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Nuclear Ribosomal DNA Variation and Pathogenic Specialization in *Alternaria* Fungi Known To Produce Host-Specific Toxins†

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A total of 99 strains of 11 *Alternaria* species, including 68 strains of seven fungi known to produce host-specific toxins, were subjected to analysis of restriction fragment length polymorphism (RFLP) in nuclear ribosomal DNA (rDNA). Total DNA was digested with *Xba*I, and the Southern blots were probed with a nuclear rDNA clone of *Alternaria kikuchiana*. The hybridization gave 17 different RFLPs from the 99 strains. On the basis of these RFLPs, populations of host-specific toxin-producing fungi could not be differentiated from one another nor from nonpathogenic *A. alternata*. Each population of the toxin-producing fungi carried rDNA variants. Nine different types, named A1 to A6 and B1 to B3, were detected among the toxin-producing fungi and nonpathogenic *A. alternata*. All of the populations contained the type A4 variant, and the other rDNA types were also shared by different toxin-producing fungi and *A. alternata*. In contrast, *Alternaria* species that are morphologically distinguishable from *A. alternata* could be differentiated from *A. alternata* on the basis of the rDNA RFLPs. Polymorphisms in rDNA digested with *Hae*III and *Msp*I were also evaluated in 61 *Alternaria* strains. These restriction enzymes produced 31 variations among all of the samples. The seven toxin-producing fungi and nonpathogenic *A. alternata* could not be resolved by phylogenetic analysis based on the RFLPs, although they could be differentiated from the other *Alternaria* species studied. These results provide support for the hypothesis that *Alternaria* fungi known to produce host-specific toxins are intraspecific variants of *A. alternata* specialized in pathogenicity.

There are seven plant diseases caused by *Alternaria* species in which host-specific toxins are responsible for fungal pathogenicity (28, 33). Participation of host-specific toxins in establishment of plant diseases is one of the most clearly understood mechanisms of host-selective pathogenesis (28, 33, 40, 50).

In previous years, particular species names were adopted for the following five *Alternaria* fungi known to produce host-specific toxins: *Alternaria kikuchiana*, a host-specific AK toxin producer causing black spot of Japanese pears (23, 45); *A. mali*, an AM toxin producer causing alternaria blotch of apples (32, 39, 48); *A. longipes*, an AT toxin producer causing brown spot of tobacco plants (16, 20); and two biotypes of *A. citri*, a producer of ACRL toxin, which causes brown spot of rough lemons (10, 37), and a producer of ACTG or ACT toxin, which causes brown spot of tangerines (17, 18, 34). The other two toxin-producing fungi have been identified as pathogenic variants within *A. alternata*. Grogan et al. (11) reported the occurrence of a stem canker of tomatoes and characterized the causal agent as *A. alternata* f. sp. *lycopersici* because of its morphological similarity to *A. alternata*. The tomato pathogen was later found to produce host-specific toxins, named AAL toxins (4, 5). The causal agent of *Alternaria* black spot of strawberries was designated the strawberry pathotype of *A. alternata*, because the pathogen was morphologically identical to *A. alternata* and appeared to produce host-specific toxins, named AF toxins (21, 24, 30). The chemical structures of these host-specific toxins, except for the AT toxin of *A. longipes*, have already been elucidated (4, 5, 10, 17, 18, 23, 24, 32, 48).

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† This paper is dedicated to the late Syoyo Nishimura, who provided us the opportunity to carry out this study and to learn his eminent way of thinking about host-specific toxins.

Nishimura et al. (27, 29, 31) observed that these fungi are all similar in conidial morphology, although they lack the sexual cycle. The measurements of conidial size were statistically within the range of the descriptions for the collective species *A. alternata* (Fries) Keissler (8, 25, 27, 29, 31, 41). *A. alternata* is fundamentally a ubiquitous, saprophytic fungus. Each pathogen has a distinct and limited host range due to production of a particular host-specific toxin, and thus it is possible to distinguish one type of pathogen from another (27, 29). On the basis of these features of the toxin-producing fungi, Nishimura et al. (27, 29, 31) proposed that they be classified as pathological variants (pathotypes) of *A. alternata*. However, this hypothesis has not been accepted widely because of the difficulties in taxonomic classification of species within the genus *Alternaria*, which are due partly to the lack of sexual stages (8, 25, 41, 42, 51).

The taxonomy of the genus *Alternaria* has been recently reviewed (42, 51). Identification of some *Alternaria* species still offers considerable difficulties, owing to their high variability and polymorphism, which occur even in pure cultures (42, 51). The classification of small-spored species, including host-specific toxin-producing fungi, has been particularly confused, because of the simple and convergent morphology of conidia and facultative parasitism, resulting in an ambiguous host range (42, 51).

Molecular approaches are used increasingly in taxonomy and systematics of filamentous fungi (6). DNA-DNA and rRNA–ribosomal DNA (rDNA) homology analyses have been successfully employed in bacterial taxonomy, and extension of this method to fungi is forthcoming (6). Restriction fragment length polymorphisms (RFLPs) and nucleotide sequences of nuclear rDNA have also been shown to provide valuable information for taxonomy and systematics in fungi (2, 6, 12, 13, 22, 35, 49). DNA-DNA homology analysis suggested a close relationship among *Alternaria* fungi known to produce host-specific toxins and also between them and nonpathogenic *A.*

TABLE 1. Strains of *Alternaria* species used in this study and known to produce host-specific toxins

Strain	Origin		rDNA RFLP type	
	Location	Source[a]	I[b]	II[c]
A. kikuchiana[d]				
No. 15A	Tottori	TU	A2	R3
IFO6444	Unknown[e]	IFO	A4	R2
T88-4	Tottori	THES	B2	R4
T88-8	Tottori	THES	B1	R5
T88-54	Tottori	THES	A5	NT
T88-64a	Tottori	THES	B2	R2
T88-154	Tottori	THES	A2	R3
T88-165	Tottori	THES	A4	R6
T88-186	Tottori	THES	A5	NT
91H-18	Aichi	NU	A2	R7
91H-27	Aichi	NU	A4	R8
N18	Aichi	NU	A2	R9
G16	Gifu	NU	B3	R8
G31	Gifu	NU	B3	R8
G90-A2	Gifu	NU	A1	NT
A85-2	Aichi	NU	B1	R10
85-10	Aichi	NU	B1	R10
Nu89-22	Aichi	NU	A3	NT
A. mali[f]				
M-71	Unknown	TU	A6	R15
O-159	Unknown	TU	A1	R16
AM-1	Aichi	NU	B2	R12
IFO8984	Unknown	IFO	A5	R17
Ki-441	Aomori	FTRS	A5	R18
Ku-15Ro	Aomori	FTRS	A5	NT
Ku-16Ro	Aomori	FTRS	A5	R17
Ku-16I	Aomori	FTRS	A5	NT
Ku-22I	Aomori	FTRS	A4	NT
FIVcont45	Aomori	FTRS	A5	R17
AM-91-1	Nagano	NFTES	A4	NT
AM-91-3	Nagano	NFTES	A4	NT
AM-91-4	Nagano	NFTES	A4	R19
AM-91-8	Nagano	NFTES	A5	NT
Ha-1	Nagano	NFTES	A6	R20
No. 40	Nagano	NFTES	A2	R12
No. 42	Nagano	NFTES	B2	NT
No. 64	Nagano	NFTES	A4	R21
A. longipes[g]				
AT-204	Aichi	NU	A5	R8
IFO6149	Unknown	IFO	B2	R11
IFO6381	Unknown	IFO	B3	R2
86-1	Kanagawa	JTY	A4	NT
No. 122-2	Tochigi	JTY	A5	NT
No. 122-3	Tochigi	JTY	A5	R8
ATL-5	Kagawa	JTL	A4	R2
ATL-6	Tochigi	JTL	A3	R12
ATL-7	Tochigi	JTL	A3	NT
ATL-9	Tottori	JTL	A3	R12
ATL-10	Tottori	JTL	A4	R6
ATL-11	Tochigi	JTL	A4	NT
ATL-16	Iwate	JTL	A5	R13
ATL-17	Yamagata	JTL	A5	NT
ATL-18	Yamagata	JTL	A4	NT
ATL-23	Iwate	JTL	A5	NT
ATL-26	Fukushima	JTL	A3	NT
ATL-28	Fukushima	JTL	A4	R6
A. citri				
ATCC 38962[h]	United States	ATCC	A4	R2
ATCC 38963[i]	United States	ATCC	A4	R2
A. alternata[j]				
0-187	Iwate	TU	A1	R16
M-30	Tottori	TU	A4	R22
T-32	Tottori	TU	A2	R3
NAF-1	Aichi	NU	A2	R3
NAF-2	Aichi	NU	A2	NT
NAF-3	Aichi	NU	A2	NT
NAF-4	Aichi	NU	A2	NT
NAF-5	Aichi	NU	A2	NT
NAF-6	Aichi	NU	A2	R3
A. alternata f. sp. *lycopersici*[k]				
AL-4	Mie	NU	A5	R1
AL-12	Mie	NU	A5	R1
No. 91080804	Mie	MARC	A4	R2

[a] IFO, Institute for Fermentation, Osaka, Japan; TU, Laboratory of Plant Pathology, Tottori University, Tottori, Japan; THES, Tottori Horticultural Experimental Station, Tottori, Japan; NU, Plant Pathology Laboratory, Nagoya University, Aichi, Japan; FTRS, Fruit Tree Research Station, Ibaraki, Japan; NFTES, Nagano Fruit Tree Experiment Station, Nagano, Japan; JTY, Yokohama Center, Japan Tobacco Inc., Kanagawa, Japan; JTL, Leaf Tobacco Research Laboratory, Japan Tobacco Inc., Tochigi, Japan; ATCC, American Type Culture Collection, Rockville, Md.; MARC, Mie Agricultural Research Center, Mie, Japan.

[b] RFLP type based on *Xba*I restriction patterns.

[c] RFLP type based on *Hae*III and *Msp*I restriction patterns. NT, not tested.

[d] The Japanese pear pathotype (27, 29). Produces AK toxin. Host plant, *Pyrus serotina* var. culta.

[e] Unknown, but in Japan.

[f] The apple pathotype (27, 29). Produces AM toxin. Host plant, *Malus pumila* var. domestica.

[g] The tobacco pathotype (27, 29). Produces AT toxin. Host plant, *Nicotiana* species.

[h] The rough lemon pathotype (27, 29). Produces ACRL toxin. Host plant, *Citrus jambhiri.*

[i] The tangerine pathotype (27, 29). Produces ACT or ACTG toxin. Host plant, *C. reticulata.*

[j] The strawberry pathotype (27, 29). Produces AF toxin. Host plant, *Fragaria ananassa.*

[k] The tomato pathotype (27, 29). Produces AAL toxin. Host plant, *Lycopersicon esculentum.*

alternata (19). Kuninaga and Yokozawa (19) investigated DNA base sequence homology among nine isolates of *Alternaria* fungi, including four host-specific toxin-producing fungi and nonpathogenic *A. alternata*, by DNA-DNA reassociation kinetics. Isolates of the toxin-producing fungi and nonpathogenic *A. alternata* shared high values of DNA homology that were similar to the values detected between isolates of the same species. In contrast, much lower values of DNA homology were found when the toxin-producing fungi and *A. solani* were compared (19). The sample size in that work seemed to be inadequate to provide definitive data on the genetic relationships between these fungi. We previously analyzed rDNA RFLPs of 322 isolates of *A. kikuchiana* collected from 14 locations in Japan and detected eight rDNA variations within the population (1). We supposed that populations of the host-specific toxin-producing fungi and *A. alternata* should show some of the same rDNA variants as in *A. kikuchiana* only if they could be categorized as pathological variants of *A. alternata*. The purpose of this study was to characterize *Alternaria* species by rDNA restriction patterns at the species level and to clarify genetic relationships among the host-specific toxin-producing fungi for assessment of their taxonomy and pathogenic specialization.

TABLE 2. Strains of *Alternaria* species used in this study

Strain	Origin		rDNA RFLP type	
	Location	Source[a]	I[b]	II[c]
A. alternata[d]				
IFO31188	Kagawa	IFO	A4	R2
IFO31189	Unknown[e]	IFO	A2	R2
IFO31805	Unknown	IFO	A5	R14
IFO4026	Unknown	IFO	A6	R1
ATCC 66981	United States	ATCC	A5	R23
ATCC 66982	United States	ATCC	A5	R23
ATCC 66983	United States	ATCC	A3	R12
O-94	Tottori	TU	B2	R2
A. bataticola[f]				
Ab1	Unknown	NU	C7	R24
IFO6187	Unknown	IFO	C7	R29
03-05009	Unknown	NIAR	C7	NT
03-05010	Chiba	NIAR	C7	NT
A. dauci[g]				
Ad1	Unknown	NU	C4	R25
Ad2	Unknown	NU	C3	NT
03-05012	Saitama	NIAR	C4	NT
A. panax[h]				
Apx1	Shimane	NU	C6	R26
Apx2	Shimane	NU	C6	NT
Apx11	Shimane	NU	C6	NT
Apx12	Shimane	NU	C6	R27
Apx22	Shimane	NU	C6	NT
A. porri[i]				
Ap0	Unknown	NU	C5	R28
Ap28	Unknown	NU	C5	NT
Ap29	Unknown	NU	C5	NT
A. sesami Se-1[j]	Unknown	NU	C8	R30
A. solani[k]				
A16	Shimane	SU	C1	R31
A17	Shimane	SU	C2	NT
A18	Shimane	SU	C1	NT
A425	Unknown	NU	C1	NT
A426	Unknown	NU	C2	NT
AsII	Mie	NU	C1	NT
IFO7516	Kanagawa	IFO	C2	NT

[a] IFO, Institute for Fermentation, Osaka, Japan; ATCC, American Type Culture Collection, Rockville, Md.; TU, Laboratory of Plant Pathology, Tottori University, Tottori, Japan; NU, Plant Pathology Laboratory, Nagoya University, Aichi, Japan; NIAR, National Institute of Agricultural Resources, Ibaraki, Japan; SU, Laboratory of Plant Pathology, Shimane University, Shimane, Japan.
[b] RFLP type based on *Xba*I restriction patterns.
[c] RFLP type based on *Hae*III and *Msp*I restriction patterns. NT, not tested.
[d] Nonpathogenic.
[e] Unknown, but in Japan.
[f] Host plant, *Ipomoea batatas.*
[g] Host plant, *Daucus carota.*
[h] Host plant, *Panax ginseng.*
[i] Host plant, *Allium cepa.*
[j] Host plant, *Sesamum indicum.*
[k] Host plants, members of the family Solanaceae.

MATERIALS AND METHODS

Fungal strains. A total of 99 strains, including 68 strains from seven *Alternaria* fungi producing host-specific toxins, 8 strains of nonpathogenic *A. alternata*, and 23 strains from an additional six *Alternaria* species, were used in this work. Their origins and host plants are shown in Tables 1 and 2. The strains were maintained on potato sucrose agar.

RFLP probe. λ phage clone Alt1 was used as a hybridization probe for detection of nuclear rDNA variation in *Alternaria* species. Alt1 was selected as a nuclear rDNA clone containing two copies of the entire rDNA unit from a genomic library of *A. kikuchiana* 15A (46).

DNA extraction. Fungal strains were grown in 50-ml portions of potato dextrose broth in 100-ml Erlenmeyer flasks at 28°C. Total DNA of each strain was prepared from the resulting mycelia by the method of Adachi et al. (1).

Recombinant λ phage DNA was isolated by the plate lysate method (38).

Hybridization. Fungal DNA was digested to completion with restriction endonucleases (Toyobo) by using standard methods (38). Total DNA was digested with restriction enzymes that recognize specific six-nucleotide-long sequences and fractionated in a 0.8% agarose gel (LO3; Takara). Total DNA was also cleaved with restriction enzymes that recognize four-nucleotide-long sequences and separated in 1.2 and 3.5% agarose gels (Nusive 3:1; FMC). The fractionated DNA was transferred to a Hybond N+ nylon membrane (Amersham) by the alkaline transfer method (36).

The Alt1 probe was labeled by randomly primed incorporation of [α-^{32}P]dCTP (ICN Biochemicals Inc.) with a random-primer DNA-labeling kit (U.S. Biochemical Corp.) (9). Hybridization was carried out in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4 [pH 7.7], and 1 mM EDTA) containing 5× Denhardt's solution (38), 0.5% sodium dodecyl sulfate, 100 μg of sonicated salmon sperm DNA per ml, and 50% formamide at 42°C. Hybridized blots were washed at 65°C; the final wash was done with 0.1× SSPE–0.1% sodium dodecyl sulfate.

Statistical analysis. The levels of relatedness between rDNA RFLP types were determined by the method of Nei and Li (26). RFLP types were evaluated for the presence or absence of hybridized fragments at specific positions. Data from separate hybridizations of *Hae*III and *Msp*I digests with the Alt1 probe were combined for analysis. The similarity coefficient (F) for the rDNA types was estimated from the RFLP data by using the formula $F = 2N_{xy}/(N_x + N_y)$, where N_{xy} is the number of restriction fragments shared by two rDNA types, x and y, and N_x and N_y are the total numbers of restriction fragments in rDNA types x and y, respectively (26). A dendrogram was constructed from the similarity coefficient data by using the unweighted pair group method with arithmetic average clustering (44).

RESULTS

Polymorphisms of rDNA restriction patterns in *Alternaria* species. Restriction endonuclease *Xba*I was used to cleave total DNA of each strain, because our previous study on rDNA RFLPs in an *A. kikuchiana* population demonstrated that it gave the best pattern variability (1). The *Xba*I-digested DNA was probed with a nuclear rDNA clone, Alt1, isolated from *A. kikuchiana* (46). The hybridization produced 17 different patterns from 99 strains of *Alternaria* species studied (Fig. 1). We also used restriction enzyme *Eco*RI to detect RFLPs in rDNA. *Eco*RI restriction patterns were not as polymorphic among the strains as those obtained with *Xba*I and gave no subgroups within *Xba*I RFLP types (data not shown).

The distribution of rDNA variation within the *Alternaria* species used is shown in Table 3. On the basis of the distribution patterns of the rDNA variants, *Alternaria* fungi known to produce host-specific toxins could not be differentiated from one another nor from nonpathogenic *A. alternata*. Populations

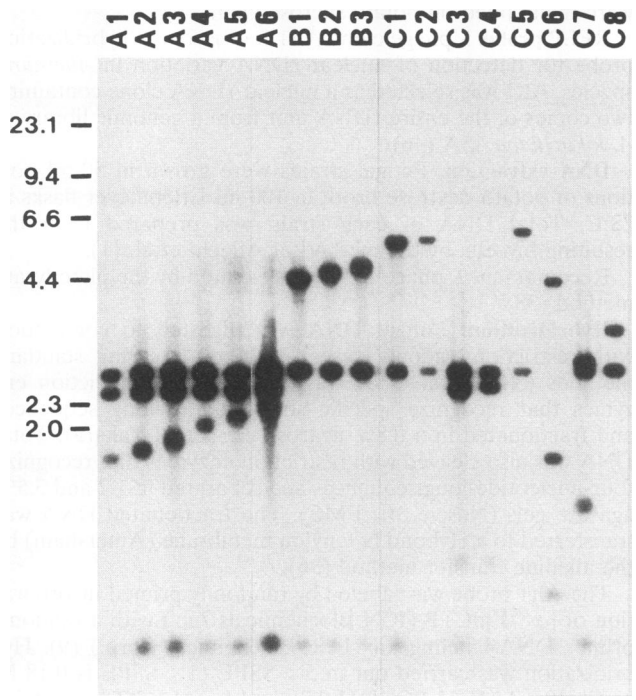

FIG. 1. RFLPs of representative strains of rDNA types in *Alternaria* species. Total DNA was cleaved with *Xba*I and fractionated in a 0.8% agarose gel. The Southern blot was hybridized with ^{32}P-labeled Alt1 DNA. The lane designations correspond to the rDNA types in Tables 1, 2, and 3. The sizes (in kilobases) of marker DNA fragments (*Hin*dIII-digested λ DNA) are indicated on the left.

of the toxin-producing fungi, except for *A. citri*, contained multiple rDNA variants (Table 3). We could use only one strain each of the two biotypes of *A. citri*, pathogenic to tangerines and rough lemons, because these have never occurred in Japan. Nine types of rDNA, A1 to A6 and B1 to B3, were detected among populations of the toxin-producing fungi and nonpathogenic *A. alternata*, and eight of them were identical to rDNA types observed previously in *A. kikuchiana* (1). Type B1 rDNA occurred only in *A. kikuchiana*, but the other eight types were shared by two or more fungi producing different host-specific toxins. Type A4 rDNA was found in all populations of the seven toxin-producing fungi and also in nonpathogenic *A. alternata* (Table 3). Thus, *Xba*I RFLPs could not differentiate the toxin-producing fungi, correlating with the original species designation and pathogenicity.

In contrast, populations of six *Alternaria* species that are morphologically distinguishable from *A. alternata* did not carry the same rDNA variants possessed by *A. alternata* and the toxin-producing fungi (Fig. 1 and Table 3). Eight rDNA types, C1 to C8, were detected among 24 strains of these six species. Although populations of *A. dauci* and *A. solani* contained some rDNA variants, all of the eight types were species specific (Table 3).

We employed restriction enzymes *Hae*III and *Msp*I for grouping of strains of *Alternaria* species in more detail, because such enzymes could provide more polymorphisms in rDNA restriction patterns. We analyzed the RFLPs in 61 strains, including 30 strains of seven fungi producing host-specific toxins, 8 strains of nonpathogenic *A. alternata*, and 8 strains of six other species. Total DNA digested with each enzyme was separated in both 1.2 and 3.5% agarose gels for exact evaluation of the banding patterns. *Hae*III and *Msp*I produced 7 to 9 and 10 to 12 resolvable fragments, respectively, in the strains studied. In total, *Hae*III and *Msp*I gave 27 and 33 different resolvable fragments, respectively. Strains were evaluated for the presence or absence of hybridized fragments with specific electrophoretic mobility. On the basis of the complete set of data, 31 variant types of rDNA, named R1 to R31, were identified (Table 4). As examples, banding patterns obtained with various rDNA types are shown in Fig. 2. Twenty-three RFLP types, R1 to R23, were detected in populations of the toxin-producing fungi and *A. alternata* (Tables 1, 2, and 4). Among them, seven types were shared by different toxin-producing fungi and nonpathogenic *A. alternata*. Strains of *Alternaria* species that are morphologically different from *A. alternata* carried different rDNA types, R24 to R31 (Table 2).

Phylogenetic analysis of *Alternaria* species. We inferred the phylogenetic relationships among strains of *Alternaria* species from RFLPs in *Hae*III- and *Msp*I-digested rDNA. A cluster analysis of the RFLP data was used to calculate the similarity

TABLE 3. Distribution of rDNA variation in *Alternaria* species measured by RFLP analysis using *Xba*I

Species	No. of strains[a] with rDNA type[b]:																
	A1	A2	A3	A4	A5	A6	B1	B2	B3	C1	C2	C3	C4	C5	C6	C7	C8
A. kikuchiana	1	4	1	3	2		3	2	2								
A. mali	1	1		5	7	2		2									
A. longipes			4	6	6			1	1								
A. citri[c]				1													
A. citri[d]				1													
A. alternata strawberry pathotype	1	7		1													
A. alternata f. sp. *lycopersici*				1	2												
Nonpathogenic *A. alternata*			1	1	3	1		2									
A. bataticola																4	
A. dauci												1	2				
A. panax															5		
A. porri														3			
A. sesami																	1
A. solani										4	3						

[a] Strains used are shown in Tables 1 and 2.
[b] See Fig. 1.
[c] The causal agent of brown spot of rough lemons.
[d] The causal agent of brown spot of tangerines.

TABLE 4. Data set showing presence and absence of restriction fragments at 60 positions hybridizing to a nuclear rDNA clone of *A. kikuchiana*

RFLP type	Presence or absence[a] of restriction fragments as determined by restriction enzyme:	
	*Hae*III	*Msp*I
R1	1111110100 0000000000 1000000	1101010000 1111100000 0000000000 100
R2	1111110100 0000100000 0000000	1101010000 1111100000 0000010000 000
R3	1111110100 0100000000 0000000	1101010000 1111100000 0100000000 000
R4	1111110100 0000001000 0100000	1101010000 1111100000 0000000100 000
R5	1111110100 0010010000 0000000	1101010000 1111100000 0010001000 000
R6	1111110100 0001000000 0000000	1101010000 1111100000 0001000000 000
R7	1111110101 1000000000 0000000	1101010000 1111100000 1000000000 000
R8	1111110100 0000000001 0000000	1101010000 1111100000 0000000001 000
R9	1111110100 1000000000 0000000	1101010000 1111100000 1000000000 000
R10	1111110100 0100000000 0000000	1101010000 1111100000 0100010000 000
R11	1111110100 0100100000 0000000	1101010000 1111100000 0100010000 000
R12	1111110100 0010000000 0000000	1101010000 1111100000 0010000000 000
R13	1111110100 0000000100 0000000	1101010000 1111100000 0000000001 000
R14	1111110100 0000000001 0010000	1101010000 1111100000 0000000001 010
R15	1111111000 0000000000 0010000	1101010000 1111100000 0000000000 001
R16	1111110100 1000000000 0000000	1101010000 1111100010 0000000000 000
R17	1111110100 0000000001 0000000	1101010000 1111100000 0000000010 000
R18	1111111000 0000001000 0000000	1101010000 1111100000 0000000100 000
R19	1111110100 0000100000 0000000	1101010000 1111100000 0001000000 000
R20	1111110100 0000000000 0010000	1101010000 1111100000 0000000000 001
R21	1111110100 0000000001 0000000	1101010000 1111100000 0000100000 000
R22	1111110100 1000000000 0000000	1101010000 1111100000 0000010000 000
R23	1111110100 0000001000 0000000	1101010000 1111100000 0000000100 000
R24	1111110000 0000000000 0000100	1101011001 1111000000 0000000000 001
R25	1111110000 0000000000 0000001	1101011000 1111111000 0000000000 000
R26	1111110110 0000000000 0000000	1011100010 1111000001 0000000000 000
R27	1111110110 0000000000 0000000	1101100010 1111000000 1000000000 000
R28	1111110000 0000000000 0000001	1101010101 1111001000 0000000000 000
R29	1111110000 0000000000 0001010	1101010101 1111000000 0000000000 001
R30	1111110000 0000000000 0001000	1101010101 1111010000 0000000000 000
R31	1111110000 0000000000 0000001	1101010100 1111100100 0000000000 000

[a] 1, presence; 0, absence.

coefficients between the RFLP types, and a dendrogram was constructed by using the unweighted pair group method with arithmetic average clustering (Fig. 3). The dendrogram did not identify any genetic clusters among the *Alternaria* fungi known to produce host-specific toxins, corresponding to the original species and pathogenicity designations; all of the toxin-producing fungi clustered in a single genetic group together with nonpathogenic *A. alternata* at a similarity level of 84% (Fig. 3). However, the other species that are morphologically distinguishable from *A. alternata* were grouped in other clusters on the dendrogram. RFLP types detected in *A. bataticola* and *A. sesami* and in *A. dauci*, *A. porri*, and *A. solani* clustered together at similarity levels of 84 and 86%, respectively.

DISCUSSION

The smallest species group level of the genus *Alternaria* has been prescribed on the basis of the following differential diagnostic criteria: arrangement of conidia on the host and in culture; conidial shape, color, septation, and measurements; surface ornamentation of conidia; nature of the conidial beak or secondary conidiophores; conidiophore characters; formation of chlamydospores or gemmae; and unique culturing characters (42, 51). The genus can be generally divided into two groups, small spored and large spored. The small-spored group, including the host-specific toxin-producing fungi, is more difficult to differentiate between similar species than the large-spored group, because of simplicity and intraspecific variability of conidial morphology, even in pure cultures. Phytopathological characters, such as host specificity, have also been used as criteria for identification of *Alternaria* specimens. Although host specificity seems to provide a good identification match in some cases, such pathology indicators seem to have caused confusion in the taxonomy of the genus (42).

During many past years, particular *Alternaria* species names have been adopted for five of the seven fungi whose pathogenicity and host specificity are dependent on host-specific toxins. Nishimura et al. (27, 29, 31) observed that the seven toxin-producing fungi all have conidial morphology similar to that of a collective species, *A. alternata*. Regardless of similarity in conidial morphology, however, they have distinct host ranges. Thus, Nishimura et al. (27, 29, 31) proposed that all of these fungi be given the valid and correct name *A. alternata*, independently of their pathogenicity, and be designated as different pathotypes of *A. alternata*, corresponding to their host specificity. Yu (51) reported that isolates of *A. kikuchiana*, *A. mali*, *A. alternata* f. sp. *lycopersici*, and the strawberry pathotype of *A. alternata* displayed enough characteristics in common with standard stock cultures of *A. alternata*. His comparison of conidia from in vitro cultures showed that their measurements agree with the published description of *A. alternata* (8, 25, 41, 51).

Simmons (42) opposed the hypothesis proposed by Nishimura et al. (27, 29, 31), mainly because of the lack of an adequate description of patterns of conidial catenulation. Simmons (42) reported that, in addition to conidial size range, three-dimensional sporulation patterns are critical to the differentiation of similar species in the small-spored group. On

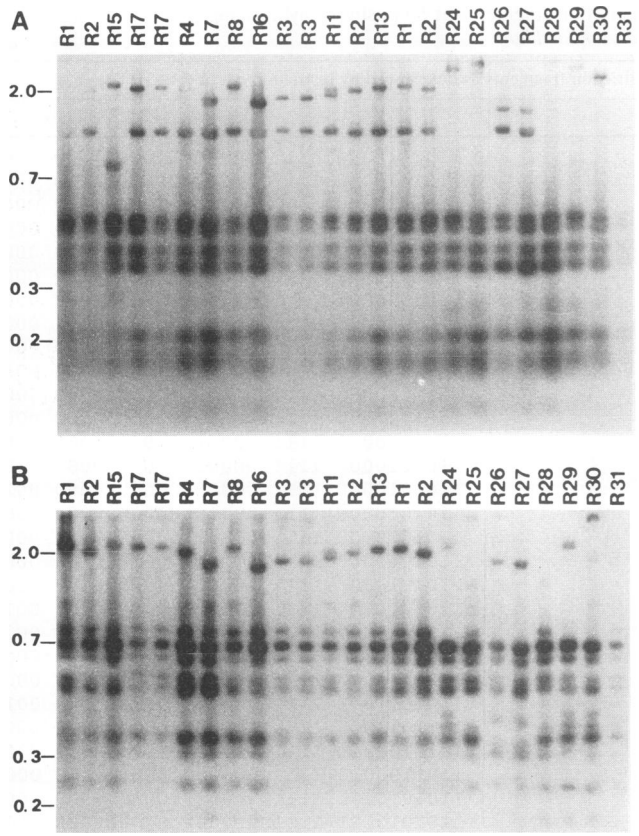

FIG. 2. RFLPs of representative strains of rDNA types in *Alternaria* species. Total DNA was cleaved with *Hae*III (A) or *Msp*I (B) and fractionated in a 3.5% agarose gel. The Southern blot was hybridized with ^{32}P-labeled Alt1 DNA. The lane designations correspond to the rDNA types in Tables 1 and 2. As an example, the hybridization patterns of 19 of 31 types are shown. The sizes (in kilobases) of marker DNA fragments are indicated on the left.

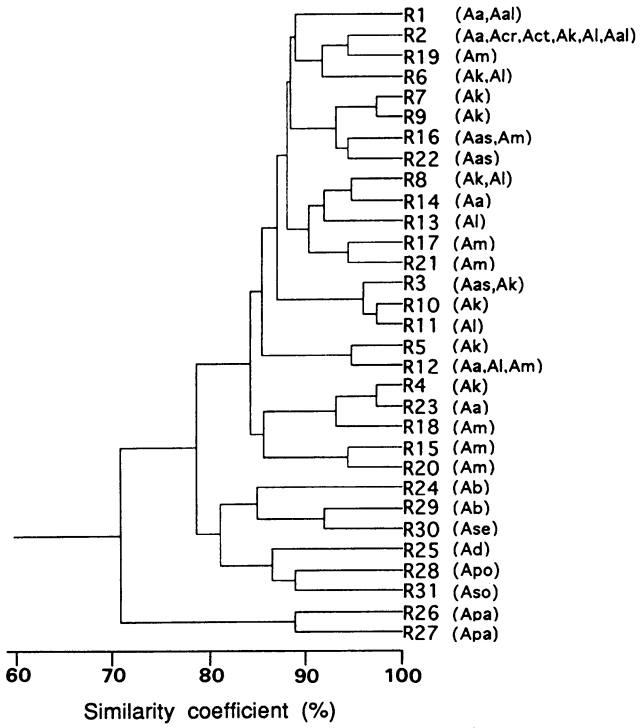

FIG. 3. Dendrogram showing the level of genetic relatedness of 31 rDNA RFLP types (R1 to R31) detected in *Alternaria* species. The 31 types were identified on the basis of the data set of RFLP profiles shown in Table 4. An unweighted pair group method with arithmetic average clustering dendrogram was constructed from the similarity coefficients calculated from the RFLP data. *Alternaria* species carrying respective rDNA types are shown in parentheses. Abbreviations: Aa, *A. alternata*; Ak, *A. kikuchiana*; Am, *A. mali*; Al, *A. longipes*; Aas, the strawberry pathotype of *A. alternata*; Aal, *A. alternata* f. sp. *lycopersici*; Acr, *A. citri* causing brown spot of rough lemons; Act, *A. citri* causing brown spot of tangerines; Ab, *A. bataticola*; Ase, *A. sesami*; Ad, *A. dauci*; Apo, *A. porri*; Aso, *A. solani*; Apa, *A. panax*.

the basis of his criteria, *A. alternata* conidia are produced in a loose, three-dimensional tuft of branching chains under specific conditions of growth and *Alternaria* fungi whose conidia are produced in a single linear chain under the same conditions are another taxon, even if their conidia show the same size range. Furthermore, *A. longipes* has been reported to form larger-sized conidia than *A. alternata* (42, 51).

Our results based on *Xba*I RFLP analysis of nuclear rDNA, however, demonstrated the close relationships among *A. alternata* and all of the host-specific toxin-producing fungi: specific RFLP patterns were not detected among the fungi, correlating with the original species designation and host specificity. In some cases, strains of different toxin-producing fungi shared the same rDNA variation, although single populations producing the same toxins contained various rDNA variants. In contrast, *Alternaria* species that are morphologically distinguishable from *A. alternata* revealed rDNA RFLPs different from those of *A. alternata*. Among nine rDNA types detected in populations of the toxin-producing fungi and *A. alternata*, eight types were identical to RFLP types previously detected in an *A. kikuchiana* population (1). The eight variant types have been reported to differ in the lengths and presence of the restriction sites in spacer DNA outside the coding regions for rRNAs (1). On the basis of the restriction profiles of an additional type A6 rDNA, type A6 probably differs from the other eight types in a similar manner. These results strongly suggest that the seven toxin-producing fungi and *A. alternata* should not be differentiated from one another at the species level.

Phylogenetic analysis based on *Hae*III and *Msp*I polymorphisms in rDNA indicated that all of the toxin-producing fungi and *A. alternata* cluster in a single genetic group. Previous studies of filamentous fungi have shown that rDNA RFLPs can represent genetic relationships within and between fungal species (2, 6, 13, 22, 49). We previously observed that rDNA RFLP types reflected the multilocus genotypes of individuals of *A. kikuchiana* measured by DNA fingerprinting with nuclear repetitive DNA sequences (1). Thus, this study provides strong support for the hypothesis of Nishimura et al. (27, 29, 31) that *Alternaria* fungi producing host-specific toxins should be characterized as pathotypes of *A. alternata* which are intraspecific variants specialized in pathogenicity, depending on host-specific toxins. Morphological differences previously reported among the toxin-producing *Alternaria* fungi (42, 51), if any, should be evaluated as intraspecific variability. To establish a firmer basis for the pathotype hypothesis, we are now comparing nucleotide sequences of the intergenic transcribed spacer regions of rDNA among *Alternaria* fungi, because these sequences have been reported to provide reliable information for classification of fungal species (12, 35).

The length and restriction site polymorphisms in nuclear rDNA repeats have precedents in other eukaryotic species, including fungi (3, 6). However, *A. alternata* appeared to carry a relatively high level of variation in the rDNA units. In *Saccharomyces cerevisiae*, detailed analysis has shown that variant forms of rDNA differ by a number of small insertions, deletions, and point mutations in the intergenic region in rDNA repeats (43). We are now further characterizing the variations in *Alternaria* rDNA by comparing the nucleotide sequences of the intergenic regions.

A. alternata is perhaps the most cosmopolitan of fungal species. It grows on many substrates and grows as a saprophyte on the surfaces of living roots, leaves, seeds, and other plant parts. *A. alternata* also grows as a weak pathogen causing opportunistic disease in a number of crops, because all fungi belonging to *A. alternata* possess a general aggressiveness, that is, an ability to penetrate plant tissues or artificial membranes (27, 29, 31). These characteristics suggest that the pathogens appear in an agroecosystem after nonpathogenic, saprophytic strains acquire the ability to produce host-specific toxins effective against certain susceptible host genotypes. Koch et al. (15) proposed the hypothesis that a direct relationship between phylogenetic and pathogenicity groupings within species might be expected if pathogenicity is controlled by many genes distributed throughout the fungal genome; an indirect relationship might result from geographical or mating isolation of individuals having the same pathogenicity type if pathogenicity is controlled by only a few genes. Since no direct correlations between rDNA variant distribution and host-specific pathogenicity were observed in populations of the toxin-producing fungi, pathogenic specialization within *A. alternata* might be controlled by a small number of genes involved in host-specific toxin biosynthesis.

There are many fungi containing intraspecific variations, designated formae speciales and races, that differ in host specificity. However, knowledge of the chemical or molecular basis of pathogenic specialization within any single species is very limited (7). Thus, *Alternaria* fungi producing host-specific toxins seem to be good models for studying the potential and development of pathogenic specialization in phytopathogenic fungi, because their host-specific toxins have already been well characterized as chemical determinants of host-specific pathogenicity (29, 33). To assess the molecular basis of pathogenic specialization of *Alternaria* pathogens, we have established gene manipulation techniques with *Alternaria* fungi and are now attempting to clone the genes required for host-specific toxin biosynthesis (14, 47).

With the development of molecular technology, new molecular markers have become available for fungal taxonomy and systematics. The simple and often convergent morphology of fungi has frequently led to confusion and difficulty in the classification of similar species. Classification of the genus *Alternaria* at the species level has also been faced with considerable difficulties (42, 51). RFLPs and nucleotide sequence polymorphisms in nuclear rDNA might provide useful markers for classification of *Alternaria* species, as shown in other fungi (2, 12, 13, 22, 35, 49). Phylogenetic analysis based on *Hae*III and *Msp*I RFLPs suggested genetic similarity between *A. bataticola* and *A. sesami* and also among *A. dauci*, *A. porri*, and *A. solani*, although the sample size might not be enough to provide a definitive conclusion. More detailed analysis of their genetic relationships by using more strains is in progress. We assume that such technology could simplify the systematic classification of species within the genus *Alternaria* and also other fungal genera.

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REFERENCES

1. **Adachi, Y., H. Watanabe, K. Tanabe, N. Doke, S. Nishimura, and T. Tsuge.** 1993. Nuclear ribosomal DNA as a probe for genetic variability in the Japanese pear pathotype of *Alternaria alternata*. Appl. Environ. Microbiol. **59**:3197–3205.
2. **Anderson, J. B., M. Petsche, and M. L. Smith.** 1987. Restriction fragment polymorphisms in biological species of *Armillaria mellea*. Mycologia **79**:69–76.
3. **Arnheim, N.** 1983. Concerted evolution in multigene families, p. 38–61. *In* M. Nei and R. K. Koehn (ed.), Evolution of genes and proteins. Sinauer Associates, Sunderland, Mass.
4. **Bottini, A. T., J. R. Bowen, and D. G. Gilchrist.** 1981. Phytotoxins. II. Characterization of a phytotoxic fraction from *Alternaria alternata* f. sp. *lycopersici*. Tetrahedron Lett. **22**:2723–2726.
5. **Bottini, A. T., and D. G. Gilchrist.** 1981. Phytotoxins. I. A 1-aminodimethylheptadecapentol from *Alternaria alternata* f. sp. *lycopersici*. Tetrahedron Lett. **22**:2719–2722.
6. **Bruns, T. D., T. J. White, and J. W. Taylor.** 1991. Fungal molecular systematics. Annu. Rev. Ecol. Syst. **22**:525–564.
7. **de Wit, P. J. G. M.** 1992. Molecular characterization of gene-for-gene systems in plant-fungus interactions and the application of avirulence genes in control of plant pathogens. Annu. Rev. Phytopathol. **30**:391–418.
8. **Ellis, M. B.** 1971. Dematiaceous hyphomycetes. Commonwealth Mycological Institute, Kew, England.
9. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
10. **Gardner, J. M., Y. Kono, J. H. Tatum, Y. Suzuki, and S. Takeuchi.** 1985. Structure of the major component of ACRL-toxins, host-specific pathotoxic compounds produced by *Alternaria citri*. Agric. Biol. Chem. **49**:1235–1238.
11. **Grogan, R. G., K. A. Kimble, and I. Misaghi.** 1975. A stem canker disease of tomato caused by *Alternaria alternata* f. sp. *lycopersici*. Phytopathology **65**:880–886.
12. **Guadet, J., J. Julien, J. F. Lafay, and Y. Brygoo.** 1989. Phylogeny of some *Fusarium* species determined by large-subunit rRNA sequence comparison. Mol. Biol. Evol. **6**:227–242.
13. **Hibbett, D. S., and R. Vilgalys.** 1991. Evolutionary relationships of *Lentinus* to the Polyporaceae: evidence from restriction analysis of enzymatically amplified ribosomal DNA. Mycologia **83**:425–439.
14. **Kimura, N., and T. Tsuge.** 1993. Gene cluster involved in melanin biosynthesis of the filamentous fungus *Alternaria alternata*. J. Bacteriol. **175**:4427–4435.
15. **Koch, R., K. Song, T. C. Osborn, and P. H. Williams.** 1991. Relationship between pathogenicity and phylogeny based on restriction fragment length polymorphism in *Leptosphaeria maculans*. Mol. Plant-Microbe Interact. **4**:341–349.
16. **Kodama, M., T. Suzuki, H. Otani, K. Kohmoto, and S. Nishimura.** 1990. Purification and bioassay of host-selective AT-toxin from *Alternaria alternata* causing brown spot of tobacco. Ann. Phytopathol. Soc. Jpn. **56**:628–636.
17. **Kohmoto, K., Y. Itoh, N. Shimomura, Y. Kondoh, H. Otani, M.**

Kodama, S. Nishimura, and S. Nakatsuka. 1993. Isolation and biological activities of two host-specific toxins from the tangerine pathotype of *Alternaria alternata*. Phytopathology **83**:495–502.

18. **Kono, Y., J. M. Gardner, Y. Suzuki, and S. Takeuchi.** 1986. Studies on host-selective toxins produced by a pathotype of *Alternaria citri* causing brown spot disease of mandarins. Agric. Biol. Chem. **50**:1597–1606.
19. **Kuninaga, S., and R. Yokozawa.** 1987. Taxonomic study of plant pathogens on the basis of DNA sequence homology. I. Genetic relatedness among species of the genus *Alternaria*. Ann. Phytopathol. Soc. Jpn. **53**:368–369. (In Japanese.)
20. **Lucas, G. B.** 1975. Disease of tobacco. Biological Consulting Associates, Raleigh, N.C.
21. **Maekawa, N., M. Yamamoto, S. Nishimura, K. Kohmoto, M. Kuwata, and Y. Watanabe.** 1984. Studies on host-specific AF-toxins produced by *Alternaria alternata* strawberry pathotype causing Alternaria black spot of strawberry. I. Production of host-specific toxins and their biological activity. Ann. Phytopathol. Soc. Jpn. **50**:600–609.
22. **Moody, S. F., and B. M. Tyler.** 1990. Use of nuclear DNA restriction fragment length polymorphisms to analyze the diversity of the *Aspergillus flavus* group: *A. flavus*, *A. parasiticus*, and *A. nomius*. Appl. Environ. Microbiol. **56**:2453–2461.
23. **Nakashima, T., T. Ueno, H. Fukami, T. Taga, H. Masuda, K. Osaki, H. Otani, K. Kohmoto, and S. Nishimura.** 1985. Isolation and structures of AK-toxin I and II, host-specific phytotoxic metabolites produced by *Alternaria alternata* Japanese pear pathotype. Agric. Biol. Chem. **49**:807–815.
24. **Nakatsuka, S., K. Ueda, T. Goto, M. Yamamoto, S. Nishimura, and K. Kohmoto.** 1986. Structure of AF-toxin II, one of the host-specific toxins produced by *Alternaria alternata* strawberry pathotype. Tetrahedron Lett. **27**:2753–2756.
25. **Neergaard, P.** 1945. Danish species of *Alternaria* and *Stemphylium*. Einar Munksgard, Copenhagen.
26. **Nei, M., and W. H. Li.** 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA **76**:5269–5273.
27. **Nishimura, S.** 1980. Host-specific toxins from *Alternaria alternata*: problem and prospects. Proc. Jpn. Acad. Ser. B **56**:362–366.
28. **Nishimura, S., and K. Kohmoto.** 1983. Host-specific toxins and chemical structures from *Alternaria* species. Annu. Rev. Phytopathol. **21**:87–116.
29. **Nishimura, S., K. Kohmoto, and H. Otani.** 1979. The role of host-specific toxins in saprophytic pathogens, p. 133–146. *In* J. M. Daly and I. Uritani (ed.), Recognition and specificity in plant host-parasite interaction. Japan Scientific Societies Press, Tokyo.
30. **Nishimura, S., N. Maekawa, M. Kuwata, and K. Kohmoto.** 1979. Characterization of host-specific toxin produced by *Alternaria alternata* causing Alternaria black spot of strawberry. Ann. Phytopathol. Soc. Jpn. **45**:108. (In Japanese.)
31. **Nishimura, S., M. Sugihara, K. Kohmoto, and H. Otani.** 1978. Two different phases in pathogenicity of the *Alternaria* pathogen causing black spot disease of Japanese pear. J. Fac. Agric. Tottori Univ. **13**:1–10.
32. **Okuno, T., Y. Ishita, K. Sawai, and T. Matsumoto.** 1974. Characterization of alternariolide, a host-specific toxin produced by *Alternaria mali* Roberts. Chem. Lett. **1974**:635–638.
33. **Otani, H., and K. Kohmoto.** 1992. Host-specific toxins of *Alternaria* species, p. 123–156. *In* J. Chelkowski and A. Visconti (ed.), *Alternaria*: biology, plant diseases and metabolites. Elsevier, Amsterdam.
34. **Pegg, K. G.** 1966. Studies of a strain of *Alternaria citri* Pierce, the causal organism of brown spot of emperor mandarin (*Citrus reticulata*). Queensland J. Agric. Anim. Sci. **23**:15–28.
35. **Peterson, S. W., and C. P. Kurtzman.** 1991. Ribosomal RNA sequence divergence among sibling yeast species. Syst. Appl. Microbiol. **14**:124–129.
36. **Reed, K. C., and D. A. Mann.** 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. **13**:7207–7221.
37. **Ruehle, G. D.** 1937. A strain of *Alternaria citri* Ellis & Pierce causing a leaf spot of rough lemon in Florida. Phytopathology **27**:863–865.
38. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
39. **Sawamura, K.** 1962. Studies on spotted diseases of apples. I. The causal agent of *Alternaria* blotch. Bull. Tohoku Natl. Agric. Exp. Stn. **23**:163–175.
40. **Scheffer, R. P., and R. S. Livingston.** 1984. Host-selective toxins and their role in plant diseases. Science **223**:415–419.
41. **Simmons, E. G.** 1967. Typification of *Alternaria*, *Stemphylium*, and *Ulocladium*. Mycologia **59**:67–92.
42. **Simmons, E. G.** 1992. *Alternaria* taxonomy: current status, viewpoint, challenge, p. 1–35. *In* J. Chelkowski and A. Visconti (ed.), *Alternaria*: biology, plant diseases and metabolites. Elsevier, Amsterdam.
43. **Smolik-Utlaut, S., and T. D. Petes.** 1983. Recombination of plasmids into the *Saccharomyces cerevisiae* chromosome is reduced by small amounts of sequence heterogeneity. Mol. Cell. Biol. **3**:1204–1211.
44. **Sneath, P. H. A., and R. R. Sokal.** 1973. Numerical taxonomy: the principles and practice of numerical classification. W.H. Freeman & Co., San Francisco.
45. **Tanaka, S.** 1933. Studies on black spot disease of Japanese pear (*Pyrus serotina* Rehd.). Mem. Coll. Agric. Kyoto Imp. Univ. **28**:1–31.
46. **Tsuge, T., H. Kobayashi, and S. Nishimura.** 1989. Organization of ribosomal RNA genes in *Alternaria alternata* Japanese pear pathotype, a host-selective AK-toxin-producing fungus. Curr. Genet. **16**:267–272.
47. **Tsuge, T., S. Nishimura, and H. Kobayashi.** 1990. Efficient integrative transformation of the phytopathogenic fungus *Alternaria alternata* mediated by the repetitive rDNA sequences. Gene **90**:207–214.
48. **Ueno, T., T. Nakashima, Y. Hayashi, and H. Fukami.** 1975. Structures of AM-toxin I and II, host-specific phytotoxic metabolites produced by *Alternaria mali*. Agric. Biol. Chem. **39**:1115–1122.
49. **Vilgalys, R., and D. Gonzalez.** 1990. Ribosomal DNA restriction fragment length polymorphisms in *Rhizoctonia solani*. Phytopathology **8**:151–158.
50. **Yoder, O. C.** 1980. Toxins in pathogenesis. Annu. Rev. Phytopathol. **18**:103–129.
51. **Yu, S. H.** 1992. Occurrence of *Alternaria* species in countries of the Far East and their taxonomy, p. 37–62. *In* J. Chelkowski and A. Visconti (ed.), *Alternaria*: biology, plant diseases and metabolites. Elsevier, Amsterdam.