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RFLP analysis for species separation in the genera *Bipolaris* and *Curvularia*

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Restriction fragment length polymorphism (RFLP) of the total DNA of *Bipolaris* and *Curvularia* species was analysed using arbitrarily chosen genomic clones of DNA from *Curvularia lunata* and *Bipolaris maydis* as probes. Clear differences among species in both genera, resulting in different banding positions, were obtained with some probe-enzyme combinations. Intraspecific polymorphism in banding positions with these probe-enzyme combinations was slight. These analyses allow discrimination between the species. DNA fingerprinting with intrageneric probes is a potentially useful tool for species separation and identification in *Bipolaris* and *Curvularia* when coupled with another characteristic such as conidial morphology. *Curvularia aeria* comb. nov. was proposed for *Curvularia lunata* var. *aeria* on the basis of differences in RFLP banding patterns and differences in conidial morphology.

Key Words——*Bipolaris*; *Curvularia*; *Curvularia aeria* comb. nov.; genomic DNA; RFLP analysis.

Introduction

Bipolaris and *Curvularia* species are the most common plant pathogenic fungi on graminea plants and are frequently detected on other cellulotic substrates. They have attracted strong interest because of their pathogenicity to important graminea plants and their ability to produce various secondary metabolites and enzymes. Species separation in the two genera has been based principally on conidial morphology, and many species have been proposed (Ellis, 1966, 1971, 1976; Alcorn, 1983; Sivanesan, 1987). But conidial morphology is often inconsistent and variable within isolates or with circumstantial conditions, and species delimitation is sometimes arbitrary and vague (Tsuda and Ueyama, 1987). Additional biological characters, such as mating reactions, have been used successfully to overcome the lack of useful morphological characters (Tsuda, 1992b; Tsuda and Ueyama, 1987). The difficulty of production of teleomorphs is well known, however, and the identification of species in both genera has often proved difficult, although these genera are usually readily distinguishable from similar genera such as *Drechslera* and *Exserohilum* by comparison of conidial morphology (Sivanesan, 1987; Tsuda, 1992a).

Clearly, new approaches are needed to improve the taxonomy of *Bipolaris* and *Curvularia*, and changes should reflect their genetic relations. Recently, the phylogenetic relationships reflected in similarities at the level of DNA base sequences have been adopted for comparison among many phenotypically uncertain organisms (Baum, 1992). To evaluate such similarities, DNA base compositions, DNA homologies have been studied and compared (Mullaney and Klich, 1990; Metzenberg, 1991). Among these restriction fragment length polymorphism (RFLP), which is determined by the combination of DNA fragments produced by digestion with specific enzymes and identified by Southern hybridization, has been adopted for many organisms. Such studies aimed mainly to elucidate the phylogenetic relationships between particular groups of organisms. Such close examination of DNA sequences by modern techniques has not yet been reported for members of *Bipolaris* and *Curvularia*.

The present study was conducted on *Bipolaris* and *Curvularia* species to examine whether analysis of chromosomal DNA utilizing nonspecifically (arbitrarily) selected probe sequences from the standard isolates would be sensitive enough (1) to distinguish these species; (2) to furnish useful taxonomic criteria; and (3) to allow construction of an identification key.

Materials and Methods

Strains used Strains of *Bipolaris* and *Curvularia* species isolated or given to us were identified as listed in Table 1. Additional strains of *Bipolaris maydis* (Nisikado et Miyake) Shoem. used for the study of intraspecific polymorphism are shown in Table 2.

Mycelial preparation
Each strain was inoculated in modified V8 liquid medium (180 ml V8-juice and 2 g $CaCO_3$ in 10 l deionized water) in a Sakaguchi flask. Cultures were incubated at 25°C on a reciprocal shaker for 3 days. The mycelia were harvested by filtration through cheese cloth and lyophilized.

Table 1. *Bipolaris* and *Curvularia* species used in this study.

Species Isolates	Origin		Locality of collection
B. oryzae (Breda de Haan) Shoem.			
KU-13	Stock culture		Kyoto Univ.
HA2	Stock culture		Leach, C.M.
90-1	seed	*Oryza sativa* L.	Iwakura, Kyoto
B. maydis (Nisikado et Miyake) Shoem.			
He5R13	Ascosporic isolate		Kyoto Univ.
HITO7711	leaf	*Zea mays* L.	Hitoyoshi, Kumamoto
59	leaf	*Z. mays*	Kuchiki, Shiga
B. kusanoi (Nisikado) Shoem.			
Tsuruga-1	inflorescence	*Eragrostis pilosa* (L.) P. Beauv.	Tsuruga, Fukui
Tsuruga-4	inflorescence	*E. pilosa*	Tsuruga, Fukui
B. hawaiiensis (M. B. Ellis) Uchida et Aragaki			
B1-1	seed	*O. sativa*	Myanmar
B1-2	seed	*O. sativa*	Myanmar
B1-3	seed	*O. sativa*	Myanmar
B. spicifer (Bainer) Shoem.			
Tajimi	seed	*Sorghum* sp.	Nishinasuno, Tochigi
B. australiensis (M. B. Ellis) Tsuda et Ueyama			
Cg151-1	seed	*Chloris gayana* Kunth	Nagano, Nagano
Cg148-2	seed	*C. gayana*	Nagano, Nagano
B. heveae (Petch) Shoem.			
Bangka-1	leaf	*Hevea brasiliensis* (Eilld. ex Adr. Juss.) Muell. Arg.	Bangka, Indonesisa
B. chloridis (Alcorn) Alcorn			
Kata-2	seed	*C. gayana*	Nagano, Nagano
760-24	Stock culture		Alcorn, J.L.
B. cynodontis (Marig.) Shoem.			
Suzu-tomari-1	leaf	*Eragrostis cilianensis* (Allioni) Vignolo-Lutati	Daieicho, Tottori
Cyn A	leaf	*Cynodon dactylon* (L.) Pers.	Sakyo, Kyoto
B. bicolor (Mitra) Shoem.			
El1-1	leaf	*Eleusine indica* Gaertn.	Sakyo, Kyoto
Kara 10.11-22	leaf	*Panicum coloratum* L.	Nishigoshi, Kumamoto
Okusa 81.1-2	leaf	*P. dichotomiflorum* Mickx.	Daigo, Kyoto
B. zeicola (Stout) Shoem.			
Tokachi 2-2	leaf	*Z. mays*	Tokachi, Hokkaido
Shihoro 1-1	leaf	*Z. mays*	Shihoro, Hokkaido
29R-2	Stock culture		Cornell Univ.
Race-1	Stock culture		Cornell Univ.
B. cookei (Sacc.) Shoem.			
Yamashina-1	leaf	*Sorghum* sp.	Yamashina, Kyoto
HG In-740	Stock culture		Natl. Inst. Agric. Sci.
B. victoriae (Meeha et Murphy) Shoem.			
HV2	Stock culture		Cornell Univ.
HV3	Stock culture		Cornell Univ.
B. sorokiniana (Sacc. in Sork.) Shoem.			
Hadaka hirai	leaf	*Hordeum vulgare* L.	Yamashirocho, Tokushima
Inumugi yama	leaf	*Bromus catharticus* Vahl	Yamashina, Kyoto
Kusakibi daigo	leaf	*P. dichotomiflorum*	Daigo, Kyoto
B. papendorfii (van der Aa) Alcorn			
D-1	leaf	*O. sativa*	Indonesia

B. coicis (Nisikado) Shoem.			
Daigo-1	leaf	*Coix lacryma-jobi* L.	Daigo, Kyoto
Kawada-1	laef	*C. lacryma-jobi*	Yamashina, Kyoto
1021-2	Stock culture		Nishihara, N.
C. lunata (Wakker) Boedijn			
Tef-1	leaf	*E. tef* (Zyccagni) Trotter	Sakyo, Kyoto
I73, 1-2	seed	*O. sativa*	Indonesia
Shin-1	leaf	*Canna* sp.	Kobe, Hyogo
C. lunata var. *aeria* (Batista et al.) M. B. Ellis			
B29-1	seed	*O. sativa*	Myanmar
B29-1 Sec	seed	*O. sativa*	Myanmar
B91-5	seed	*O. sativa*	Myanmar
B30-1	seed	*O. sativa*	Myanmar
I98	seed	*O. sativa*	Indonesia
I54	seed	*O. sativa*	Indonesia
Ohisatuma1-1	seed	*Elusine indica*	Satumacho, Kagoshima
C. gladiolii (Parmelee et Luttrell) Boerema et Hamers			
Kagoshima-1	leaf	*Gladiolus* x *hortulanus* L. H. Bailey	Kagoshima
Kagoshima-2	leaf	*G.* x *hortulanus*	Kagoshima
Kagoshima-3	leaf	*G.* x *hortulanus*	Kagoshima
Demachi1-1	leaf	*G.* x *hortulanus*	Kamikyo, Kyoto
C. trifolii (Kauffm.) beodijn			
Nasuno 1	leaf	*Trifolium repens* L.	Nasunocho, Tochigi
Nasuno 2	leaf	*Trifolium repens* L.	Nasunocho, Tochigi
Nasuno 2-2	leaf	*T. repens*	Nasunocho, Tochigi
Iwate 2	leaf	*T. repens*	Morioka, Iwate
C. akaii Tsuda et Ueyama			
He5	Ascospore isolate		Kyoto Univ.
Roku2-1	leaf	*Themeda triandra* Forssk. subsp. *japonica* T. Koyama	Rokujizo, Kyoto

Total DNA preparation
Total DNA was used for large scale screening of RFLP. A modification of the DNA mini-preparation procedure (Liu et al., 1990 based on Mettler, 1987) was used: Mycelia were frozen in liquid nitrogen and powdered with a pestle and mortar. The mycelial powder was then transferred in 50-ml centrifuge tube and mixed with three volumes of extraction buffer made of 1% sarkosyl, 0.25 M sucrose, 50 mM NaCl, 20 mM EDTA, 50 mM Tris-HCl (pH 8.0) and 10 mM mercaptoethanol.
After centrifugation at room temperature for 30 min, one volume of phenol/chloroform (v/v) was added, and the mixture was emulsified with a spoon. After centrifugation at 15,000 *g* for 15 min, the aqueous phase was collected, and one-tenth volume of 3.0 M Na-acetate was added. The DNA was precipitated with six-tenth volume of isopropanol, spooled out with a glass rod, rinsed in 70% ethanol and dissolved in TE buffer containing RNase A (3 μg/ml).
This solution was again subjected to phenol/chloroform extraction, and the DNA was precipitated with ethanol and dissolved in a suitable volume of TE buffer. The quantity of DNA was determined from its band intensity on an EtBr-stained agarose gel in comparison with a standard DNA sample. This method yielded 100–150 μg of DNA per g of fresh mycelia.

RFLP probes
Nuclear DNA from *B. maydis* HITO7711 and *C. lunata* (Wakker) Boedijin Shin-1 was digested either with *Hin*dIII or *Eco*RI. DNA fragments ranging between 0.5 and 4.0 kb in size were collected by fractionation in a 10–40% sucrose gradient, and ligated into the plasmid vector, pUC119. The ligated plasmids were used to transform *E. coli* DH5α cells. Clones with an insert were screened by the X-gal/IPTG procedure, and clones with an insert larger than 0.5 kb were selected by the rapid disruption method (Maniatis et al., 1982). The plasmid DNAs were mini-prepared by the alkaline lysis method (Maniatis et al., 1982), and the sizes of the inserts were determined by agarose gel electrophoresis of the plasmids digested with *Hin*dIII or *Eco*RI, the same enzyme being used as in the initial DNA digestion. *Hae*III-digested ϕX174 DNA was used as a size marker. The clones are designated as B (55B106, 55B130) and C (CL1), indicating *Bipolaris* or *Curvularia* genome, respectively. Plasmids carrying *B. maydis* structural genes GPD1 and TRP1 (Van Wert and Yoder, 1992) were also used as probes.

Southern blot analysis
DNA extraction, digestion, transfer to membranes, and hybridization to detect polymorphism in total genomic DNA was previously described by Liu et al. (1990).

Table 2. List of *B. maydis* isolates used in this study.

Isolate No.	Host plant		Locality of collection
1.	*Z. mays*	leaf	Kuchiki, Shiga
2.	do.	do.	Sagara, Kumamoto
3.	do.	do.	Nishigoshi, Kumamoto
4.	*Imperata cyrindrica* (L.) P. Beauv. var. *koenigii* (Rez.) Durand et Shinz		Daiei, Tottori
5.	*Z. mays*	leaf	Itsugi, Kumamoto
6.	do.	do.	Sagara, Kumamoto
7.	*I. cylindrica* var. *koenigii*		Daiei, Tottori
8.	*Z. mays*	leaf	Aso, Kumamoto
9.	*Bothriochloa* sp.		Nishigoshi, Kumamoto
10.	*Z. mays*	leaf	Sagara, Kumamoto
11.	*Panicum miliaceum* L.		Hayase, Yamanashi
12.	*Z. mays*	leaf	Yoshino, Nara
13.	do.	do.	Nishigoshi, Kumamoto
14.	do.	do.	Kuchiki, Shiga
15.	*Bothriochloa* sp.		Nishigoshi, Kumamoto
16.	*Z. mays*	leaf	Sagara, Kumamoto
17.	do.	do.	Sakyo, Kyoto
18.	do.	do.	Itsugi, Kumamoto
19.	do.	do.	Nishigoshi, Kumamoto
20.	do.	do.	Kuchiki, Shiga
21.	do.	do.	Kuchiki, Shiga
22.	Yoder, C.	C2	Cornell Univ.
23.	*Z. mays*	leaf	Itsugi, Kumamoto
24.	do.	do.	Sagara, Kumamoto
25.	do.	do.	Hatano, Kumamoto
26.	do.	do.	Mihara, Hyogo
27.	do.	do.	Ohto, Nara
28.	do.	do.	Yoshino, Nara

Total DNAs digested with *Bam*HI and *Hin*dIII were transferred to nylon membranes (Hybond-N$^+$, Amersham, UK) and hybridized with the above plasmids as probes.

Results

The arbitrarily chosen genomic clones of DNA from *C. lunata* Shin-1 and *B. maydis* HITO7711 were used as hybridization probes to detect restriction fragments with sequence similarity to the DNA of other species of both genera listed in Table 1. Though all probes are obviously hybridized with the genomic DNA of all species of both genera, the numbers of bands and their relative positions appear to be somewhat different. Thirteen of the 59 clones hybridized to a single or a few major restriction fragments of genomic DNA. Banding positions for 57 strains belonging to 21 species with combinations of probe CL1 and digesting enzymes *Hin*dIII and *Bam*HI are summarized in Figs. 1a and 1b.

Clear differences among the species, resulting in different banding positions, were obtained with all probes. The discriminatory potential of a probe, however, is strongly dependent on the enzyme. For example, *B. australiensis* (M. B. Ellis) Tsuda et Ueyama, *B. hawaiiensis* (M. B. Ellis) Uchida et Aragaki, and *B. spicifer* (Bainier) Subram., which have similar cultural characteristics with easily distinguishable conidial morphology, gave identical banding positions when digested with *Bam*HI, but different positions with *Hin*dIII probed with CL1 (Fig. 1) and 55B130 (Fig. 2). When other fragments were hybridized to chromosomal DNA from a range of species belonging to both genera, distinctive restriction patterns were also found. The intensity of hybridization bands in these strains was slightly different but not so prominent.

Intraspecific polymorphisms of banding positions within *B. maydis* were examined with selected probes including arbitrarily chosen and structural gene coding DNA fragments of the fungus. Typical banding patterns with *Bam*HI/GPDI and *Bam*HI/55B106 are shown in Figs. 3 and 4. Three different types were detected, and intraspecific polymorphisms were clearly recognized with the former combination (lane 1, 2, lane 17 and others in Fig. 3). In the case of 55B106, however, no intraspecific polymorphism was observed. A set of different species was checked for intraspecific polymorphism using other enzyme-probe combinations. It was difficult to find intraspecific polymorphism with our selected fragments (data not shown).

Discussion

Analysis of RFLPs for separate DNA fragments of total genomic DNA revealed considerable banding pattern variation among species belonging to *Bipolaris* and *Curvularia* genera. Using two restriction enzymes with 6-base recognition sequences and some arbitrarily chosen DNA fragments, we were able to recognize differences among species studied. Our results indicate some variations among individual isolates of a species with respect to RFLPs in DNA. But in these cases intraspecific polymorphism was slight and interspecific diversity was very prominent. Distance index values (Natvig et al., 1987) were calculated for some 20 probe-enzyme combinations by replacing fragments with banding patterns for some related species, i.e., *B. bicolor* (Mitra) Shoem., *B. chloridis* (Alcorn) Alcorn, and *B. cynodontis* (Marignoni) Shoem. (Table 3), and *C. lunata, C. lunata* var. *aeria* (Batista et al.) M. B. Ellis, *C. trifolii* (Kauffm.) Boedijn, *C. gladiolii* (Parmelee et Luttrell) Boerema et Hamers and *C. akaii* Tsuda et Ueyama (Table 4). In the latter case, the results concurred with our previous observations on the differences between *C. lunata* and *C. lunata* var. *aeria* (Tsuda et al., 1985). The ornamentation of the outer surface of conidia in *C. lunata* is rough, and small warts are visible. No such ornamentation is observed on the conidia of *C. lunata* var. *aeria*. Difference of distance index values between both groups was high enough for differentiation at the species level. Therefore, *C. lunata* var. *aeria* would be better treated as a separate species in *Curvularia* on the basis of differences in DNA homology and morphological characteristics.

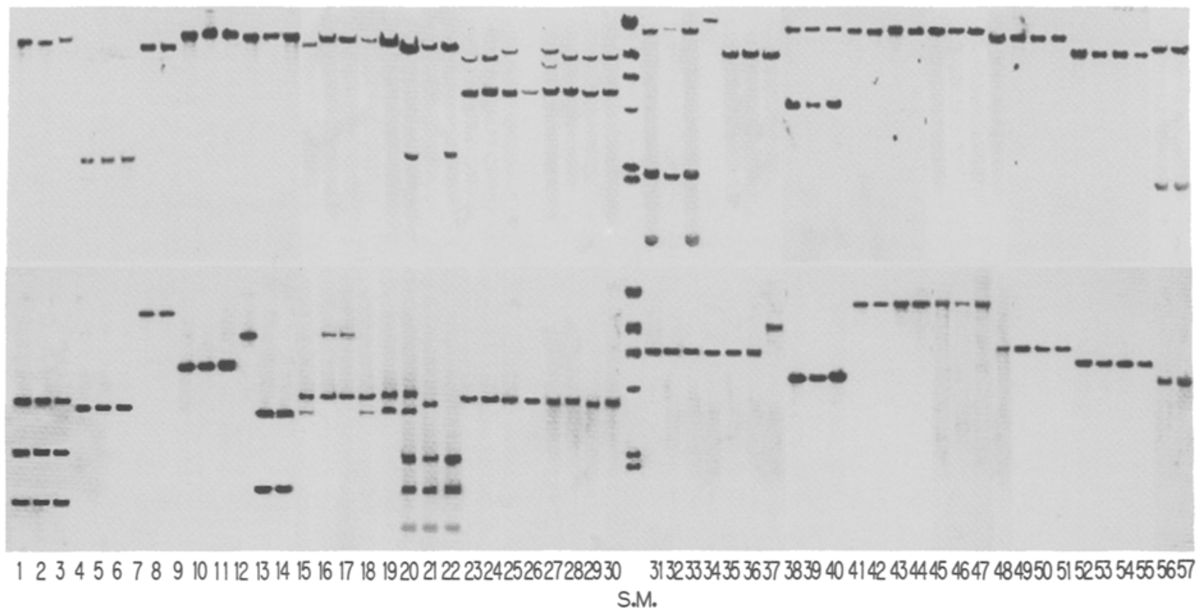

Fig. 1. Banding patterns of Southern blots of the total DNAs isolated from *Bipolaris* and *Curvularia* species, probed with *C. lunata* genomic clone CL1.
Lane: 1, *B. oryzae* KU-13, 2, *B. oryzae* HA2, 3, *B. oryzae* 90-1, 4, *B. maydis* He5R13, 5, *B. maydis* HITO7711, 6, *B. maydis* 59, 7, *B. kusanoi* Tsuruga-1, 8, *B. kusanoi* Tsuruga-4, 9, *B. hawaiiensis* B1-1, 10, *B. hawaiiensis* B1-2, 11, *B. hawaiiensis* B1-3, 12, *B. spicifer* Tajimi, 13, *B. australiensis* Cg151-1, 14, *B. australiensis* Cg148-2, 15, *B. heveae* Bangka-1, 16, *B. chloridis* Kata-2, 17, *B. chloridis* 760-24, 18, *B. cynodontis* Suzu-tomari-1, 19, *B. cynodontis* Cyn A, 20, *B. bicolor* El1-1, 21, *B. bicolor* Kara 10.11-22, 22, *B. bicolor* Okusa 81.1-2, 23, *B. cookei* Yamashina-1, 24, *B. cookei* HG In-740, 25, *B. zeicola* Tokachi 2-2, 26, *B. zeicola* Shihoro 1-1, 27, *B. zeicola* 29R-2, 28, *B. zeicola* Race-1, 29, *B. victoriae* HV2, 30, *B. victoriae* HV3, 31, *B. sorokiniana* Hadaka hirai, 32, *B. sorokiniana* Inumugi, 33, *B. sorokiniana* Kusakbi daigo, 34, *B. papendorfii* D-1, 35, *B. coicis* Daigo-1, 36, *B. coicis* Kawada-1, 37, *B. coicis* 1021-2, 38, *C. lunata* Tef-1, 39, *C. lunata* I73, 1-2, 40, *C. lunata* Shin-1, 41, *C. lunata* var. *aeria* B29-1, 42, *C. lunata* var. *aeria* B29-1 Sec, 43, *C. lunata* var. *aeria* B91-5, 44, *C. lunata* var. *aeria* B30-1, 45, *C. lunata* var. *aeria* I98, 46, *C. lunata* var. *aeria* I54, 47, *C. lunata* var. *aeria* Ohisatuma1-1, 48, *C. gladiolii* Kagoshima-1, 49, *C. gladiolii* Kagoshima-2, 50, *C. gladiolii* Kagoshima-3, 51, *C. gladiolii* Demachi1-1, 52, *C. trifolii* Nasuno 1, 53, *C. trifolii* Nasuno 2, 54, *C. trifolii* Nasuno 2-2, 55, *C. trifolii* Iwate 2, 56, *C. akaii* He5, 57, *C. akaii* Roku2-1. S.M.: Size marker, λ/*Hin*dIII. a. After treatment with *Bam*HI. b. After treatment with *Hin*dIII.

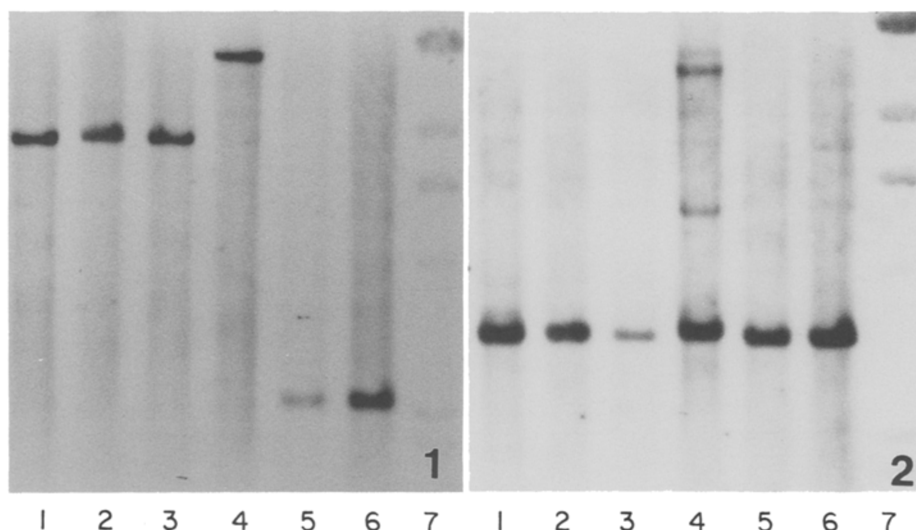

Fig. 2. Banding patterns of Southern blots of the total DNAs isolated from *B. hawaiiensis, B. australiensis* and *B. spicifer*, probed with *B. maydis* genomic clone 55B130.
Lane: 1, *B. hawaiiensis* B1-1, 2, *B. hawaiiensis* B1-2, 3, *B. hawaiiensis* B1-3, 4, *B. spicifer* Tajimi, 5, *B. australiensis* Cg151-1, 6, *B. australiensis* Cg148-2, 7, λ/*Hin*dIII.
1. After treatment with *Bam*HI. 2. After treatment with *Hin*dIII.

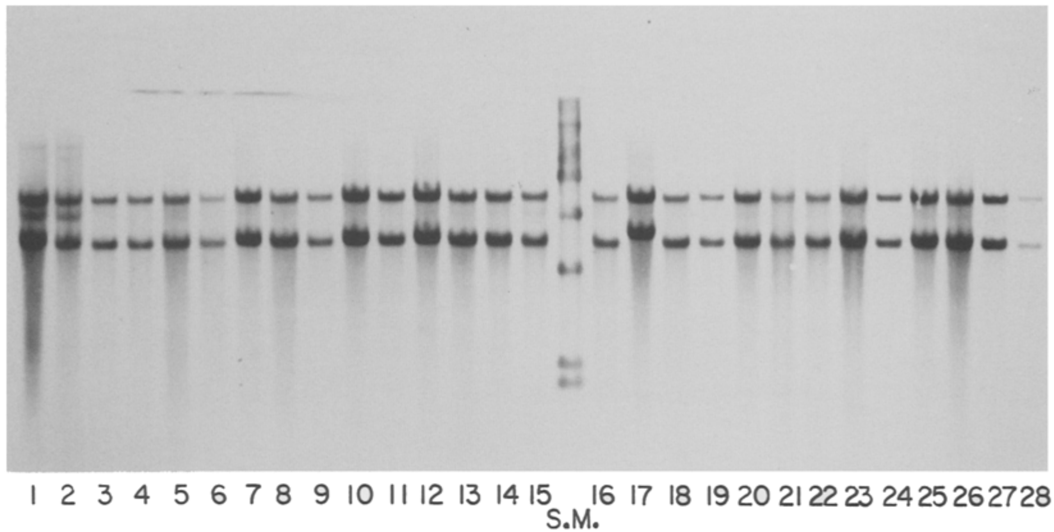

Fig. 3. Test of intraspecific variation of RFLP banding pattern of *B. maydis*. The total DNAs were probed with clone pVW6 (*B. maydis, GPD1*), after treatment with *Bam*HI.
Lane: see Table 2.
S.M.: Size marker, λ/*Hin*dIII.

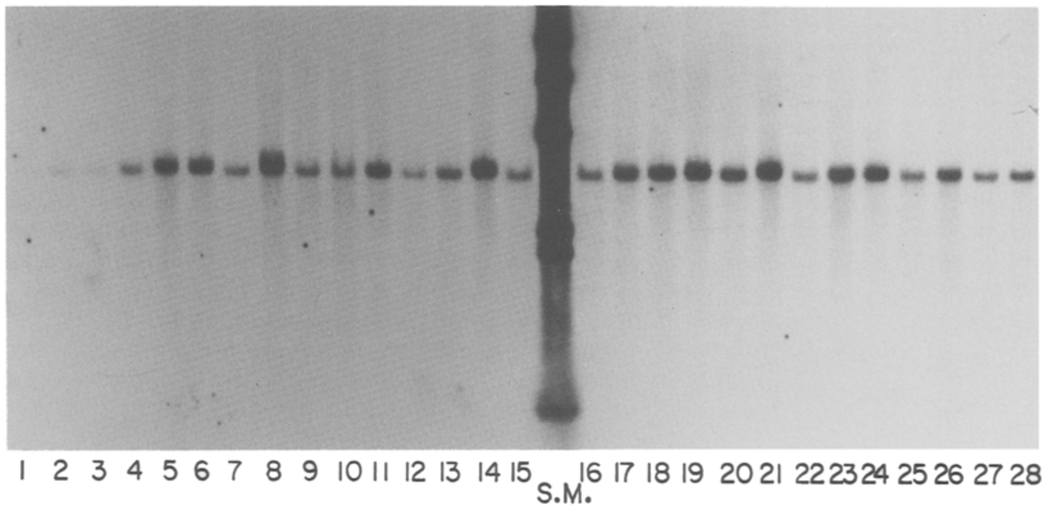

Fig. 4. Test of intraspecific variation of RFLP banding pattern of *B. maydis*. The total DNAs were probed with clone 55B106 (*B. maydis*), after treatment with *Bam*HI.
Lane: see Table 2.
S.M.: Size marker, λ/*Hin*dIII.

Table 3. Distance index* values for *B. bicolor, B. chloridis* and *B. cynodontis*.

	B. bicolor (3)	*B. chloridis* (2)	*B. cynodontis* (2)
B. bicolor	0.09–0.17	0.83–0.92	0.75–0.83
B. chloridis		0.25	0.50–0.59
B. cynodontis			0.09

Numbers of strains used are indicated in parentheses.

$$\text{* Distance index} = 1 - \frac{\text{\# banding patterns common to both strains}}{\text{total \# banding patterns compared}}$$

Table 4. Distance index values for *C. lunata, C. lunata* var. *aeria, C. trifolii, C. galdiolii* and *C. akaii*.

	C. lunata (3)	*C. lunata* var. *aeria* (7)	*C. trifolii* (3)	*C. galdiolii* (4)	*C. akaii* (2)
C. lunata	0.00–0.17	0.89–0.94	0.94–0.94	0.89–0.89	1.00
C. lunata var. *aeria*		0.00–0.17	0.95–1.00	0.89–0.92	1.00
C. trifolii			0.12–0.12	0.73–0.84	1.00
C. gladiolii				0.12–0.12	0.92
C. akaii					0.00

Numbers of strains used are indicated in parentheses.

A formal proposal for a new combination for the species is follows;

Curvularia aeria (Batista, Lima et Vasconcelos) Tsuda, comb. nov.

Illustrations; Tsuda et al., 1985, Trans. Mycol. Soc. Jpn 26: 36–37; Batista et al. 1960, Pub. Inst. Micol. Univ. Recife 263: 7; Ellis, 1966, CAB Mycol. Pap. 106: 34; Sivanesan, 1987, CAB Mycol. Pap. 158: 139.

Basinonym: *Malustela aeria* Batista, Lima et Vasconcelos, Pub. Inst. Micol. Univ. Recife 263: 7

Descriptions and other synonyms: indicated in Ellis (1966) and Sivanesan (1987).

Unfortunatley, morphological plans for separation of species in these fungal groups have been arbitrarily chosen for convenience, and their numbers are restricted. Though species separation using morphological characters and pathogenicity towards several plant pathogenic species allows routine estimation of biological species in both genera, these conventional methods suffer from several disadvantages. This is illustrated by the excessive proposal of new species differing only in showing slight variations in conidial morphology (Tsuda and Ueyama, 1987). Such differences may be explained by, e.g., the use of different sets of environmental conditions for conidiation. Even under controlled conditions, however, individual differences between isolates make decision difficult. Second, mating experiments are burdensome, even if one has isolates with enough mating ability. Third, the diagnostic features are difficult to describe objectively in the literature, which is the fundamental base of modern science. These obstacles have, thus far, prevented any meaningful comparison between isolates from different origins.

With the advent of DNA fingerprinting, many of these problems can be overcome. Fortunately, the RFLP of DNA is specific to a given clone, isolate, variant, species, and genus, with suitably chosen probe-enzyme combination(s). Thus we can use these combinations as a taxonomic plan. The numbers of probe-enzyme combinations are virtually unlimited.

By surveying other fragment probes or using a wider array of restriction enzymes, it should be possible to identify diagnostic RFLPs for fingerprinting of isolates, species, genera or other taxa. The potential of this technology is demonstrated by the present results, which allow sound species separation in the genera used in this study. The next problems are to elucidate typical RFLPs for specific species in these fungi and to consider whether it is better to separate or to combine the two genera.

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