setae morphology and temperature response on potato dextrose agar (PDA) medium (12,18,24,29). Recently, a variety of molecular approaches have been used to discriminate various *Colletotrichum* spp. (2,13,14) or to study the genetic diversity within *Colletotrichum* spp. (9,27). Some of these studies have focused on *Colletotrichum* spp. isolated from strawberry, distinguishing the three species pathogenic on strawberry (1,3,8,20,26).

Although important progress for identification of *Colletotrichum* spp. pathogenic on strawberry has been made, genetic diversity within these *Colletotrichum* spp. has been investigated to a lesser extent. Moreover, no correlation between genetic polymorphism and the two groups of pathogenicity (5) has been studied. The purpose of this work was (i) to investigate genetic polymorphism and pathogenicity of *C. acutatum*, the major pathogen isolated from strawberry in Europe, and (ii) to determine the genetic relatedness of this pathogen to representative isolates of *C. acutatum* obtained from strawberry and other hosts from various countries worldwide. In addition, isolates of *C. gloeosporioides*, observed occasionally on strawberry in Europe, were included in this work.

MATERIALS AND METHODS

Fungal isolates. The 62 isolates of *C. acutatum* isolated from strawberry were chosen from various collections representing a range of isolation dates (1978 to 1998) and geographic regions worldwide (Table 1). Nineteen *C. acutatum* isolates from other host plants were included in the study for comparison. Among these 81 isolates, 22 exhibiting a pink chromogenic coloring in culture are referred as *C. acutatum* chromogenic (Table 1). In addition, 13 isolates of *C. gloeosporioides* from strawberry originating from Europe and the United States and one isolate from mango used as an outgroup were studied. All isolates were single-conidia cultures. Stock cultures were maintained on silica gel at 4°C as described by Perkins (22). Cultures were initiated by transferring silica gel particles from the stock cultures to PDA (Difco Laboratories, Detroit, MI) plates to produce fungal material for molecular analysis and pathogenicity tests. All isolates were identified using classical taxonomic criteria such as conidial shape and growth on PDA (5).

Corresponding author: B. Denoyes-Rothan; E-mail address: denoyes@bordeaux.inra.fr

Publication no. P-2002-1213-03R

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 2003.

Isolation of genomic DNA. Fungal mycelium was produced in 200 ml of liquid medium (24 g/liter of potato dextrose broth [PDB, Difco Laboratories], 1 g/liter of yeast extract [Difco Laboratories], and 1 g/liter of casein hydrolysate [Difco Laboratories]). Cultures were shaken at 100 rpm for 5 days at 27°C. Mycelium was harvested by filtration through mesh sieves (40 µm), washed with sterile water, and deposited onto Whatman paper to remove excess water. Mycelium was ground to a fine powder in liquid nitrogen using a mortar and pestle and stored at –80°C. Three grams of mycelium powder was transferred to a 50-ml tube, and DNA was extracted using a method adapted from Saghai-Maroof (23) by the addition of two chloroform/octanol phases and the addition of 10 mg/ml RNase A (Sigma Chemicals, St Quentin Fallavier, France). DNA concentration was assessed using spectrometric measurement (GeneQuant II, Pharmacia Biotech, Cambridge, UK).

PCR amplification. All 95 *Colletotrichum* isolates were analyzed using random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) (30,31). Amplifications were carried out in 15-µl reaction mixtures containing 30 ng of genomic DNA, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.6), 200 µM each of dATP, dTTP, dCTP, and dGTP, 0.2 µM ten-base primers (Operon Technologies, Rungis, France), 0.2 mg/ml bovine serum albumin, 0.05% (wt/vol) polyoxyethylene-ether W1 (Sigma Chemicals, St Quentin Fallavier, France), and 0.7 units of *Taq* DNA polymerase (Gibco BRL, Life Technologies, Cergy Pontoise, France). A Perkin-Elmer Cetus DNA thermal cycler (Model 9600, Perkin-Elmer Cetus Instruments Division, Courtaboeuf, France) was used with the following program: 1 min denaturation at 95°C, 45 cycles of 10 s at 95°C, 15 s at 37°C, and 2 min at 72°C and a final extension step of 5 min at 72°C. Twenty RAPD primers were tested on six isolates (three strawberry isolates per species of *Colletotrichum*). Four primers (OPA13 [CAGCACCCAC], OPB07 [GGTGACGCAG], OPO02 [ACGTAGCGTC], and OPQ16 [AGTGCAGCCA]) that revealed clear and polymorphic patterns were selected for further RAPD analysis. All RAPD-PCR were performed twice. Universal PCR primers (ITS1, TCCGTAGGTGAACCTGCGG and ITS4, TCCTCCGCTTATT-

TABLE 1. Origin of the 95 analyzed single spore isolates of *Colletotrichum* spp. used in this study

Isolates	Geographic origin	Host plant species	Date of isolation	Color on potato dextrose agar (PDA)[a]	RAPD[b] genetic group
C. acutatum from strawberry					
1079a	France	Strawberry	1989	Orange	CA-clonal
1267b	France	Strawberry	1988	Orange	CA-clonal
1341a	France	Strawberry	1990	Orange	CA-clonal
162a	France	Strawberry	1990	Orange	CA-clonal
1641a	France	Strawberry	1995	Orange	CA-clonal
2033a	France	Strawberry	1995	Orange	CA-clonal
2050a	France	Strawberry	1992	Orange	CA-clonal
2077a	France	Strawberry	1995	Orange	CA-clonal
2081a	France	Strawberry	1995	Orange	CA-clonal
2102c	France	Strawberry	1996	Orange	CA-clonal
2184a	France	Strawberry	1992	Orange	CA-clonal
2218a	France	Strawberry	1992	Orange	CA-clonal
318a	France	Strawberry	1995	Orange	CA-clonal
528a	France	Strawberry	1990	Orange	CA-clonal
66b	France	Strawberry	1992	Orange	CA-clonal
688b	France	Strawberry	1990	Orange	CA-clonal
703a	France	Strawberry	1996	Orange	CA-clonal
Cha-s	France	Strawberry	1994	Orange	CA-clonal
Delorgueil.a	France	Strawberry	1992	Orange	CA-clonal
F1a	France	Strawberry	1985	Orange	CA-clonal
F2b	France	Strawberry	1985	Orange	CA-clonal
F2c	France	Strawberry	1985	Orange	CA-clonal
F5a	France	Strawberry	1985	Orange	CA-clonal
F7a	France	Strawberry	1984	Orange	CA-clonal
V1a	France	Strawberry	1995	Orange	CA-clonal
494a	France	Strawberry	1990	Chromogenic	CA-variable
F3c	France	Strawberry	1984	Orange	CA-variable
F3e	France	Strawberry	1984	Orange	CA-variable
All.F2a	Germany	Strawberry	1995	Orange	CA-clonal
87.8.41	Italy	Strawberry	1994	Orange	CA-clonal
Cuneo.a	Italy	Strawberry	1994	Orange	CA-clonal
Dana	Italy	Strawberry	1994	Orange	CA-clonal
2020a	Poland	Strawberry	1995	Orange	CA-clonal
1159.5c	Spain	Strawberry	1988	Orange	CA-clonal
1159.2a	Spain	Strawberry	1988	Orange	CA-variable
Coll.11a	Switzerland	Strawberry	1996	Orange	CA-clonal
Suisse.a	Switzerland	Strawberry	1993	Orange	CA-clonal
Coll.15a	Switzerland	Strawberry	1996	Chromogenic	CA-variable
382a	United States	Strawberry	…	Orange	CA-clonal
388a	United States	Strawberry	…	Orange	CA-clonal
394a	United States	Strawberry	…	Orange	CA-clonal
Ca1B	United States	Strawberry	1984	Orange	CA-clonal
CalA.a	United States	Strawberry	1984	Orange	CA-clonal
CA-OH1	United States	Strawberry	…	Orange	CA-clonal
CT6	United States	Strawberry	1991	Orange	CA-clonal
Fla411	United States	Strawberry	1992	Orange	CA-clonal

(*continued on next page*)

[a] Orange or chromogenic = isolates exhibiting orange or pink chromogenic coloring on PDA culture, respectively.
[b] RAPD = random amplified polymorphic DNA.
[c] Other strawberry representatives included 138 clonal isolates from Israel (8).

GATATGC) were used for amplification of the ITS1 and ITS2 regions between the small and large nuclear rDNA including the 5.8S rDNA from representative isolates of *C. acutatum* and *C. gloeosporioides*, which were selected according to RAPD analysis (one isolate per subgroup) (Table 2). For rDNA amplification, denaturation for 5 min at 95°C was followed by 40 cycles consisting of 30 s at 95°C, 30 s at 50°C, and 1.5 min at 72°C. The amplification products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide and viewed under UV light. All ITS-PCR experiments were repeated at least four times with consistent results being achieved.

ITS2 sequencing procedure. PCR amplified rDNA products using the primer pair ITS1 and ITS4 (32) resulted in a product of approximately 560 bp, which was extracted from agarose gels using the Jetsorb kit (Genomed GmbH, Germany). The Big Dye Terminator DNA sequencing kit (Perkin-Elmer Inc., Branchburg, NJ) was used for determining the sequence of the ITS2 regions (32). The sequence was determined using an ABI prism 377 DNA sequencer (Applied Biosystem Inc., Foster City, CA) and was performed at the Molecular Biology Center, Rehovot, Israel.

Pathogenicity tests. Pathogenicity of a subset of 38 European isolates of *C. acutatum* and *C. gloeosporioides* selected according to RAPD analysis, plus the isolate Goff from the United States, was assessed. To assign *C. acutatum* isolates to pathogenicity groups 1 or 2, two strawberry cultivars (Belrubi and Elsanta), were chosen as differential hosts (4). Cv. Belrubi is resistant to pathogenicity group 2 isolates and sensitive to pathogenicity group 1 isolates. Cv. Elsanta is susceptible to isolates in both pathogenicity groups and exhibits different severity of symptoms depending on the aggressiveness of isolates. Three 6-week-old plants originating from micropropagation were inoculated with each isolate by spraying a conidial suspension adjusted to 2×10^6 conidia per ml, as previously described in a controlled environment chamber (27 ± 2°C, 90 to 95% relative humidity, and 14-h light) (5). Disease response was recorded 4 weeks after inoculation and based on a 0 to 5 disease severity scale where 0 = no symptom and 5 = dead plant (5).

TABLE 1. (*continued from preceding page*)

Isolates	Geographic origin	Host plant species	Date of isolation	Color on potato dextrose agar (PDA)[a]	RAPD[b] genetic group
Goff	United States	Strawberry	1988	Orange	CA-clonal
J.Jenkins	United States	Strawberry	1989	Orange	CA-clonal
L.Levigne	United States	Strawberry	1989	Orange	CA-clonal
La30	United States	Strawberry	1998	Orange	CA-clonal
LLB10	United States	Strawberry	1988	Orange	CA-clonal
LLB5	United States	Strawberry	1986	Orange	CA-clonal
MD16	United States	Strawberry	1998	Orange	CA-clonal
Mil1a	United States	Strawberry	1983	Orange	CA-clonal
Mil-2	United States	Strawberry	1984	Orange	CA-clonal
MS1	United States	Strawberry	1998	Orange	CA-clonal
NC2	United States	Strawberry	1998	Orange	CA-clonal
OKU1	United States	Strawberry	1992	Orange	CA-clonal
Cooley1b	United States	Strawberry	1990	Chromogenic	CA-variable
Cooley2	United States	Strawberry	1990	Chromogenic	CA-variable
NY3	United States	Strawberry	1998	Chromogenic	CA-variable
TUT-5954[c]	Israel	Strawberry	1996	Orange	CA-clonal
C. acutatum from various hosts					
G4a	France	Lupin	1984	Orange	…
Myrtille.a	France	Bilberry	1995	Chromogenic	CA-variable
Patriot	France	Raspberry	1995	Chromogenic	CA-variable
Coll.14a	Switzerland	Blackberry	…	Orange	…
Coll.4a	Switzerland	Bilberry	1996	Chromogenic	CA-variable
Coll36	Switzerland	Blackberry	…	Chromogenic	CA-variable
120V.2II	United States	Raspberry	1993	Chromogenic	…
BB Pop#11	United States	Blueberry	1994	Chromogenic	CA-variable
BB Pop#19	United States	Blueberry	1994	Chromogenic	CA-variable
BJS Tomato	United States	Tomato	1997	Chromogenic	CA-variable
CA-MIL	United States	Blackberry	1987	Chromogenic	CA-variable
Clemson SF-21	United States	Peach	1993	Chromogenic	CA-variable
DA3	United States	Dogwood	1988	Chromogenic	CA-variable
Dolly BB#25	United States	Blueberry	1995	Chromogenic	CA-variable
Nantana A1	United States	Grape	1995	Chromogenic	CA-variable
NC87-22	United States	Apple	1988	Chromogenic	CA-variable
RB-Ant-91E	United States	Raspberry	1993	Chromogenic	CA-variable
Tomato ARK	United States	Tomato	1988	Chromogenic	CA-variable
Wanda's tomato	United States	Tomato	1996	Chromogenic	CA-variable
C. gloeosporioides					
9.89r2	France	Strawberry	1989		
9.89n1	France	Strawberry	1989		
ArkP1	United States	Strawberry	1982		
CG55a	United States	Strawberry	…		
CG63a	United States	Strawberry	…		
CG162a	United States	Strawberry	…		
86.16	United States	Strawberry	…		
89.15	United States	Strawberry	…		
1159.5d	Spain	Strawberry	1988		
1159.5e	Spain	Strawberry	1988		
Na1a	Japan	Strawberry	1978		
Shi1a	Japan	Strawberry	1992		
Na8a	Japan	Strawberry	1993		
Manguier.a	Reunion	Mango	1981		

Analysis of RAPD data. Only RAPD fragments clearly visible and observed in both experiments were considered for analysis. Presence or absence of fragments was scored as 1 or 0, respectively. Genetic similarities between all pairs of isolates were computed using the formula given by Nei and Li (21): $S = 2b_{ij}/(b_i + b_j)$, where b_{ij} is the number of fragments shared by two isolates, i and j, and b_i and b_j are the total number of fragments found in isolates i and j, respectively. Dissimilarities were computed as genetic distance = 1 – S, and the data were used to construct a dendogram using the unweighted pair-group method with arithmetic average (UPGMA) (25). Distance calculations and construction of the dendogram were performed using Splus 3.1 software (Statistical Sciences Inc., Seattle, WA). Bootstrap analysis (6) was used to support the major groups observed in the dendogram. One hundred genetic dissimilarity matrixes were computed using the Splus software by generating 100 binary matrixes of the same size as the original RAPD data sets by random sampling with replacement. Data were analyzed with the programs Neighbor and Consense of the PHYLIP software package (Phylogeny Inference Package, J. Felsenstein, University of Washington) and used to construct the consensus UPGMA dendogram. Mean genetic dissimilarities between genetic groups were calculated as the arithmetical mean of all pairs of isolate of the two groups. Since the summary table of RAPD data is very large (69 markers), it was not convenient to include this table in the manuscript. All relevant information is available upon request from the corresponding author.

Phylogenetic analysis of sequence data. Analyses of internal transcribed spacer (ITS) sequences were carried out using the program package ARB (28). Alignment of sequences was performed with the implemented ARB automated alignment tool, and alignments were refined manually. Phylogenetic analyses were performed by applying ARB parsimony, distance matrix, and maximum-likelihood methods. Branching order was similar in all methods used. To determine the robustness of phylogenetic trees, analyses were performed on the original data set and a data set from which highly variable positions were removed by use of a 50% conservation filter for the members of *Colletotrichum* to reduce potential tree artifacts that may result from multiple base changes. All sequences used in this study were aligned according to 155 bases in the ITS2 region and used for phylogenetic analysis. Analysis was conducted on sequences from representative isolates of *C. acutatum* from strawberry (Cha-s, France; 1267b, France; F3c, France; F3e, France; Cooley2, United States; Coll.15a, Switzerland; and TUT-5954, Israel), tomato (BJS Tomato and Tomato ARK, United States), blackberry (Coll.14a, France), raspberry (120V.211, United States), grape (Nantana A.1, United States), and bilberry (Myrtille.a, France), of *C. gloeosporioides* from strawberry (1159.5d, Spain), and a number of *Colletotrichum* sequences previously sequenced by the authors (9,11) as well as a number retrieved from GenBank (Table 2). As previously shown (9), DNA sequence analysis of the ITS2 region was more informative and showed a greater interspecies divergence than the ITS1 region alone. Complete ITS1-2 sequences of the isolates were submitted to GenBank with accession numbers appearing in Table 2.

Analyses of pathogenicity experiments. Six plants of each of the cvs. Belrubi and Elsanta were inoculated with the tested isolates. For statistical analysis, the means of disease rating from two experiments were pooled for each cultivar and isolate. Isolates of *C. acutatum* were assigned to pathogenicity group 1 when disease ratings were similar on cvs. Belrubi and Elsanta, and to pathogenicity group 2 when cv. Belrubi was found to be much more resistant (disease rating <1) than cv. Elsanta (disease rating ≥1), according to Student's *t* test ($P = 0.05$).

TABLE 2. Internal transcribed spacer (ITS) 2 sequences of *Colletotrichum* isolates used in this study

Species	Isolate	Host	EMBL accession
C. graminicola	DR1[a]	*Poa annua*	AF 059676
C. linicola	CBS 172.51[b]	…	AB 046609
C. dematium	IMI-080025[c]	…	AB 046608
C. gloeosporioides	CG 231[d]	*Fragaria × ananassa*	AF 272780
C. gloeosporioides	AVO-37-4B[d]	*Persea americana*	AF 207792
C. gloeosporioides	APL 7[d]	*Malus domestica*	AF 272779
C. acutatum	IMI 348494[d]	*Fragaria × ananassa*	AF 272785
C. acutatum	TUT 5954[d]	*Fragaria × ananassa*	AF 207794
C. acutatum	PCN 5[d]	*Carya illinoensis*	AF 272786
C. acutatum	PCH 8[d]	*Prunus persica*	AF 272788
C. acutatum	ANE-HV83C[d]	*Anemone coronaria*	AF 272782
C. acutatum	STR 3[d]	*Fragaria × ananassa*	AF 272784
C. acutatum	ALM-US-4[d]	*Prunus dulcis*	AF 207793
C. acutatum	IMI 223120[d]	*Anemone coronaria*	AF 272783
C. acutatum	APL 2[d]	*Malus domestica*	AF 272787
C. acutatum	ANE-NL12A[d]	*Anemone coronaria*	AF 272781
C. acutatum	IMI 345026[d]	*Fragaria × ananassa*	AF 272789
Colletotrichum	ALM-KSH-10[d]	*Prunus dulcis*	AF 207791
C. gloeosporioides	1159.5d[e]	*Fragaria × ananassa*	AF 489568
C. acutatum	Myrtille.a[e]	*Vaccinium myrtillus*	AF 489567
C. acutatum	Tomato ARK[e]	*Lycopersicon esculentum*	AF 489560
C. acutatum	F3c[e]	*Fragaria × ananassa*	AF 489566
C. acutatum	F3e[e]	*Fragaria × ananassa*	AF 489561
C. acutatum	Nantana A1[e]	*Vitis* sp.	AF 489559
C. acutatum	120V.211[e]	*Rubus* sp.	AF 489556
C. acutatum	Coll.15a[e]	*Fragaria × ananassa*	AF 489557
C. acutatum	Cooley2[e]	*Fragaria × ananassa*	AF 489558
C. acutatum	Coll.14a[e]	*Rubus* sp.	AF 489563
C. acutatum	BJS Tomato[e]	*Lycopersicon esculentum*	AF 489565
C. acutatum	1267b[e]	*Fragaria × ananassa*	AF 489562
C. acutatum	Cha-s[e]	*Fragaria × ananassa*	AF 489564

[a] Isolate sequenced by E. A. Travanty, L. M. Buttonow, R. T. Kane, S. B. Martin, J. S. Gregos, and D. P. Maxwell (*unpublished*) (EMBL Accession No. AF 059676).
[b] Isolate sequenced by Moriwaki and Tsukiboshi, (*unpublished*, host not described) (EMBL Accession No. AB 046609).
[c] Isolate sequenced by Moriwaki and Tsukiboshi, (*unpublished*, host not described (EMBL Accession No. AB 046608).
[d] Isolates sequenced by Freeman et al. (10).
[e] Isolates sequenced by authors.

RESULTS

RAPD polymorphism. Four RAPD primers were used to amplify DNA from 95 *Colletotrichum* isolates. Sixty-nine reproducible RAPD fragments were obtained. Each primer yielded an average of 17 RAPD fragments, ranging in size from 450 to 3,200 bp. Representative RAPD banding patterns obtained using primer OPC02 and OPA13 are shown in Figure 1. UPGMA cluster analysis using the 69 RAPD fragments yielded a dendogram (Fig. 2). The 95 isolates clustered into two distinct groups (Fig. 2), CA and CG, *C. acutatum* and *C. gloeosporioides*, respectively. The 81 isolates of *C. acutatum* displayed 49 fragments. None were monomorphic or group CA-specific, whereas one was specific to all *C. acutatum* isolates except for isolate 120V-II. Three isolates, 120V-II from raspberry, Coll.14a from blackberry, and G4a from lupin displayed the most divergent patterns within group CA and showed 10, 5, and 5 specific fragments for each isolate, respectively. Within the CA group, two subgroups were distinguished that encompassed the 78 remaining *C. acutatum* isolates, CA-clonal (54 isolates) and CA-variable (24 isolates). The 54 CA-clonal isolates collected from strawberry during the years 1983 to 1998 in North America and Europe and displaying a salmon-orange coloring in culture on PDA, clustered into one clade, present in 100% of the bootstrap-generated trees. All these isolates displayed identical banding patterns with a total of 11 monomorphic fragments, six of which were subgroup CA-clonal-specific and five of which were present in the RAPD patterns of several isolates in subgroup CA-variable. The 24 CA-variable isolates clustered into a more diverse genetic group and were collected from 1984 to 1998 from various host plants in North America and Europe (Table 1). These isolates, except for isolates F3e, F3c, and 1159-2a, exhibited a pink-chromogenic coloring in PDA culture. The RAPD patterns of the 24 isolates in subgroup CA-variable displayed 22 fragments, eight of which were monomorphic. Five monomorphic fragments were specific to subgroup CA-variable. The CA-variable isolates of *C. acutatum* could be separated into two subgroups present in 99% of generated trees according to bootstrap analysis. One of them included all the isolates from tomato (three isolates), bilberry (three isolates), and grape (one isolate) and displayed four specific fragments; the other one included isolates from strawberry, peach, blackberry, dogwood, raspberry, bilberry, and apple and displayed two specific fragments.

The 14 isolates of *C. gloeosporioides* displayed 23 RAPD fragments, four of which were monomorphic. Among the four monomorphic fragments, three were specific to this group and one was also present in the RAPD patterns of two *C. acutatum* isolates, 120V-II and Coll.14a. The isolate Manguier.a from mango displayed the most divergent pattern within group CG containing six specific fragments. Within group CG, the 13 isolates from strawberry clustered into a subgroup that was present in 99% of the dendograms generated by bootstrap (Fig. 2). In this subgroup, two Japanese isolates, Na8a and Shi1a, were the most divergent, and all four European isolates, 1159.5d, 1159.5e, 9.89n1, and 9.89r2, clustered into a distinct subgroup which was well supported by bootstrap analysis (75% of generated trees).

Sequence and phylogenetic analysis. Sequence analyses were conducted on representative isolates of CA-clonal, CA-variable, and CG chosen according to RAPD analysis (Table 2). Representatives of CA-clonal from strawberry included the isolates TUT-5954 from Israel and Cha-s and 1267b from France. Representatives of CA-variable included: strawberry isolates F3c and F3e from France, Coll.15a from Switzerland, and Cooley2 from the United States; tomato isolates BJS Tomato and Tomato ARK from the United States; blackberry isolate Coll.14a from France;

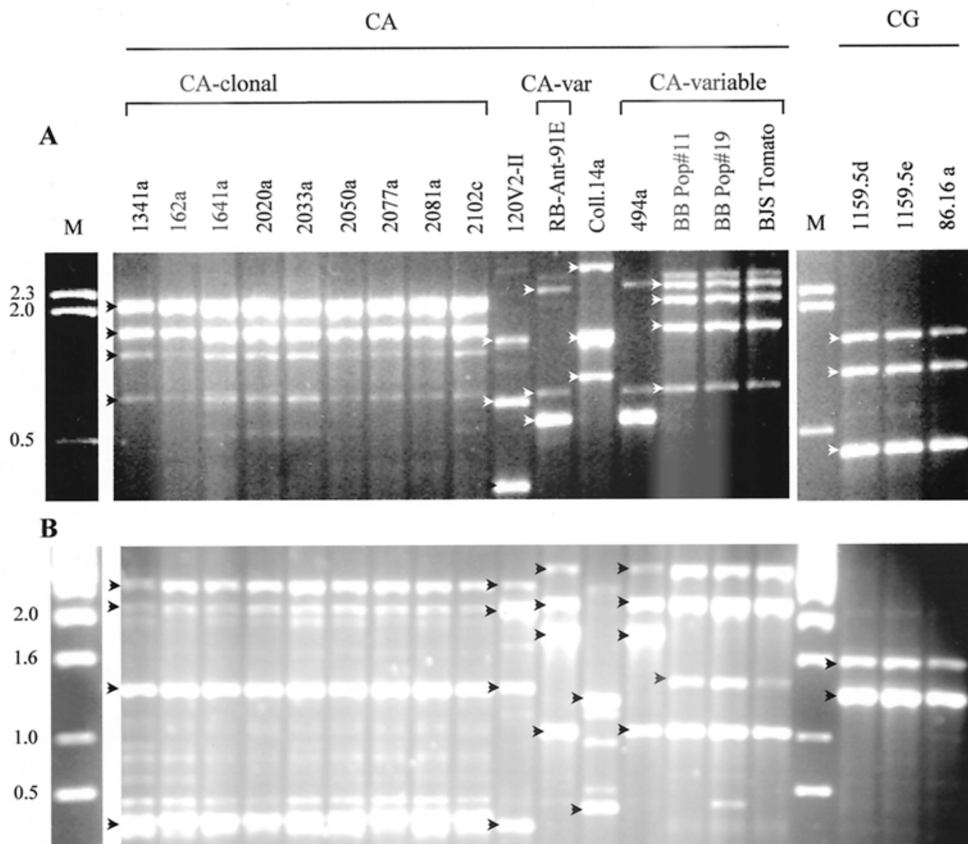

Fig. 1. Random amplified polymorphic DNA (RAPD) patterns of *Colletotrichum acutatum* (CA, 19 isolates) including subgroups CA-clonal (12 isolates) and CA-variable (five isolates), and isolates 120V2-II and Coll.14a, and RAPD patterns of *C. gloeosporioides* (CG, three isolates) obtained with **A,** primer OPC02 and **B,** primer OPA13. Lane M = DNA markers in kilobases.

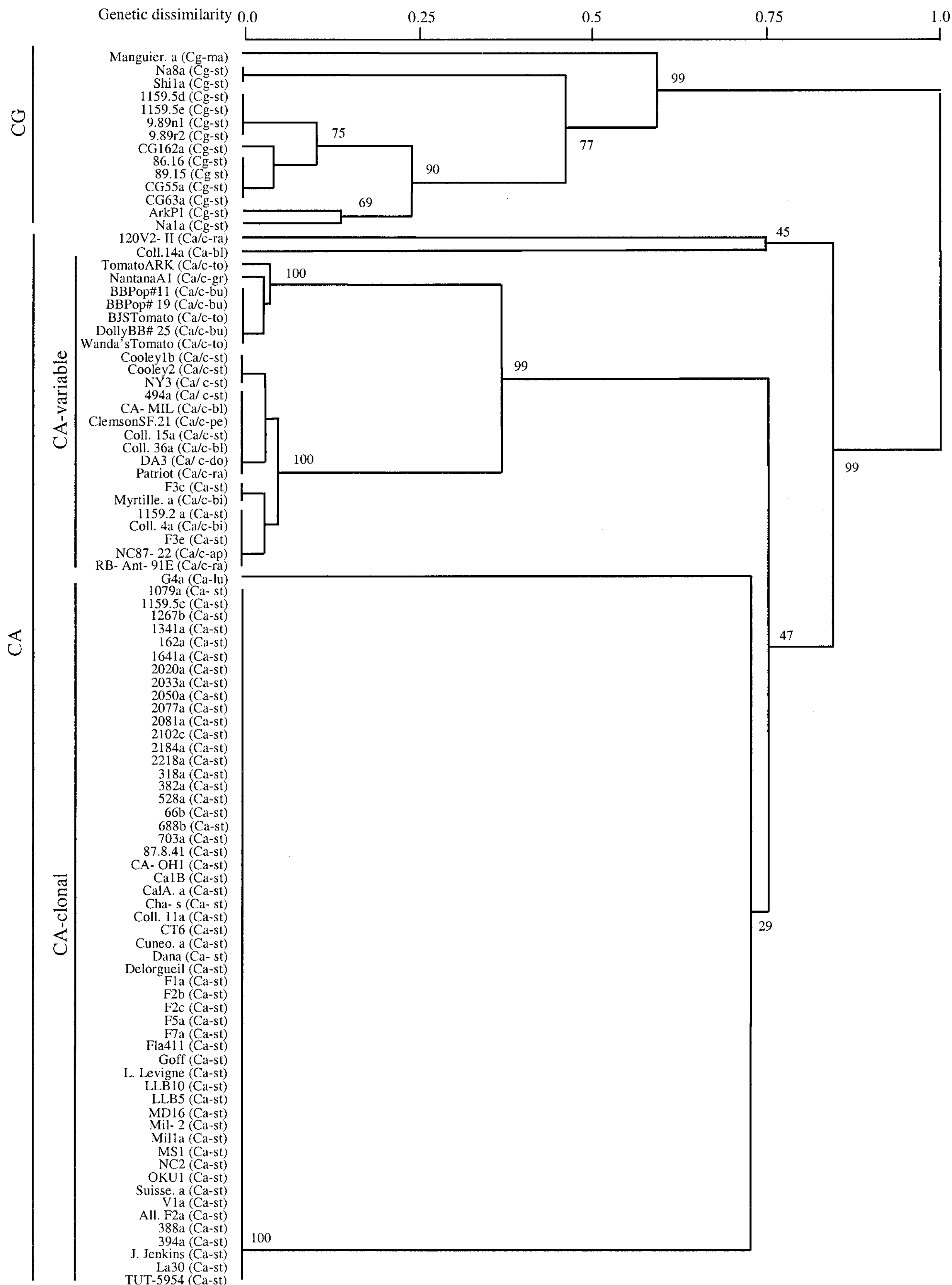

Fig. 2. Dendogram generated by unweighted pair-group method with arithmetic average cluster analysis of random amplified polymorphic DNA (RAPD) data from 95 isolates of two *Colletotrichum* species, *C. acutatum* and *C. gloeosporioides*. Numbers at major nodes indicate the number of times the group of isolates at the left of the node occurred among the sample of 100 bootstrap-generated dendograms. CA = *C. acutatum* group; CG = *C. gloeosporioides* group; Ca = *C acutatum*; Ca/c = *C. acutatum* exhibiting pink chromogenic coloring in potato dextrose agar culture; and Cg = *C. gloeosporioides*. Host plants from the isolates are indicated: ap = apple; bi = bilberry; bl = blackberry; bu = blueberry; do = dogwood; gr = grape; pe = peach; ra = raspberry; st = strawberry; to = tomato; lu = lupin; and ma = mango. Scored bands are indicated by arrows.

raspberry isolate 120V.2II from the United States; grape isolate Nantana A1 from the United States; and bilberry isolate Myrtille.a from France. The representative isolate 1159.5d of *C. gloeosporioides* from strawberry from Spain was also sequenced.

Phylogenetic analysis was performed using the ITS2 region (Table 2; Fig. 3). Comparative analysis of ITS2 sequences produced a phylogenetic tree (Fig. 3) that supported the previous analysis published by Freeman et al. (9) and Sreenivasaprasad et al. (28). Analysis of sequences of the ITS2 fragments supported the phylogeny inferred from the RAPD dendogram grouping the CG isolates separately from the CA isolates. This analysis confirmed that 24 of the *C. acutatum* isolates, 12 from this study, were grouped separately from reference cultures of the species *C. gloeosporioides*, *C. dematium*, and *C. graminicola.* In addition, based on ITS2 sequence analysis two *C. acutatum* clusters were revealed, which corresponded to the CA-clonal and CA-variable subgroups previously identified by RAPD-PCR (Fig. 2). The three representative CA-clonal isolates were grouped together (nearly identical sequence), but apart from the other subgroup of CA-variable isolates. The CA-variable isolates were further grouped into subsets indicating the diversity of these populations (Fig. 3). The analysis was performed on ITS2 sequences of 12 isolates mentioned above, as well as 18 additional sequences of isolates available in GenBank (Table 2). It should be noted that sequences from submissions to GenBank were not independently confirmed in this study.

Pathogenicity testing. *C. acutatum* isolates were assigned to pathogenicity group 1 when disease ratings were similar on cvs. Belrubi and Elsanta, or to pathogenicity group 2 when cv. Belrubi was found to be resistant (disease rating of <1), while cv. Elsanta was susceptible (disease rating fo ≥1) (Table 3). All the 35 *C. acutatum* isolates inoculated onto strawberry caused at least one developing lesion on cv. Elsanta (disease rating of ≥1), and were therefore considered as pathogenic on strawberry. Two isolates from group CA-clonal (i.e., isolates F7a and F2b) displayed a very low virulence level and were not assigned to any pathogenic group. The remaining 29 CA-clonal group isolates tested were assigned to pathogenicity group 1 (15 isolates) or 2 (14 isolates). Isolates collected during the years 1988 to 1994 displayed higher aggressiveness than those collected at the beginning of the 1980s (Table 3). All four inoculated CA-variable isolates, collected from 1984 to 1990, were assigned to pathogenicity group 1. They displayed a relatively high level of aggressiveness. The four *C. gloeosporioides* isolates tested for pathogenicity produced only a few necrotic symptoms or none at all on both inoculated cultivars.

DISCUSSION

The main objective of this study was to investigate genetic diversity and pathogenicity of a large representative population of *C. acutatum* isolated from strawberry (62 isolates) mainly from Europe and the United States. Results based on RAPD and ITS2 sequence analyses provide clear genetic evidence of at least two well characterized groups among the tested isolates of *C. acutatum* from strawberry. Subgroup CA-clonal was homogeneous, containing isolates only from strawberry that exhibited identical RAPD patterns and nearly identical ITS2 sequence data. The other larger genetic subgroup, CA-variable, included isolates from various hosts including strawberry that exhibited variable RAPD patterns and ITS2 sequence. These two main subgroups were well supported by bootstrap analysis of RAPD markers and sequence phylogeny. According to RAPD analysis, isolates 120V.2II, Coll.14a, and G4a of *C. acutatum* could not be assigned to either of these two subgroups, CA-clonal and CA-variable. This suggests an extreme variation already known to exist in the broad species of *C. acutatum* (9,18,29), and therefore additional subgroups such as that of isolate NL-12A from the ornamental anemone (11) may exist. However, all tested isolates of *C. acutatum*, including these three exceptions, were grouped within the *C. acutatum* species, which is confirmed by using ITS2 sequence analysis.

Past studies on *C. acutatum* were more focused on species identification, which is of paramount importance since *C. acutatum* is subjected to statutory quarantine requirements (16) than on genetic diversity. However, by using mtDNA, rDNA RFLPs, and isozymes the existence of one probable clonal population of *C. acutatum* on strawberry was suggested by Buddie et al. (3). Since these markers discriminate more efficiently between species than between subgroups within one species (17), we found it necessary to use RAPD markers to discriminate between subgroups within *C. acutatum*. This study confirms the existence of one CA-clonal group clearly distinct from other *C. acutatum* genetic subgroups such as CA-variable. In addition, the CA-clonal group, which included 54 of 62 strawberry isolates is the most prevalent group from this host in France, Israel, and probably in other European countries (3,7). This subgroup also includes isolates from the United States. The larger genetic group, CA-variable, included eight additional *C. acutatum* strawberry isolates and 15 *C. acutatum* isolates from other hosts producing orange to red or chromogenic pigment.

Freeman and Rodriguez (10) suggested two genetic distinct groups that were differentiated according to their ability to produce a red pigment (chromogenic). In this study, no clear correlation was found between genetic grouping and pigmentation since

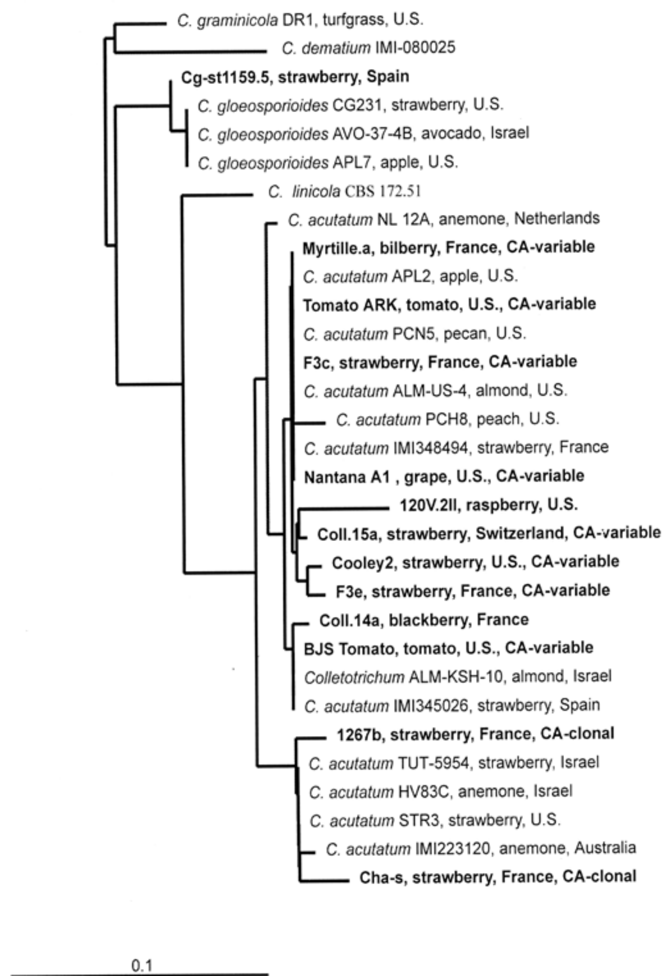

Fig. 3. Internal transcribed spacer 2-based phylogenetic tree of *Colletotrichum* isolates and published sequences. The tree was produced using the neighbor-joining algorithm. The orders of branching were similar in all tree construction approaches used (described in text). Scale bar indicates estimated 10% sequence divergence.

20 of 23 *C. acutatum* isolates in subgroup CA-variable were chromogenic, whereas none of the 53 isolates within group CA-clonal possessed this trait. These results further support the existence of a polymorphic *C. acutatum* species, which include the chromogenic isolates (9,12,27).

The low level of genetic polymorphism observed in subgroup CA-clonal containing only isolates from strawberry compared to subgroup CA-variable is consistent with a possible specialization of these isolates on strawberry, as suggested previously for *C. acutatum* isolates from almond (8) or for *C. gloeosporioides* isolates from mango (13,14). Since *C. acutatum* is reported to be a polymorphic, polyphageous fungus (29), the low level of genetic polymorphism observed among isolates in group CA-clonal may thus be a consequence of a recent epidemic of clonal line(s) pathogenic on strawberry. The hypothesis of specialization of isolates on strawberry is strengthened by isolates in group CA-clonal being very aggressive, at least on some strawberry cultivars such as Elsanta, i.e., disease rating of 5 for the most aggressive isolates, compared with isolates in group CA-variable with a rating of 2.8 for the most aggressive isolates. However, CA-clonal isolates would have to be inoculated on other hosts to determine whether there are any differential levels of virulence compared to non CA-clonal isolates to confirm or reject the hypothesis of host specialization of CA-clonal isolates on strawberry.

Sreenivasaprasad et al. (26) suggested that European isolates of *C. acutatum* infecting strawberry were discrete from the United States *C. acutatum* isolates from a variety of hosts. In this study, no correlation was found between genetic grouping and geographical origin of *C. acutatum* isolates from strawberry (Table 1). This may be due to the large number of *C. acutatum* isolates from strawberry analyzed in our work. The lack of correlation between genetic grouping and geographical origin of *C. acutatum* isolates suggests that the pathogen may have been recently spread worldwide from a single or few sources of origin. Since large numbers of strawberry plants of the everbearing-types were imported at the beginning of the 1980s from the southwestern United States (5), introduction of *C. acutatum* into Europe may have occurred through contamination of traded plant material. This hypothesis is further supported by the occurrence of North American and European isolates from strawberry exhibiting identical RAPD patterns and ITS sequence data within both subgroups, CA-clonal and CA-variable. Furthermore, the *C. acutatum* species on strawberry is prevalent in Europe and various parts of the United States including California. Freeman and Katan (7) who

TABLE 3. Disease rating[a] of European *Colletotrichum* strains isolated from strawberry

Isolates	Date of isolation	Geographic origin	Genetic group	Disease rating Belrubi	Disease rating Elsanta
C. acutatum					
F2b	1985	France	CA-clonal	0.2	0.8
F7a	1984	France	CA-clonal	0.3	0.5
Isolates of pathogenic group 1[b]					
Goff	1988	United States	CA-clonal	4.1	4.0
1079a	1989	France	CA-clonal	1.3	1.8
528a	1990	France	CA-clonal	0.8	2.4
162a	1990	France	CA-clonal	1.8	2.2
66b	1992	France	CA-clonal	1.8	1.8
Cuneo.a	1994	Italy	CA-clonal	1.2	1.7
Cha-s	1994	France	CA-clonal	4.8	5.0
2020a	1995	Poland	CA-clonal	3.8	4.5
2077a	1995	France	CA-clonal	3.1	2.8
1641a	1995	France	CA-clonal	3.4	4.0
2033a	1995	France	CA-clonal	4.1	4.7
2081a	1995	France	CA-clonal	4.5	5.0
318a	1995	France	CA-clonal	4.5	4.8
703a	1996	France	CA-clonal	3.0	3.6
2102c	1996	France	CA-clonal	5.0	5.0
F3c	1984	France	CA-variable	2.7	2.6
F3e	1984	France	CA-variable	1.2	1.8
1159.2a	1988	Spain	CA-variable	2.2	2.8
494a	1990	France	CA-variable	1.7	1.8
Isolates of pathogenic group 2[b]					
F1a	1985	France	CA-clonal	0.0	1.7
F2c	1985	France	CA-clonal	0.0	2.0
F5a	1985	France	CA-clonal	0.3	2.0
1267b	1988	France	CA-clonal	0.0	2.3
1159.5c	1988	Spain	CA-clonal	0.0	4.4
1341a	1990	France	CA-clonal	0.0	5.0
688b	1990	France	CA-clonal	0.4	5.0
Delorgueil.a	1992	France	CA-clonal	0.3	4.3
2184a	1992	France	CA-clonal	0.5	4.5
2218a	1992	France	CA-clonal	0.0	4.6
2050a	1992	France	CA-clonal	0.2	5.0
Suisse.a	1993	Switzerland	CA-clonal	0.7	4.8
V1a	1995	France	CA-clonal	0.0	4.8
All.F2a	1995	Germany	CA-clonal	0.5	5.0
C. gloeosporioides					
9.89r2	1989	France	CGI	0.0	0.8
9.89n1	1989	France	CGI	0.2	0.8
1159.5d	1988	Spain	CGI	0.0	0.4
1159.5e	1988	Spain	CGI	0.5	0.3

[a] Disease rating was based on a 0 to 5 disease severity scale where 0 = no symptom and 5 = dead plant. Each mean was calculated according to symptoms recorded from six plants of cvs. Belrubi and Elsanta from two experiments. Results were pooled.

[b] Isolates of *C. acutatum* were assigned to pathogenicity group 1 or group 2 according to their pathogenicity on cvs. Belrubi and Elsanta.

observed identical RAPD patterns in isolates of the pathogen originating from both countries also suggested introduction of *C. acutatum* from the United States into Israel.

Using pathogenicity testing, 35 *C. acutatum* European isolates, collected from strawberry and representing the CA-clonal and CA-variable genetic subgroups were assigned to pathogenicity groups 1 and 2, as previously described (5). Assignment of *C. acutatum* isolates from strawberry to genetic subgroups and to pathogenicity groups was not correlated (Table 3). Within each group, isolates varied in aggressiveness (Table 3). Isolates collected after 1988 or 1994 displayed significantly higher levels of aggressiveness. The introduction of *C. acutatum* into Europe most likely occurred at the beginning of the 1980s (5). Therefore, variation in pathogenicity cannot be explained by host specialization of this group. The analysis of genetic polymorphism and variation in pathogenicity among CA-clonal isolates suggests that isolates within this subgroup are closely related genetically but not identical, which may be further studied with additional molecular markers such as amplified fragment length polymorphisms (AFLPs). A possible explanation for the pathogenic differentiation within CA-clonal is the adaptation and disease incidence of these isolates on resistant strawberry cultivars with major resistance genes such as the Californian cultivar, e.g., Chandler, and resistant European cultivar, e.g., Belrubi (7). This hypothesis is supported by the occurrence of highly virulent isolates assigned to pathogenicity group 1 which overcame the major dominant resistance gene of Chandler in 1994, in the Val de Loire (Central France) (4).

For the first time, four European isolates of *C. gloeosporioides* from strawberry that have been rarely observed were included in a study of genetic polymorphism and pathogenic variability. RAPD patterns of all 14 isolates of *C. gloeosporioides* showed high level of polymorphism as previously described (10). Because isolates of *C. gloeosporioides* from strawberry collected in Europe and the United States were closely related genetically, it is possible that *C. gloeosporioides* isolates pathogenic on strawberry were also introduced into Europe from North America through plant trade, as suggested for *C. acutatum*. Dissemination of *C. gloeosporioides* and *C. acutatum* through plant exchange between Europe and the United States, and genetic and pathogenic variation within both species, could be further investigated using Californian and European *C. acutatum* and *C. gloeosporioides* isolates.

Whereas high levels of virulence were reported elsewhere for isolates of *C. gloeosporioides* from the southeastern United States (15), European isolates of *C. gloeosporioides* collected from strawberry exhibited low virulence (Table 3) and could even be considered as nonpathogenic. This may be due to a difference between European and southeastern United States isolates or to the temperate climatic conditions occurring in Europe. The latter hypothesis is supported by the fact that optimal growth of *C. gloeosporioides* is obtained in vitro at higher temperatures than that for *C. acutatum* (24). Alternatively, low levels of virulence of *C. gloeosporioides* on strawberry observed in Europe could be related to limited epidemics of this species on strawberry in this region.

RAPD fragments specific to the different genetic groups of *C. acutatum*, CA-clonal and CA-variable, may be very useful for designing specific PCR-primers for rapid and reliable detection and for differentiation between the subgroups pathogenic on strawberry. This is of particular interest for detection of *C. acutatum* and identification of the genetic groups, CA-clonal or CA-variable, for epidemiological studies. Moreover, using additional primers or other markers such as AFLPs would increase the chance of detecting avirulence genes that are related to pathogenic variability. This could be used to discriminate between the two pathogenicity groups in group CA-clonal, and to differentiate between the highly virulent United States isolates of *C. gloeosporioides* compared to the predominantly mildly virulent isolates in Europe.

ACKNOWLEDGMENTS

We thank Alain Baudry, L. Black, E. Bosshard, C. Chandler, D. Cooley, M. Ellis, J. Gondran, M. Maltoni, M. Meyer, R. D. Milholland, J. G. Nourrisseau, and K. Okayama for providing isolates of *Colletotrichum* spp. This research was supported in part by a grant from the "Région Aquitaine" (PDZR) and European FEDER for strawberry research awarded to B. Denoyes-Rothan and by Research Grant Award IS 2825-97 from BARD, The United States–Israel Binational Agricultural Research and Development Fund awarded to S. Freeman.

LITERATURE CITED

1. Bonde, M. R., Peterson, G. L., and Maas, J. L. 1991. Isozyme comparisons for identification of *Colletotrichum* species pathogenic to strawberry. Phytopathology 81:1523-1528.
2. Brown, A. E., Sreenivasaprasad, S., and Timmer, L. W. 1996. Molecular characterization of slow-growing orange and key lime anthracnose strains of *Colletotrichum* from citrus as *C. acutatum*. Phytopathology 86:523-527.
3. Buddie, A. G., Martinez-Culebras, P., Bridge, P. D., Garcia, M. D., Querol, A., Cannon, P. F., and Monte, E. 1999. Molecular characterization of *Colletotrichum* strains derived from strawberry. Mycol. Res. 103:385-394.
4. Denoyes-Rothan, B. 1997. Etude des relations hôte-pathogène chez le couple *Fraragaria* × *ananassa* et *Colletotrichum* spp. Ph.D. thesis. Université de Bordeaux II, France.
5. Denoyes, B., and Baudry, A. 1995. Species identification and pathogenicity study of French *Colletotrichum* strains isolated from strawberry using morphological and cultural characteristics. Phytopathology 85:53-57.
6. Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.
7. Freeman, S., and Katan, T. 1997. Identification of *Colletotrichum* species responsible for anthracnose and root necrosis of strawberry in Israel. Phytopathology 87:516-521.
8. Freeman, S., Katan, T., and Shabi, E. 1998. Characterization of *Colletotrichum* species responsible for anthracnose diseases of various fruits. Plant Dis. 82:596-605.
9. Freeman, S., Minz, D., Maymon, M., and, Zveibil, A. 2001. Genetic diversity within *Colletotrichum acutatum sensu* Simmonds. Phytopathology 91:586-592.
10. Freeman, S., and Rodriguez, R. J. 1995. Differentiation of *Colletotrichum* species responsible for anthracnose of strawberry by arbitrarily primed PCR. Mycol. Res. 99:501-504.
11. Freeman, S., Shabi, E., and Katan, T. 2000. Characterization of *Colletotrichum acutatum* causing anthracnose disease of anemone. Appl. Environ. Microbiol. 66:5267-5272.
12. Gunnell, P. S., and Gubler, W. D. 1992. Taxonomy and morphology of *Colletotrichum* species pathogenic to strawberry. Mycologia 84:157-165.
13. Hayden, H. L., Pegg, K. G., Aitken, E. A. B., and Irwin, J. A. G. 1994. Genetic relationship as assessed by molecular markers and cross-infection among strains of *Colletotrichum gloeosporioides*. Aust. J. Bot. 42:9-18.
14. Hodson, A., Mills, P. R., and Brown, A. E. 1993. Ribosomal and mitochondrial DNA polymorphisms in *Colletotrichum gloeosporioides* isolated from tropical fruits. Mycol. Res. 97:329-335.
15. Howard, C. M., Maas, J. L., Chandler, C. K., and Albregts, E. E. 1992. Anthracnose of strawberry caused by the *Colletotrichum* complex in Florida. Plant Dis. 76:976-981.
16. Journal Officiel de la République Française. 1993. Arrété du 2 septembre 1993 relatif aux exigences sanitaires des végétaux, produits végétaux et autres objets. 4 novembre 1993. Ed. Ministère de l'Agriculture et de la Pêche.
17. Kohn, L. M. 1992. Developing new characters for fungal systematics: An experimental approach for determining the rank of resolution. Mycologia 84:139-153.
18. Lenné, J. M. 1977. A study of the biology and taxonomy of the genus *Colletotrichum*. Ph.D. thesis. University of Melbourne, Australia.
19. Maas, J. L. 1987. Compendium of Strawberry Diseases. The American Phytopathological Society, St. Paul, MN.
20. Martinez-Culebras, V., Barrio, E., Garcia, M. D., and Querol, A. 2000. Identification of *Colletotrichum* species responsible for anthracnose of strawberry based on the internal transcribed spacers of the ribosomal region. FEMS Microbiol. Lett. 189:97-101.
21. Nei, M., and Li, W. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. 76:5269-5273.
22. Perkins, D. D. 1962. Preservation of *Neurospora* stock cultures with

anhydrous silica gel. Can. J. Microbiol. 8:591-594.
23. Saghai-Maroof, M. A., Soliman, K. M., Jorgensen, R. A., and Allard, R. W. 1984. Ribosomal DNA spacer-length polymorphism in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. USA 81:8014:8018.
24. Smith, B. J., and Black, L. L. 1990. Morphological, cultural, and pathogenic variation among *Colletotrichum* species isolated from strawberry. Plant Dis. 74:69-76.
25. Sokal, R. R., and Michener, C. D. 1958. A statistical method for evaluating systematic relationships. Univ. Kans. Sci. Bull. 38:1409-1438.
26. Sreenivasaprasad, S., Brown, A. E., and Mills, P. R. 1992. DNA sequence variation and interrelationships among *Colletotrichum* species causing strawberry anthracnose. Physiol. Mol. Plant Pathol. 41:265-281.
27. Sreenivasaprasad, S., Mills, P. R., Meehan, B. M., and Brown, A. E. 1996. Phylogeny and systematics of 18 *Colletotrichum* species based on ribosomal DNA spacer sequences. Genome 39:499-512.
28. Strunk, O., Ludwig, W., Gross, O., Reichel, B., Stuckmann, N., May, M., Nonhoff, B., Lenke, M., Ginhart, T., Vilbig, A., and Westran, R. 1998. ARB–A software environment for sequence data. Technische Universität München, Munich, Germany.
29. Sutton, B. C. 1980. The Coelomycetes Fungi Imperfecti with Pycnidia, Acervuli and Stromata. CMI, Kew, Surrey, UK.
30. Welsh, J., and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18:7213-7218.
31. Williams, J. G. K., Kubelick, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531-6535.
32. White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols, A Guide to Methods and Applications. M. A. Innis, D. H. Gelfand, and J. J. Sninsky, eds. Academic Press, San Diego, CA.