APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1993, p. 3197–3205
0099-2240/93/103197-09$02.00/0

Vol. 59, No. 10

Nuclear Ribosomal DNA as a Probe for Genetic Variability in the Japanese Pear Pathotype of *Alternaria alternata*

YOSHIHIKO ADACHI,[1] HIROYUKI WATANABE,[2] KENTARO TANABE,[1]† NORIYUKI DOKE,[1] SYOYO NISHIMURA,[1]‡ AND TAKASHI TSUGE[1]*

School of Agricultural Sciences, Nagoya University, Chikusa, Nagoya 464-01,[1] and Tottori Horticultural Experiment Station, Daiei-Cho, Tottori 689-22,[2] Japan

Received 24 May 1993/Accepted 21 July 1993

A restriction fragment length polymorphism analysis of nuclear ribosomal RNA genes (rDNA) was used to measure the amount and distribution of genetic variability in populations of the Japanese pear pathotype of *Alternaria alternata* on both micro- and macrogeographical scales. A total of 322 isolates were obtained from 13 areas in Aichi, Gifu, and Tottori Prefectures in central and western Japan. The restriction fragment length polymorphism analysis revealed that the pathogen populations contained at least eight rDNA variants. The eight variant types differed in the lengths and in the presence of the restriction sites in spacer DNA outside the coding regions for rRNAs. A total of 271 isolates were classified into the eight types. The remaining 51 isolates were determined to have mixed rDNA types. Single pear fields typically contained two to five types of rDNA variants. The frequencies of rDNA variants in 11 populations in Tottori Prefecture were compared; in this prefecture orchards containing the susceptible pear are common. Except for one collection site, there were no significant differences in the composition of the rDNA variants among the populations. This suggests that dispersal of inocula has occurred frequently in Tottori Prefecture. In contrast, significantly different distributions were observed in the three prefectures, indicating that gene flow between prefectures might be limited by geographical isolation. DNA fingerprints resulting from hybridization with a moderately repetitive DNA sequence of the fungus revealed greater genetic variability and geographical differences in genetic population structure even within the same rDNA type.

At least seven plant diseases caused by the filamentous fungus *Alternaria alternata* (Fries) Keissler involve host-specific toxins as pathogenicity factors of the pathogens (14, 23). The fact that host-specific toxins participate in the establishment of plant diseases in a host-selective manner is one of the most clearly understood mechanisms of fungal pathogenesis (14, 23, 29, 39).

The *Alternaria* pathogens that produce host-specific toxins can be classified in the species *A. alternata* because of morphological similarity at the asexual stage, although the sexual stage is unknown (22, 23). Each pathogen has a distinct and limited host range, and it is possible to distinguish one type of pathogen from another. *A. alternata* is fundamentally a ubiquitous saprophytic fungus, and it is now believed that *A. alternata* pathogens appear in agroecosystems after they acquire the ability to produce host-specific toxins against certain susceptible host genotypes (22, 23, 39). Thus, the *A. alternata* pathogens seem to be good models for studying the ecology and evolution of fungal pathogens by population genetics.

Knowledge of the amount and distribution of genetic variability in plant pathogen populations is a prerequisite to studies of gene flow, natural selection, and host-pathogen coevolution in agroecosystems. Restriction fragment length polymorphisms (RFLPs) have been used as useful markers for evaluating genetic variability in natural populations (6, 17, 18). An important application of this technique in population biology is in identifying individuals and assessing genetic relatedness within and between populations (6, 7). RFLP analyses of nuclear and mitochondrial genomes have recently been used for taxonomic, phylogenetic, and population studies of phytopathogenic fungi (6, 17, 18).

Genetic variability of phytopathogenic fungi at the population level is of great interest in the field of plant pathology because it is a window into the process of speciation. The genetic population structure of pathogens may indicate their potential for development of pathogenic specialization and fungicide resistance. As an extension of population genetics studies of *A. alternata* pathogens, we used RFLPs of the nuclear rRNA genes (rDNA) to infer genetic variability in the Japanese pear pathotype of *A. alternata*. The Japanese pear pathotype, which produces host-specific AK-toxins, is the causal agent of black spot of certain Japanese pear cultivars, including the commercially important cultivar Nijisseiki (20, 24, 35). This disease first occurred in the 1900s, just after cultivation of the Nijisseiki pear was initiated, and has been the most critical problem in pear cultivation since then (23, 35).

We previously detected a relatively high level of variation in nuclear rDNA in Japanese pear pathotype populations, even within a single pear orchard (34). The sample size in the previous work, however, was too small to provide definitive data on the genetic variability of the pathogen. In this paper we describe a more extensive use of RFLPs in rDNA for estimating the amount and distribution of genetic variability in Japanese pear pathotype populations on micro- and macrogeographical scales. In addition, DNA fingerprints resulting from hybridization with a moderately repetitive DNA sequence of the fungus were used to confirm geographical

* Corresponding author.
† Present address: Central Research Laboratories, Dainippon Ink and Chemicals, Inc., Sakura, Chiba 285, Japan.
‡ Deceased.

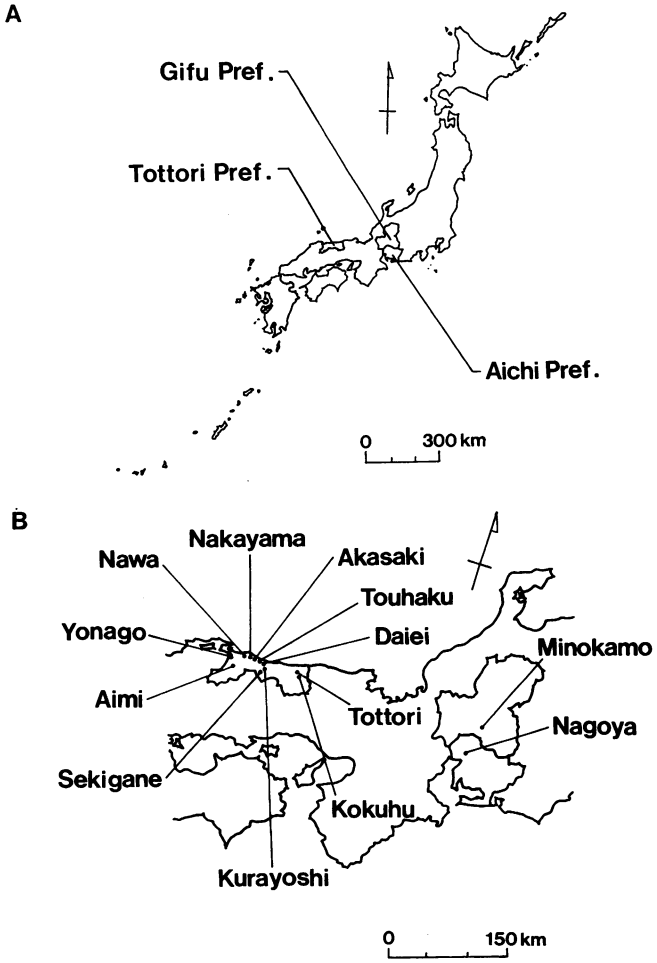

FIG. 1. Geographical origins of field isolates of the Japanese pear pathotype of *A. alternata*. (A) Locations of Aichi, Gifu, and Tottori Prefectures in Japan. (B) Distribution of the sampling sites in the three prefectures.

differences in the population structure detected by the rDNA RFLP analysis.

MATERIALS AND METHODS

Fungal isolates. Laboratory strain 15A of the Japanese pear pathotype of *A. alternata* was used to select the RFLP probes employed in this study. Field isolates of the Japanese pear pathotype were obtained from naturally occurring lesions on leaves of Japanese pear cultivar Nijisseiki plants. Diseased leaves were collected from 13 sites in Tottori, Aichi, and Gifu Prefectures in western and central Japan (Fig. 1). Tottori Prefecture has had many widely distributed orchards of Nijisseiki pear. A total of 11 pear fields in this prefecture were sampled for infections of the Japanese pear pathotype in 1988. In Gifu Prefecture, commercially managed orchards of Nijisseiki pear have been limited to Minokamo City. Pathogen isolates were obtained from one field in Minokamo City in 1990. The areas of the pear fields in Tottori and Gifu Prefectures that were sampled were 200 to 600 m^2, and the fields contained 10 to 30 pear plants that were 20 to 30 years old. Three experimental fields (H1, P1, and P2) at Nagoya University (Nagoya City, Aichi Prefecture, Japan) were also sampled for the pathogen in 1989. These fields were located linearly in the order P1-H1-P2 and were ca. 50 m apart. Nijisseiki pear has been cultivated since 1981 in fields P1 and P2 and since ca. 1960 in field H1. Field H1 contained six Nijisseiki plants, and field P1 contained ca. 20 plants. Field P2 contained several vegetable crops and only one pear tree.

Diseased leaves were taken from as many different branches and trees as possible at each field. A single sample was removed from each leaf, even if the leaf had more than one discrete lesion. Lesions on the leaves were cut into ca. 4-mm^2 pieces with a sterile blade, wetted by immersion in 70% ethanol for 15 s, and surface sterilized by immersion in 1% sodium hypochlorite for 3 min. After they were rinsed with sterile distilled water, the leaf pieces were placed on potato-sucrose agar in petri dishes and incubated at 28°C for 2 days. After 2 days the growing fungal mycelia were transferred to fresh potato-sucrose agar dishes. After incubation at 28°C for 5 days, the growing fungi were identified by light microscopy, and their pathogenicity for pears was tested. A total of 322 isolates were used in this study.

Assay for pathogenicity. Each isolate was cultured on a potato-sucrose agar plate at 28°C for 5 days, and then agar blocks (ca. 15 mm^3) bearing cultured mycelia were cut from the plate. Detached young leaves of the susceptible cultivar Nijisseiki and the resistant cultivar Chojuro of Japanese pear were slightly wounded at the center of the lamina with a needle. An agar block was placed on the wounded portion of each leaf, and 1 drop of distilled water was added. After incubation at 25°C for 20 h, pathogenicity was evaluated; a characteristic veinal necrosis occurred on Nijisseiki leaves but not on Chojuro leaves.

RFLP probes. λ phage clones Alt1 and AAR9 were used as hybridization probes for RFLP analyses. Both of these clones were selected from a genomic library of Japanese pear pathotype strain 15A. Alt1 was isolated as a nuclear rDNA clone containing two copies of the entire rDNA unit (36). AAR9 was isolated from the genomic library by the method of Hamer et al. (11) and was found to carry a repetitive DNA sequence dispersed in the fungal chromosomes (unpublished data).

DNA extraction. Fungal DNA was prepared from the cultured mycelia by a method described previously (1, 37, 40), with some modifications. Fungi were grown in 50-ml portions of potato-sucrose broth in 100-ml Erlenmeyer flasks at 25°C for 3 days on an orbital shaker (120 rpm) and were harvested by filtration through filter paper. The mycelia were ground in liquid nitrogen with a mortar and pestle. The resulting powder was suspended in lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM EDTA, 1% *N*-lauroylsarcosine) and heated at 65°C for 10 min. The supernatant obtained by centrifugation was treated with 300 μg of proteinase K (Merck) per ml at 37°C for 1 h and then brought to 2 M potassium acetate. The resulting solution was placed on ice for 30 min and centrifuged. The supernatant which was obtained was brought to 2 M ammonium acetate, and the DNA was precipitated with an equal volume of isopropyl alcohol. The DNA precipitate was dissolved in TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) and treated with 20 μg of RNase A (Sigma) per ml at 37°C for 1 h. The DNA was extracted sequentially with water-saturated phenol (28), phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol), and chloroform-isoamyl alcohol (24:1, vol/vol). The DNA was then precipitated with 0.75 volume of a polyethylene glycol solution (25% polyethylene glycol 8,000, 2.5 M NaCl)

on ice for 10 min. The DNA pellet was dissolved in TE buffer (pH 7.5).

Recombinant λ phage DNA was isolated by the plate lysate method (28).

Hybridization. Fungal DNA was digested to completion with restriction endonucleases (Toyobo) and electrophoresed in an agarose gel by using standard methods (28). The fractionated DNA was transferred to Hybond N+ nylon membranes (Amersham) by the alkaline transfer method (26).

Hybridization with rDNA clone Alt1 and detection of specific sequences were performed by using a nonradioactive DNA labeling and detection kit (Boehringer Mannheim) according to the manufacturer's recommendations. A nonradioactive rDNA probe was prepared by randomly primed incorporation of digoxigenin-labeled dUTP (9).

The AAR9 probe used for DNA fingerprinting 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], 1 mM EDTA) containing 5× Denhardt's solution (28), 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 1× SSPE–0.1% sodium dodecyl sulfate.

Statistical analysis. The statistical significance of differences in the frequency distribution of rDNA variants among the pathogen populations was tested by using a χ^2 test for heterogeneity (10, 16, 33, 38). Since *A. alternata* is haploid, we calculated the χ^2 value by using the following formula:

$$\chi^2 = N\sum_{i=1}^{k}\sigma^2 P_i/\overline{P_i}$$

where N is the number of isolates and $\overline{P_i}$ *and* $\sigma^2 P_i$ are the mean and weighted variance of the frequencies of the ith allele (10, 16, 33, 38). The genetic contingency χ^2 is a function of the total sample size and the mean and weighted variances. To compare the population structures on geographical scales, it was necessary to make pairwise comparisons of rDNA variant frequencies between all possible pairs of populations. For the pairwise comparisons, the χ^2 test described above was employed.

The levels of DNA relatedness between isolates were determined from DNA fingerprints by the method of Nei and Li (21). The presence of comigrating restriction fragments was determined for all isolates. All fragments were given equal weight. The proportion of DNA fragments shared by any two isolates was expected to be positively correlated with the degree of genetic relatedness of the isolates (21). Thus, the similarity coefficient (F) for the isolates was estimated from the RFLP data by using the following formula:

$$F = 2N_{xy}/(N_x + N_y)$$

where N_{xy} is the number of restriction fragments shared by isolates x and y and N_x and N_y are the total numbers of restriction fragments in isolates x and y, respectively (21). A dendrogram was constructed from the similarity coefficient data by using the unweighted pair group method with arithmetic average clustering (UPGMA) (32).

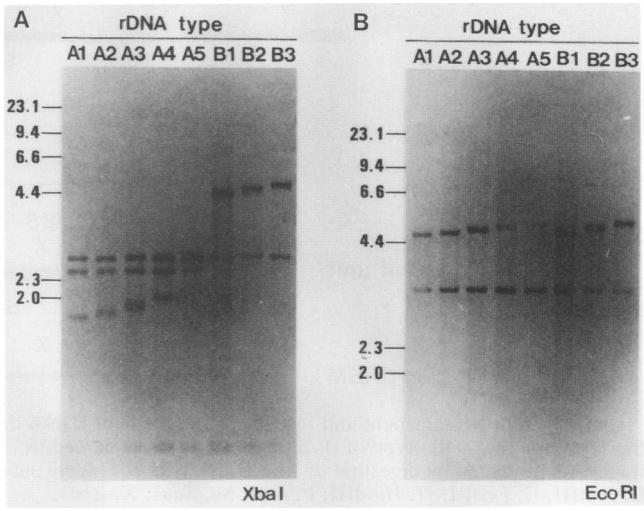

FIG. 2. RFLPs of representative isolates of the eight rDNA variants of the Japanese pear pathotype of *A. alternata*. Total DNA was digested with *Xba*I (A) or *Eco*RI (B) and fractionated on a 0.8% agarose gel. The Southern blot was hybridized with digoxigenin-labeled Alt1 DNA (Fig. 3). The lane designations correspond to the rDNA types in Table 1. The sizes of marker DNA fragments (*Hin*dIII-digested λ DNA) (in kilobases) are indicated on the left.

RESULTS AND DISCUSSION

Variation in rDNA in the Japanese pear pathotype of *A. alternata*. Hybridization of *Xba*I-digested fungal DNAs with the Alt1 probe showed that the pathogen population contains at least eight rDNA variants (Fig. 2). The variant types of rDNA were organized in two families, designated types A and B, on the basis of the RFLPs. The type A rDNAs had *Xba*I fragments that were ca. 0.7, 2.5, and 2.8 kb long, which correspond to the pABM4, pABM2, and pABM1 subclones of Alt1, respectively (Fig. 2A and 3). Type B rDNA was distinguished from type A rDNA by the absence of the 2.5-kb fragment (Fig. 2A). Type A rDNA had five variants (types A1 to A5); a type-specific fragment ranged from 1.7 to 2.3 kb long. Type B rDNA had three variants (types B1 to B3); each variant had a specific 4.7-, 4.9-, or 5.1-kb fragment.

Type A2 rDNA produced the same *Xba*I digestion pattern as Alt1. Although Alt1 contains an additional, small *Xba*I fragment corresponding to the pABM5 insert (Fig. 3), the fragment was difficult to discern in some type A2 individuals because of the lower sensitivity of the nonradioactive hybridization system used. Thus, this fragment was ignored in the evaluation of rDNA types.

The 0.7- and 2.8-kb *Xba*I fragments were always present in the eight rDNA variants, indicating that the three *Xba*I sites in regions of the 18S and 28S rRNA genes are conserved in the variants (Fig. 3). Thus, the polymorphisms of the *Xba*I digests were explained on the basis of the presence or absence of *Xba*I recognition sites presumably in the intergenic regions between the 3′ end of the 28S rDNA and the 5′ end of the 18S rDNA.

*Eco*RI digestion of total DNA produced two fragments that hybridized to Alt1 in all rDNA variants, indicating that the rDNA units contain two *Eco*RI sites (Fig. 2B). Since the smaller fragment was always detected at ca. 3.2 kb in all rDNA types, the two *Eco*RI sites are located in the 5.8S and 28S rDNAs in all rDNA variants (Fig. 3). The longer

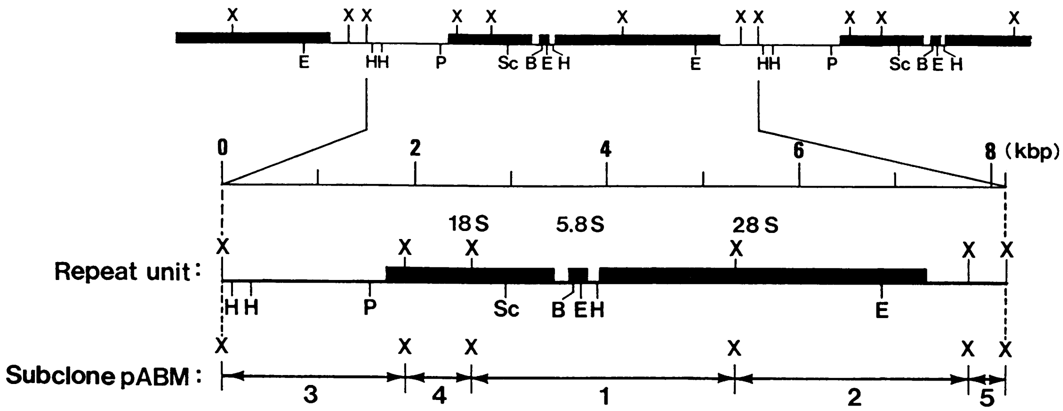

FIG. 3. Gene arrangement and restriction site maps of rDNA deduced for rDNA clone Alt1 possessing the nuclear rDNA repeat unit of the Japanese pear pathotype of *A. alternata*. Alt1 was cloned from a genomic library of Japanese pear pathotype strain 15A (36). The five fragments produced by digestion of Alt1 DNA with *Xba*I were subcloned and designated the pABM series of plasmids (36). Abbreviations: B, *Ban*III; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Sc, *Sac*I; X, *Xba*I.

fragments, however, were different in the variants. From these results, the polymorphisms in *Eco*RI digests may be explained on the basis of differences in the lengths of the intergenic regions. Since the polymorphisms were not detected when types A2 and B1 were compared and when types A4 and B2 were compared, the members of each of these pairs of types probably do not differ in intergenic region length (Fig. 2B).

Although the length and restriction site polymorphisms in the intergenic regions between rDNA repeats have precedents in eukaryotic species (2, 8, 25, 27), including fungi (reviewed in reference 6), the Japanese pear pathotype population of *A. alternata* appeared to exhibit 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 spacer DNA (31). We are now trying to further characterize the variations in the *Alternaria* rDNAs by comparing the nucleotide sequences of the intergenic regions.

Distribution frequency of rDNA variants. The Table 1 shows the distribution of the eight rDNA variants in the sampled areas; a total of 322 isolates were examined. Of these 322 isolates, 271 were classified as having one of the eight rDNA variants, and 51 seemed to have mixed rDNA types. Type A2, A4, B1, B2, and B3 variants occurred frequently in the population, but type A1, A3, and A5 variants were very rare (one or two cases each) (Table 1).

The total DNAs of all of the mixed-rDNA variants contained two types of rDNA units (data not shown). An analysis of single-spore isolates obtained from some of the mixed-rDNA isolates showed that about one-half of them resulted from double infections of individuals in single lesions; the remaining cases might have been true mixed-

TABLE 1. Frequencies of occurrence of eight rDNA variants in 15 geographical populations of the Japanese pear pathotype of *A. alternata* in Japan

Site[a]	Sampling year	No. of isolates having the following rDNA types[b]:									Total no. of isolates
		A1	A2	A3	A4	A5	B1	B2	B3	Mixed	
Aichi Prefecture											
Nagoya H1	1989	0	25	1	2	0	2	0	14	1	45
Nagoya P1	1989	0	32	0	0	0	18	1	2	2	55
Nagoya P2	1989	0	27	0	3	0	0	0	0	1	31
Gifu Prefecture: Minokamo City	1990	1	0	0	5	0	9	2	23	6	46
Tottori Prefecture											
Yonago	1988	0	1	0	6	0	2	4	0	4	17
Aimi	1988	0	0	0	1	0	7	4	0	4	16
Nawa	1988	0	1	0	2	1	2	3	0	4	13
Nakayama	1988	0	0	0	1	0	7	3	0	6	17
Akasaki	1988	0	12	0	0	0	6	1	0	9	28
Touhaku	1988	0	1	0	1	0	7	2	0	4	15
Kurayoshi	1988	0	0	0	4	0	2	2	0	2	10
Sekigane	1988	0	1	0	1	0	2	2	0	3	9
Tottori	1988	0	0	0	3	1	1	0	0	0	5
Daiei	1988	0	1	0	0	0	1	3	0	2	7
Kokufu	1988	0	0	0	1	0	2	2	0	3	8
Total		1	101	1	30	2	68	29	39	51	322

[a] See Fig. 1.
[b] See Fig. 2.

TABLE 2. χ^2 values for comparisons between distributions of rDNA variants in geographical populations of the Japanese pear pathotype of *A. alternata*

Population[a]	χ^2 values with the following populations:				
	Nagoya P1	Nagoya P2	Minokamo	Tottori 10[b]	Akasaki
Nagoya H	26.50 (5)[c,d]	15.43 (4)[c]	33.86 (5)[c]	89.35 (5)[c]	17.01 (5)[c]
Nagoya P1		37.88 (4)[c]	58.33 (5)[c]	66.64 (5)[c]	1.29 (3)
Nagoya P2			62.93 (5)[c]	79.35 (4)[c]	14.36 (3)[c]
Minokamo City				65.00 (5)[c]	39.88 (5)[c]
Tottori 10[b]					40.44 (4)[c]

[a] See Fig. 1.
[b] Pooled populations from 10 areas in Tottori Prefecture (all Tottori Prefecture populations, except the Akasaki population).
[c] $P < 0.01$.
[d] The numbers in parentheses are degrees of freedom.

rDNA isolates carrying two types of rDNA units in their genomes (unpublished data). In this study, the mixed-rDNA variants were eliminated from further analyses.

All fields contained two or more types of rDNA variants (Table 1). Since no two populations had completely different sets of rDNA variants, the genetic population structures had to be recognized by differences in the frequencies of shared variants. For statistical analyses, five rDNA types (types A2, A4, B1, B2, and B3) and one composite type (pooled types A1, A3, and A5) were used, since types A1, A3, and A5 occurred very rarely.

Distribution of rDNA variants on a microgeographical scale. The amount and distribution of rDNA variation in pathogen populations were studied on a microgeographical scale by using the samples obtained from experimental fields H1, P1, and P2 at Nagoya University as a model system. A total of six rDNA variants were detected (Table 1). The three fields contained the type A2 variant as the most common variant (about 56, 60, and 90% of the individuals in the H1, P1, and P2 populations, respectively). The P2 population from a single pear tree had a simple genetic structure. The pairwise analysis revealed a significant difference in the distribution of the rDNA variants between fields ($P < 0.01$) (Table 2). This difference was due to the distribution of the semiprevalent and rare rDNA variants (types A3, A4, B1, B2, and B3).

It has been reported that *Alternaria* conidia are dispersed abundantly in the atmosphere and are transported widely by wind and also by various biotic and abiotic factors (3, 4). Since it is difficult to envisage strong selection pressures that maintain different frequencies of rDNA variants in three fields within tens of meters of each other, the differences may have been due to distinctive original inocula introduced with the pear seedlings. We suppose that field populations on pear trees are initially established from the original inoculum sources introduced with the pear seedlings and then modified annually by pathogen dispersal and selection by various biotic and abiotic factors. Further analyses of variability within small areas and variability between seasons and years are needed to address the problems of the frequency of pathogen dissemination on a microgeographical scale and the nature of selection pressures.

Distribution of rDNA variants in Tottori Prefecture. Tottori Prefecture (area, about 3,500 km^2) has had many widely distributed orchards of Nijisseiki pear since the late 1950s. The frequencies of the rDNA variants in the 11 populations in Tottori Prefecture studied were compared (Fig. 1). Five variants (types A2, A4, A5, B1, and B2) were detected in the prefecture. Types A4, B1, and B2 were dominant in 10 populations; the exception was the Akasaki population. No significant differences were detected among the 10 similar populations ($\chi^2 = 44.06$; df = 36; $0.1 < P < 0.5$). On the other hand, the type A2 variant accounted for ca. 60% of the individuals in the Akasaki population, and the distribution of rDNA variants in the Akasaki population was significantly different from the composite distribution determined for the other populations (Table 2, Tottori 10 population) ($\chi^2 = 40.44$; df = 4; $P < 0.005$). Although the reasons for this difference in the Akasaki population are not known, the similarity of the data for the other populations suggests that inoculum dispersal and gene flow have occurred frequently in Tottori Prefecture.

Previous studies have shown that rDNA RFLPs can represent genetic distances within and between fungal species (reviewed in reference 6). Since *A. alternata* is a haploid imperfect fungus, rDNA polymorphisms were assumed to be correlated closely with levels of genetic relatedness among individual isolates within the pathogen population. To determine whether the variation in rDNA data reflects genetic diversity at other genetic loci, nuclear DNA fingerprints resulting from hybridization with the AAR9 probe were used to examine dispersed genomic regions. AAR9 was a genomic clone of strain 15A carrying a moderately repetitive DNA sequence dispersed in the fungal chromosomes; the AAR9 probe hybridized to 11 *Pst*I fragments (ranging in size from 0.7 to 10 kb) in strain 15A.

Examples of the DNA fingerprints are shown in Fig. 4. We found 1 to 16 *Pst*I fragments in each of the 104 isolates from the Tottori populations examined. A total of 46 different resolvable fragments ranging in size from 0.7 to 15 kb were found in the population. The 104 isolates produced 46 different fingerprints. There were 6, 7, 2, 17, and 14 fingerprint types within the type A2, A4, A5, B1, and B2 rDNA variants, respectively. A cluster analysis of the fingerprint data was used to calculate the similarity coefficients between the fingerprint types, and a dendrogram was constructed by using UPGMA (Fig. 5). On the dendrogram, most of the type A2 isolates (15 of 17 isolates), most of the type B1 isolates (38 of 39 isolates), and most of the type B2 isolates (24 of 26 isolates) were distributed in distinct single clusters corresponding to the rDNA types; groups 1 to 3 on the dendrogram consisted of type B1, B2, and A2 rDNA isolates, respectively. A total of 11 of the 20 type A4 isolates produced one fingerprint type (Fig. 5, type TA4-6). These results indicated that ca. 85% of the individuals in the Tottori population clustered according to rDNA type. Characteristic fingerprints for collection sites in the prefecture were not observed for these clusters (data not shown).

On the other hand, the remaining isolates, which did not belong to groups 1 to 3, produced distinct fingerprints

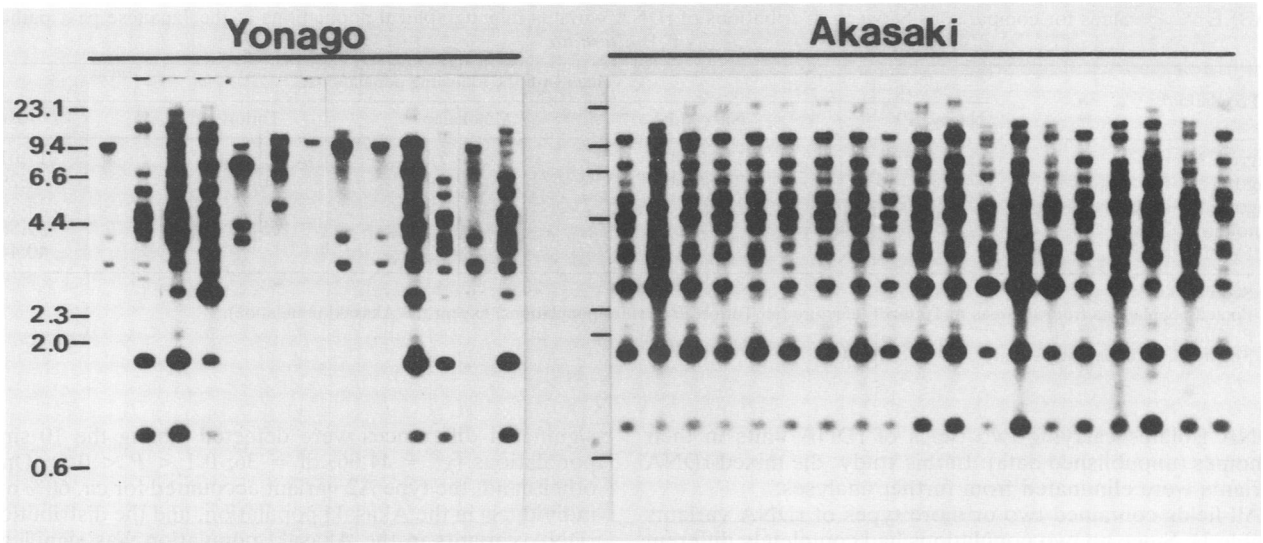

FIG. 4. AAR9 DNA fingerprints of the Japanese pear pathotype isolates of *A. alternata* collected from Yonago and Akasaki in Tottori Prefecture. Total DNA was digested with *Pst*I and fractionated on a 0.8% agarose gel. The Southern blots were hybridized with ^{32}P-labeled AAR9 DNA. The AAR9 clone, which contained a moderately repetitive DNA sequence dispersed in the fungal chromosomes, was isolated from a genomic library of Japanese pear pathotype strain 15A. The sizes of marker DNA fragments (*Hin*dIII-digested λ DNA) (in kilobases) are indicated on the left.

regardless of rDNA type, although they were rarely isolated in the prefecture (Fig. 5). The numbers of restriction fragments that hybridized to the AAR9 probe were smaller in these isolates than in the group 1 to 3 isolates. Although AAR9 hybridized to 12 to 16 fragments in isolates belonging to groups 1 to 3, the fingerprints of the remaining isolates contained 1 to 8 fragments. We hope to analyze the genetic population structure further by using additional clones of repetitive elements obtained from the exceptional isolates to determine whether similar results are obtained. However, the rDNA types reflected the multilocus genotypes of individuals in the Tottori population in the majority of cases. This seems to support our hypothesis based on the rDNA RFLP analysis results that inoculum dispersal and gene flow have occurred frequently in Tottori Prefecture.

Inoculum dissemination in Tottori Prefecture may have been caused by a number of factors that occurred during pear cultivation in this area during a period of about 50 years. Since many orchards containing Nijisseiki pear trees are widely distributed in the prefecture, natural factors, especially wind, could aid pathogen dispersal over relatively short distances. In addition, artificial factors could play an important role in inoculum dissemination. The pear trees have been propagated vegetatively by grafting on clonal rootstocks. Seedling companies have produced numerous Nijisseiki seedlings by grafting scions from a limited number of mother plants in Tottori Prefecture and have supplied the seedlings to farmers throughout the prefecture. This may have resulted in wide dissemination of pathogen inoculum with seedlings. Furthermore, the field populations in the prefecture have been exposed to strong selection pressures from continuous efforts to control the disease, mainly by fungicides. The efforts to control the disease have been conducted uniformly all around the prefecture and have been based on annual guidelines, which include the kinds of fungicides to be used and the frequency and timing of application. These practices may have led to the genetic uniformity of the pathogen in the pear fields in Tottori Prefecture.

Distribution of rDNA variants on a macrogeographical scale. Minokamo City (Gifu Prefecture) is ca. 35 km from Nagoya City (Aichi Prefecture). The type A2 rDNA variant was dominant in Nagoya City populations, but in the Minokamo City population the type B3 rDNA variant was dominant (Table 1). The pairwise analysis revealed highly significant differences in rDNA variant distribution between the populations (Table 2), suggesting that there is little gene flow between the populations. In Aichi and Gifu Prefectures, the distribution of commercially managed orchards of Nijisseiki pear has been very limited. Such a distribution of pear fields seems to cause a strong geographical isolation of pathogen populations because of restricted pathogen dispersal over relatively long distances.

Minokamo City and Nagoya City are ca. 250 km from Tottori Prefecture. When all of the populations from the three prefectures were used to analyze the heterogeneity of the frequency distributions of the rDNA variants, there was a highly significant difference among the populations (χ^2 = 165.73; df = 25; $P < 0.005$). This indicates that there is little gene flow between the prefectures. Of the 15 possible combinations in the pairwise analysis, differences in 14 combinations were significant ($P < 0.01$) (Table 2). However, no significant difference was observed in one comparison, surprisingly, the comparison between the Nagoya P1 (Aichi Prefecture) and Akasaki (Tottori Prefecture) populations (χ^2 = 1.29; df = 3; $0.5 < P < 0.9$). While we have found no obvious explanation for the similarity of the Nagoya P1 and Akasaki populations, we suppose that DNA fingerprinting with the AAR9 probe might reveal significant genetic differences among isolates from different geographical locations, even among isolates having the same rDNA type.

The DNA fingerprints of type B1 rDNA isolates were compared, because type B1 isolates were widely distributed in the three prefectures (Table 1). AAR9 hybridized to 4 to

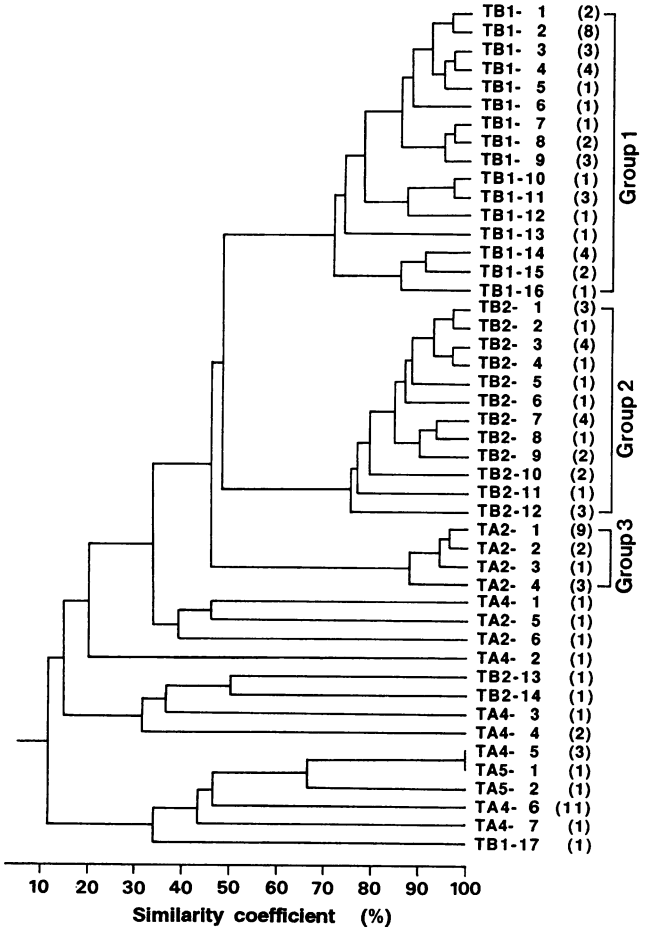

FIG. 5. Dendrogram showing the levels of genetic relatedness among 104 isolates of the Japanese pear pathotype of *A. alternata* obtained from Tottori Prefecture. The 104 isolates were divided into 46 fingerprint types on the basis of their AAR9 fingerprints. Similarity coefficients were calculated from the fingerprints by the method of Nei and Li (21). A dendrogram was constructed from the similarity coefficients by using UPGMA (32). The numbers in parentheses are the number of isolates belonging to each fingerprint type. The fingerprint type designations indicate the geographical origin (T, Tottori Prefecture), the rDNA type (A2 to B2), and the code number.

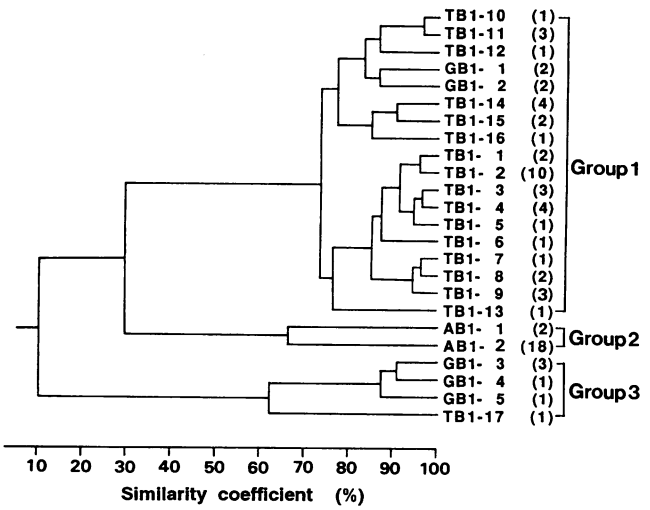

FIG. 6. Dendrogram showing the levels of genetic relatedness among 68 isolates of the Japanese pear pathotype of *A. alternata* in the rDNA type B1 population. The 68 isolates were divided into 24 fingerprint types on the basis of their AAR9 fingerprints. Similarity coefficients were calculated from the fingerprints by the method of Nei and Li (21). A dendrogram was constructed from the similarity coefficients by using UPGMA (32). The numbers in parentheses are the number of isolates belonging to each fingerprint type. The fingerprint type designations indicate the geographical origin (A, G, and T, Aichi, Gifu, and Tottori Prefectures, respectively), the rDNA type (B1), and the code number.

17 *Pst*I fragments in each of 68 isolates belonging to the type B1 population. A total of 30 different resolvable fragments (lengths, 0.7 to 15 kb) were detected, and the type B1 population was classified into 24 fingerprint types. On the basis of the fingerprints, a dendrogram was constructed by using UPGMA (Fig. 6). The dendrogram identified three genetic groups at a similarity level of more than 60% (Fig. 6). In group 1, 38 of 42 isolates were from Tottori Prefecture, and the remaining 4 isolates were from Gifu Prefecture. Group 2 consisted of 20 isolates, all of which were from Aichi Prefecture. Group 3 contained five isolates and one isolate from Gifu and Tottori Prefectures, respectively. Although all of the isolates from the Aichi population were in a single genetic cluster (group 2), members of both the Tottori and Gifu populations were in two distinct genetic groups (groups 1 and 3) (Fig. 6). However, no fingerprint type was shared by isolates from the two prefectures, and the isolates from the prefectures clustered in groups according to their geographical origins. Therefore, the dendrogram suggests that there is a correlation between genetic similarity and geographical location in the type B1 rDNA population.

On the basis of the rDNA RFLPs, there was no significant difference in rDNA variant distribution between the Nagoya P1 (Aichi Prefecture) and Akasaki (Tottori Prefecture) populations. However, AAR9 fingerprints of the type B1 rDNA population showed that a genetic cluster of the Nagoya population was distinguishable from the cluster of the Tottori population that included the Akasaki isolates, even if they shared the same rDNA type. We also compared the levels of genetic relatedness of type A2 rDNA isolates between the two prefectures (data not shown). Our results confirmed differences in genetic population structure between the two populations.

The samples used were collected in different years (1988, 1989, and 1990 from Tottori, Aichi, and Gifu Prefectures, respectively). We suppose that differences in population structure between the prefectures may have been due to differences in the sampling years, not to differences in geographical locations. We have monitored the genetic structures of some geographical populations over multiple years. So far, considerable changes in the populations from year to year have not been detected. This seems to confirm our hypothesis that the differences in population structure between prefectures have been caused by geographical isolation of the pathogen populations, resulting in restricted pathogen dissemination. However, much more research is needed to confirm this hypothesis.

Recently, it has been suggested that hypervariable repetitive DNA may be useful for DNA fingerprinting strains belonging to a particular pathogenic type or from a geographical locale (5, 10, 12, 13, 15, 16, 19, 30). We have recently isolated additional nuclear repetitive sequences of the *Alter-*

naria pathogen and have sampled additional populations to make more precise estimates of within-, between-, and among-population variability and to estimate rigorously the rates of gene flow between pathogen populations over time and space. Data obtained with RFLP markers, including DNA fingerprints, should provide a firmer basis for such estimates and might also be useful for understanding the impact of natural and artificial stresses on pathogen populations and the fitness characters of the pathogen.

A. alternata includes seven pathogenic variants, called pathotypes, which produce distinct host-specific toxins and infect different host plants (22, 23). Our data for the Japanese pear pathotype suggest that there is a large amount of genetic variability in *A. alternata*. We are now comparing genetic population structures within and among nonpathogenic populations and seven pathogenic populations of *A. alternata* to determine the genetic potential of pathogenic specialization in the fungus depending on host-specific toxins. Furthermore, RFLP analysis will be used to assess the development of fungicide-resistant populations of the *Alternaria* pathogens in fields.

ACKNOWLEDGMENTS

We are grateful to Y. Kawamoto (Primate Science Institute of Kyoto University, Aichi, Japan) for providing the computer program software for construction of the dendrogram. We also thank K. Kawakita and H. Yoshioka of our laboratory for valuable discussions.

This work was supported by research grants from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. **Akins, R. A., and A. M. Lambowitz.** 1985. General method for cloning *Neurospora crassa* nuclear genes by complementation of mutants. Mol. Cell. Biol. **5**:2272–2278.
2. **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.
3. **Bashan, Y.** 1986. Field dispersal of *Pseudomonas syringae* pv. *tomato*, *Xanthomonas campestris* pv. *vesicatoria* and *Alternaria macrospora* by animals, people, birds, insects, mites, agricultural tools, aircraft, soil particles and water sources. Can. J. Bot. **64**:276–281.
4. **Bashan, Y., H. Levanony, and R. Or.** 1991. Wind dispersal of *Alternaria alternata*, a cause of leaf blight of cotton. J. Phytopathol. **133**:225–238.
5. **Brown, J. K. M., M. O'Dell, C. G. Simpson, and M. S. Wolfe.** 1990. The use of DNA polymorphisms to test hypotheses about a population of *Erysiphe graminis* f. sp. *hordei*. Plant Pathol. **39**:391–401.
6. **Bruns, T. D., T. J. White, and J. W. Taylor.** 1991. Fungal molecular systematics. Annu. Rev. Ecol. Syst. **22**:525–564.
7. **Burke, T., G. Dolf, A. J. Jeffreys, and R. Wolff (ed.).** 1991. DNA fingerprinting: approaches and applications. Birkhauser Verlag, Basel.
8. **Cluster, P. D., D. Marinkovic, R. W. Allard, and F. J. Ayala.** 1987. Correlations between development rates, enzyme activities, ribosomal DNA spacer-length phenotypes, and adaptation in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA **84**: 610–614.
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. **Goodwin, S. B., L. J. Spielman, J. M. Matuszak, S. N. Bergeron, and W. E. Fry.** 1992. Clonal diversity and genetic differentiation of *Phytophthora infestans* populations in northern and central Mexico. Phytopathology **82**:955–961.
11. **Hamer, J. E., L. Farrall, M. J. Orbach, B. Valent, and F. G. Chumley.** 1989. Host species-specific conservation of a family of repeated DNA sequences in the genome of a fungal plant pathogen. Proc. Natl. Acad. Sci. USA **86**:9981–9985.
12. **Kistler, F. C., E. A. Momal, and U. Benny.** 1991. Repetitive genomic sequences for determining relatedness among strains of *Fusarium oxysporum*. Phytopathology **81**:331–336.
13. **Kohli, Y., R. A. A. Morrall, J. B. Anderson, and L. M. Kohn.** 1992. Local and trans-Canadian clonal distribution of *Sclerotinia sclerotiorum* on canola. Phytopathology **82**:875–880.
14. **Kohmoto, K., H. Otani, and S. Nishimura.** 1989. Host recognition: can accessibility to fungal invasion be induced by host-specific toxins without necessitating necrotic cell death?, p. 249–265. *In* A. Graniti (ed.), Phytotoxins and plant pathogenesis. Springer-Verlag, Berlin.
15. **Levy, M., J. Romao, M. A. Marchetti, and J. E. Hamer.** 1991. DNA fingerprinting with a dispersed repeated sequence resolves pathotype diversity in the rice blast fungus. Plant Cell **3**:95–102.
16. **McDonald, B. A., and J. P. Martinez.** 1990. DNA restriction fragment length polymorphisms among *Mycosphaerella graminicola* (anamorph *Septoria tritici*) isolates collected from a single wheat field. Phytopathology **80**:1368–1373.
17. **McDonald, B. A., J. M. McDermott, S. B. Goodwin, and R. W. Allard.** 1989. The population biology of host-pathogen interactions. Annu. Rev. Phytopathol. **27**:77–94.
18. **Michelmore, R. W., and S. H. Hulbert.** 1987. Molecular markers for genetic analysis of phytopathogenic fungi. Annu. Rev. Phytopathol. **25**:383–404.
19. **Milgroom, M. G., S. E. Lipari, and W. A. Powell.** 1992. DNA fingerprinting and analysis of population structure in the chestnut blight fungus, *Cryphonectria parasitica*. Genetics **131**:297–306.
20. **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.
21. **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.
22. **Nishimura, S.** 1980. Host-specific toxins from *Alternaria alternata*: problems and prospects. Proc. Jpn. Acad. **56B**:362–366.
23. **Nishimura, S., and K. Kohmoto.** 1983. Host-specific toxins and chemical structures from *Alternaria* species. Annu. Rev. Phytopathol. **21**:87–116.
24. **Otani, H., K. Kohmoto, S. Nishimura, T. Nakashima, T. Ueno, and H. Fukami.** 1985. Biological activities of AK-toxins I and II, host-specific toxins from *Alternaria alternata* Japanese pear pathotype. Ann. Phytopathol. Soc. Jpn. **51**:285–293.
25. **Petes, T. D., and D. Bostein.** 1977. Simple Mendelian inheritance of the reiterated ribosomal DNA of yeast. Proc. Natl. Acad. Sci. USA **74**:5091–5095.
26. **Reed, K. C., and D. A. Mann.** 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. **13**:7207–7221.
27. **Saghai-Maroof, M. A., K. M. Soliman, R. A. Jorgensen, and R. W. Allard.** 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. USA **81**:8014–8018.
28. **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.
29. **Scheffer, R. P., and R. S. Livingston.** 1984. Host-selective toxins and their role in plant diseases. Science **223**:415–419.
30. **Smith, M. L., J. N. Bruhn, and J. B. Anderson.** 1992. The fungus *Armillaria bulbosa* is among the largest and oldest living organisms. Nature (London) **356**:428–430.
31. **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.
32. **Sneath, P. H. A., and R. R. Sokal.** 1973. Numerical taxonomy: the principles and practice of numerical classification. W. H. Freeman and Co., San Francisco.
33. **Snedecor, G., and M. R. Irwin.** 1933. On the chi-square test for

homogeneity. Iowa State Coll. J. Sci. **8**:75–81.
34. **Tanabe, K., T. Tsuge, and S. Nishimura.** 1990. Potential application of DNA restriction fragment length polymorphisms to the ecological studies of *Alternaria alternata* Japanese pear pathotype. Ann. Phytopathol. Soc. Jpn. **55**:361–365.
35. **Tanaka, S.** 1933. Studies on black spot disease of Japanese pear (*Pyrus serotina* Rehd.). Mem. Coll. Agric. Kyoto Imp. Univ. **28**:1–31.
36. **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.
37. **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.
38. **Workman, P. L., and J. D. Niswander.** 1970. Population studies on southwestern Indian tribes. II. Local genetic differentiation in the Papago. Am. J. Hum. Genet. **22**:24–49.
39. **Yoder, O. C.** 1980. Toxins in pathogenesis. Annu. Rev. Phytopathol. **18**:103–129.
40. **Yoder, O. C.** 1988. *Cochliobolus heterostrophus*, cause of Southern corn leaf blight, p. 93–112. *In* G. S. Sidhu (ed.), Advances in plant pathology, vol. 6. Genetics of plant pathogenic fungi. Academic Press, London.