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Restriction Enzyme Analysis of Mitochondrial DNA of the *Aspergillus flavus* group: *A. flavus*, *A. parasiticus*, and *A. nomius*

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Mitochondrial DNA restriction fragment length polymorphisms were identified that clearly distinguish *Aspergillus flavus*, *A. parasiticus*, and *A. nomius*. Mitochondrial DNAs of *A. flavus* and *A. parasiticus* were found to be circular, and their size was estimated size to be 32 kilobases. A restriction map was constructed for the mitochondrial genome of an *A. parasiticus* isolate by using four restriction endonucleases. Four genes tested were found to have the same order as in the mitochondrial genome of *A. nidulans*. The mitochondrial genome of *A. nomius* was estimated to be 33 kilobases.

The taxonomy of the *Aspergillus* Section *Flavi*, commonly referred to as the *Aspergillus flavus* group of fungi, is controversial (21). Members of this group include isolates used in Oriental food fermentations, *A. oryzae* (Ahlb.) Cohn and *A. sojae* Sakaguch & Yamada, and isolates that spoil food, *A. tamarii* Kita, *A. flavus* Link ex Fries, *A. parasiticus* Speare, and *A. nomius* Kurtzman and Hesseltine. *A. flavus*, *A. parasiticus*, and *A. nomius* are of considerable agricultural importance because of the ability of some isolates to produce aflatoxins (22, 25). Aflatoxin B_1 is the most carcinogenic natural product known. The economic importance of these fungi has prompted a search for characters that unequivocally identify individual members of this group (21, 22, 26).

Mitochondrial DNA (mtDNA) sequence diversity has been used in a number of studies to differentiate between fungi and to establish relationships between them. Nucleotide differences between individuals can be identified by using restriction endonucleases which cut the DNA at specific recognition sequences. Fragments of different size are generated when individuals differ in their DNA sequence; these are called restriction fragment length polymorphisms (RFLPs). RFLPs of mtDNA have proven to be useful for determining relatedness among the basidiomycetes (1), the oomycetes (14, 15, 28), and the ascomycetes (8, 10, 17, 19, 20, 23, 24, 39, 40, 44).

Kozlowski and Stepien (24) suggested the use of RFLP analysis of mtDNA to assist the resolution of phylogenetic relationships among the 18 "groups" of the genus *Aspergillus*. These 18 groups are not accredited taxa and serve only as convenient categories for distinguishing the strains (13). These authors examined the restriction endonuclease cleavage patterns of mtDNA from single isolates of *A. nidulans* (11) from the *A. nidulans* group, *A. wentii* from the *A. wentii* group, *A. awamori* and *A. niger* from the *A. niger* group, and *A. oryzae* and *A. tamarii* from the *A. flavus* group. Restriction fragment patterns generated by *Eco*RI and *Hin*dIII double digestion were unique for each strain. The success of this limited study revealed the potential for more detailed analysis of the taxonomy of the genus.

The major aim of this study was to identify mitochondrial markers that correlate with distinct morphological and biochemical characters of *A. flavus*, *A. parasiticus*, and *A. nomius*. A second aim was to use restriction endonucleases to investigate the sequence diversity of these fungi and to use this information to examine relationships between isolates. As part of this study we have established the size of the mitochondrial genome of these fungi and the gene order of four genes within the genome. We investigated 16 isolates of *A. flavus*, 17 isolates of *A. parasiticus*, and a 1 isolate of *A. nomius* that were collected from 14 countries, as well as 23 *A. flavus* isolates and 1 *A. nomius* isolate that were collected from southern India.

MATERIALS AND METHODS

Fungal isolates and growth conditions. The fungal isolates used in these studies are described in Table 1. International (Intl) isolates 1 to 36 were selected to represent diverse geographic origins, morphology, and mycotoxin production. The Commonwealth Scientific and Industrial Research Organisation Division of Food Processing isolates were kindly supplied and characterized morhpologically by J. I. Pitt, Commonwealth Scientific and Industrial Research Organisation, North Ryde, New South Wales, Australia. An unusual nonaflatoxigenic *A. parasiticus* isolate (CP-461) was generously provided by R. J. Cole, U.S. Department of Agriculture, Agricultural Research Service National Peanut Research Laboratory, Dawson, Ga. The Indian isolates, 1 to 25, were from a variety of sources within southern India and were collected at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT). Indian field isolates were sampled from soil and plant material by plating onto A. flavus and parasiticus (AFPA) medium (34), which selects for fungi of the *A. flavus* group. Cultures were maintained on malt extract agar at 30°C for 8 to 12 days.

Isolation of aflatoxins. Aflatoxin production was assessed after 8 days of growth on a semisynthetic liquid medium (9). Aflatoxins B_1, B_2, G_1, and G_2 were assayed by thin-layer chromatography (35).

Nucleic acid isolation from *Aspergillus* species. Mycelial mats for DNA extraction were prepared from conidia that had been grown on malt extract agar for 7 to 8 days at 30°C and had been dispersed into a liquid suspension by using

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TABLE 1. Origins and characteristics of the *Aspergillus* isolates used in this study

Identification		Geographical origin		Source[a]	Culture no.[b]	Aflatoxin produced	Sclerotia production
Label	Species	Region	Country (area)				
Intl							
1	*A. flavus*	Oceania	Solomon Islands	Shoe	FRR 3354	B_1	?
2	*A. parasiticus*	Americas	USA (Georgia)	Mutant (brown)	FRR 2570	B_1, G_2	?
3	*A. flavus*	Africa	Botswana	Sunflower seed	FRR 3384		High
4	*A. flavus*	Oceania	Australia (Sydney)	Indonesian dried fish	FRR 3240	B_1	?
5	*A. flavus*	Africa	South Africa (Pretoria)	Mutant (white)	FRR 2675	B_1	?
6	*A. flavus*	Americas	USA (California)	Peanut	FRR 3340	B_1	?
7	*A. flavus*	Asia	Japan	Buckwheat seed	FRR 3344	B_1	−
8	*A. parasiticus*	Oceania	Australia (Queensland)	Peanut	FRR 2747	B_1, G_2	−
9	*A. flavus*	Africa	South Africa	Sorghum grain	FRR 3369		−
10	*A. parasiticus*	Americas	USA		CP-461		?
11	*A. parasiticus*	Americas	USA (Texas)	Cottonseed	FRR 3282	B_1, G_2	−
12	*A. parasiticus*	Oceania	Australia (Queensland)	Peanut	FRR 2744	B_1, G_2	?
13	*A. flavus*	Africa	South Africa	Barley	FRR 3371	B	?
14	*A. flavus*	Africa	Tanzania	Peanut	FRR 3351 (IMI 39178a)	B	?
15	*A. flavus*	Africa	Nigeria	Peanut	FRR 3353	−	?
16	*A. nomius*	Americas	USA	Wheat	FRR 3339 (ATCC 15546)	B, G	?
17	*A. flavus*	Americas	USA	Peanut	FRR 3340	G?	?
18	*A. flavus*	Americas	USA	Black pepper	FRR 3342		?
19	*A. flavus*	Oceania	Australia (Queensland)	Soil	FRR 2751		?
20	*A. flavus*	Oceania	Australia (Queensland)	Peanut	FRR 2755		?
21	*A. flavus*	Oceania	South Pacific	Cellophane	FRR 2807 (IMI 124930)	?	?
22	*A. flavus*	Oceania	New Zealand	Corn	FRR 2878		?
23	*A. flavus*	Asia	Indonesia	Dried fish	FRR 3057	B	?
24	*A. flavus*	Europe	Turkey	Hazel nut	FRR 3299		?
25	*A. parasiticus*	Africa	Uganda	Peanut	FRR 2999 (NRRL 2999)	B, G	?
26	*A. parasiticus*	Africa	Mozambique	Corn	FRR 3372	B, G	?
27	*A. parasiticus*	Africa	South Africa	Sunflower seed	FRR 3385	B, G	?
28	*A. parasiticus*	Americas	USA (Hawaii)	Insect	FRR 2806 (IMI 15957)	B, G	?
29	*A. parasiticus*	Americas	USA	Soil	FRR 3281	B, G	?
31	*A. parasiticus*	Americas	USA	Peanut	FRR 3347	B, G	?
32	*A. parasiticus*	Americas	USA	Rice	FRR 3348	B, G	?
33	*A. parasiticus*	Oceania	Australia (Queensland)	Soil	FRR 2753	B, G	?
34	*A. parasiticus*	Europe	Poland	Sausage	FRR 3346	B, G	?
35	*A. parasiticus*	Africa	Libya	Soil	FRR 3358	B, G	?
36	*A. parasiticus*	Africa	Uganda	Peanut	FRR 3373	B, G	?
Indian							
1	*A. flavus*	Asia	India	Peanut seed (F1-5× NCAC 17090) Bapatla (1984)		B	−
2	*A. flavus*	Asia	India	Red soil ICRISAT (1987)		B	+
3	*A. nomius*	Asia	India	Red soil ICRISAT (1987)		B, G	+
4	*A. flavus*	Asia	India	Black soil ICRISAT (1987)		B	−
5	*A. flavus*	Asia	India	Red geocarposphere soil of peanut (TMV2) ICRISAT (1987)		B	+
6	*A. flavus*	Asia	India	Red geocarposphere soil of peanut (TMV2) ICRISAT (1987)		B	+
7	*A. flavus*	Asia	India	Red geocarposphere soil of peanut (TMV2) ICRISAT (1987)		B	+

8	*A. flavus*	Asia	India	Red geocarposphere soil of peanut (TMV2) ICRISAT (1987)	B	−
9	*A. flavus*	Asia	India	Red geocarposphere soil of peanut (EC 76446 (292) (ICRISAT(1987)		+
10	*A. flavus*	Asia	India	Peanut seed (EC 76446 (292)) ICRISAT (1987)		+
11	*A. flavus*	Asia	India	Pigeon pea seed ICRISAT (1987)	B	−
12	*A. flavus*	Asia	India	Stock culture Af-8-3-2A ICRISAT	B	−
13	*A. flavus*	Asia	India	Red geocarposphere soil of peanut (PI 337394F) ICRISAT (1987)	B	+
14	*A. flavus*	Asia	India	Red geocarposphere soil of peanut (J 11) ICRISAT (1987)	B	+
15	*A. flavus*	Asia	India	Peanut seed (EC 76446 (292)) Tirupati (1987)	B	+
16	*A. flavus*	Asia	India	Peanut seed (EC 76446 (292)) ICRISAT (1987)	B	+
17	*A. flavus*	Asia	India	Peanut seed (TMV 2) Bapatla (1986)	B	−
18	*A. flavus*	Asia	India	Peanut seed (PI 337394F) ICRISAT (1987)	B	−
19	*A. flavus*	Asia	India	Peanut seed (JL 24) ICRISAT (1987)	B	−
21	*A. flavus*	Asia	India	Pearl millet ICRISAT (1987)	B	+
22	*A. flavus*	Asia	India	Peanut pod (PI 337394F) ICRISAT (1987)	B	+
23	*A. flavus*	Asia	India	Sorghum grain ICRISAT (1987)	B	−
24	*A. flavus*	Asia	India	Peanut, pigeon field soil Jogipet (1987)	B	−
25	*A. flavus*	Asia	India	Pigeon pea seed ICRISAT (1987)	B	−

[a] Peanut genotypes include the following: F1-5× NCAC 17090; TMV 2; J II; EC 76446 (292); PI 337394 F; JL 24.
[b] FRR, CSIRO Division of Food Processing, North Ryde, Australia, culture collection; CP, National Peanut Research Laboratory, Dawson, Ga., culture collection; IMI, CAB International Mycological Institute, Kew, England, culture collection; NRRL, isolates of the U.S. Department of Agriculture Northern Regional Research Center, Peoria, Ill., culture collection; ATCC, American Type Culture collection, Rockville, Md.

Tween 20. The conidial suspensions were inoculated into 2-liter flasks containing 800 ml of malt extract broth. The suspensions containing 10^5 to 10^6 conidia per ml were shaken at 300 rpm for 24 h at 30°C. The mycelial mats were collected by vacuum filtration through Miracloth and frozen at −80°C. Mycelia (20 to 40 g [wet weight]) were frozen in liquid nitrogen and ground with a mortar and pestle to a fine frozen powder. The powder was suspended in a minimum volume of extraction buffer (20 mM Tris hydrochloride [pH 8.5], 250 mM NaCl, 25 mM EDTA [pH 8.0], 0.5% sodium dodecyl sulfate [SDS]). DNA was extracted by using procedures modified from those of Raeder and Broda (36). The slurry was homogenized in extraction buffer-phenol-chloroform (10:3:7) for 30 min at 75 rpm to extract protein. The suspension was centrifuged for 1 h at 9,000 rpm. The upper, aqueous phase was removed, added to 0.54 volume of isopropanol, and stored overnight at −20°C. The precipitated DNA and RNA were pelleted for 30 min at 3,000 rpm. The pellets were rinsed in 70% ethanol and suspended in 9 ml of 10 mM Tris–1 mM EDTA–1.2 g of CsCl per ml–3 μg of bisbenzimide per ml.

Nuclear DNA, ribosomal DNA, mtDNA, and RNA were separated by centrifuging in bisbenzimide gradients (16) at 40,000 rpm for 48 h, and DNA bands were removed under UV light (366 nm). The dye was removed from the isolated fractions by extraction three times in an equal volume of saturated isobutanol solution. The samples were precipitated with ethanol and suspended in 10 mM Tris–1 mM EDTA.

Restriction enzyme digestion. Restriction endonucleases used in this study include *Ase*I (ATTAAT), *Cla*I (ATCGAT), *Dra*I (TTTAAA), *Eco*RI (GAATTC), *Hin*dIII (AAGCTT), *Hin*fI (GANTC), *Nsi*I (ATGCAT), *Pvu*II (CAGCTG), *Rsa*I (GTAC), *Xba*I (TCTAGA), and *Xho*I (CTCGAG). DNA was incubated with a twofold excess of endonuclease (3 U/μg of DNA) at 37°C (65°C for *Taq*I) for 16 h to ensure complete digestion; the buffers recommended by the manufacturers were used. Reactions were terminated by adding 5× loading buffer (0.1% SDS, 75 mM EDTA, 50% glycerol, 0.1% bromophenol blue) or by adding EDTA (to give a final concentration of 20 mM), precipitating the DNA, and suspending it in 1× loading buffer.

Agarose gel electrophoresis. Agarose gel electrophoresis was used to separate restriction fragments ranging in size from 400 base pairs (bp) to 40 kilobase pairs (kb). DNA samples that had been digested with restriction endonucleases were fractionated by electrophoresis in 0.8% agarose gels and run in Tris-acetate-EDTA buffer (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA [pH 7.8]) at 80 mA for 3 to 4 h.

TABLE 2. mtDNA fragments from petite mutants of *S. cerevisiae* used as probes for hybridization

Probe	Origin and description of mtDNA gene fragments
E3	305–1,958-bp fragment containing the 5′ end of LrRNA gene (41)
P2	5-kb fragment of SrRNA gene (41)
DS6/A422	5.3-kb fragment containing exons 3–7 or 8 from cytochrome *c* oxidase subunit I gene (3)
DS31	4.5-kb framgent containing cytochrome *c* oxidase III gene (43)
DS400/NI	431-bp fragment containing exon 1 from apocytochrome *b* gene (32)
DS14	4.1-kb fragment containing ATPase subunit 6 gene (27)
pScm5	704-bp fragment containing 5′ terminus of cytochrome *c* oxidase subunit II gene (6)

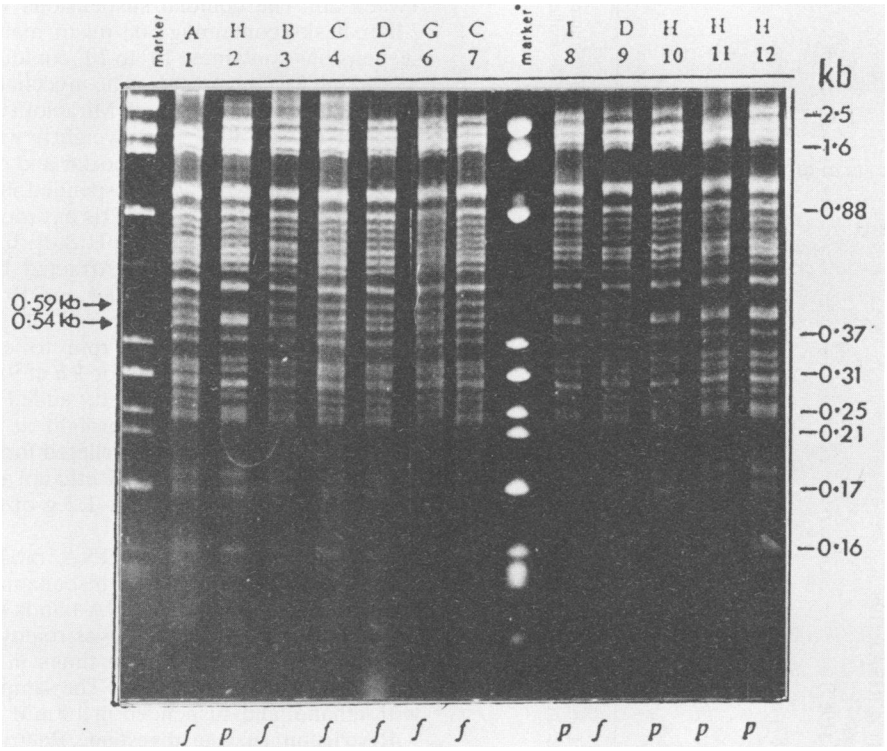

FIG. 1. Restriction fragment patterns of mtDNAs of *Aspergillus* Section *Flavi* isolates *A. flavus* (*f*) and *A. parasiticus* (*p*), digested with *Hin*fI and fractionated by polyacrylamide gel electrophoresis. The isolates shown (Intl isolates 1 through 12, corresponding to the isolates in Table 1) represent 7 of the 13 different RFLP patterns (A through M) that were observed among the *Hin*fI-digested Intl isolates 1 through 36 and Indian isolates 1 through 25 (summarized in Tables 3 and 4). The arrows indicate species-specific polymorphic bands. The 0.59-kb band (in the absence of the 0.49- and 0.48-kb bands which are characteristic of *A. nomius* isolates) is present only in *A. flavus* isolates. The 0.54-kb doublet is present only among *A. parasiticus* isolates. *Hae*III-digested phage M13mp19 DNA was used as a size marker either alone (lane marker*) or together with *Hin*dIII-digested phage λ DNA (lane marker). From top to bottom, the sizes of the phage M13mp19 bands are 2.5, 1.6, 0.88, 0.37, 0.31 (doublet), 0.25, 0.21, 0.17, 0.16, 0.12, 0.11, and 0.10 kb. Sizes of marker fragments are indicated at the right of the figure.

Bacteriophage lambda DNA digested with *Hin*dIII and phage M13mp19 DNA digested with *Hae*III were used as size markers. The gels were stained with ethidium bromide (1 μg/ml) and destained with distilled water, and the DNA bands were visualized on a UV transilluminator and photographed.

Polyacrylamide Gel Electrophoresis of mtDNA. mtDNA fragments ranging in size from 20 to 700 bp, generated by the restriction endonucleases *Ase*I, *Dra*I, *Hin*fI, and *Rsa*I, were fractionated by electrophoresis in 5% polyacrylamide gels (33) and run in Tris-borate-EDTA (TBE) buffer (50 mM Tris, 42 mM boric acid, 1 mM EDTA [pH 8.0]) at 100 V for 2 to 3 h. Separation of DNA fragments was improved by the addition of sodium acetate (50 mM) to the lower gel tank buffer. The gels were stained and photographed by following the same procedure used for agarose gels.

Hybridization probes. *Saccharomyces cerivisiae* petite mutants used for mapping the mitochondrial genomes are listed in Table 2. Radioactively labeled hybridization probes were prepared by random-primed synthesis of DNA, using *Escherichia coli* DNA polymerase I (Klenow fragment) (12).

Hybridization conditions. mtDNA was examined by Southern blot hybridization analysis (42). mtDNA that had been fractionated on agarose gels was transferred onto PAL Biodyne nylon membrane, and the DNA was fixed by baking, as specified by the manufacturer. Membranes were wetted briefly in preheated (65°C) hybridization solution (3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% SDS, 0.05 g of skim milk powder per liter) (modified from reference 37). Membrane-bound mtDNA fragments were hybridized to denatured probe (boiled for 5 min) in a minimum volume of hybridization solution in sealed plastic bags at 50°C. After hybridization, bound membranes were washed in 3× SSC–1% SDS twice for 30 min each at 45°C. Following washing, the membranes were blotted but not allowed to dry before exposure to Kodak X-Omat TM AR GBX-2 diagnostic X-ray film for 12 h to 5 days at −80°C with intensifier screens.

Membrane regeneration. The [^{32}P]DNA probes were removed from the membranes before reprobing by microwaving at the maximum setting in 0.1% SDS–0.1× SSC for 5 min. Filters were stored wet in 2× SSC prior to reprobing.

Analysis of data. The DNA relatedness between isolates was assessed by the method of Nei and Li (30). Enzyme-digested mtDNAs were separated side by side on gels, and restriction patterns were compared. Fragments which migrated the same distance during electrophoresis were considered to be identical, although in some cases the fragments may have been unrelated. The proportion of fragments in common was used to estimate the proportion of nucleotide base substitutions per nucleotide position and was calculated as a nucleotide sequence divergence value (p), $p = (-\ln F)/r$, where p is an estimate of the proportion of nucleotide base substitutions per nucleotide position, r is the number of nucleotide base pairs for the restriction endonuclease recognition site (31), and F is the proportion of DNA fragments

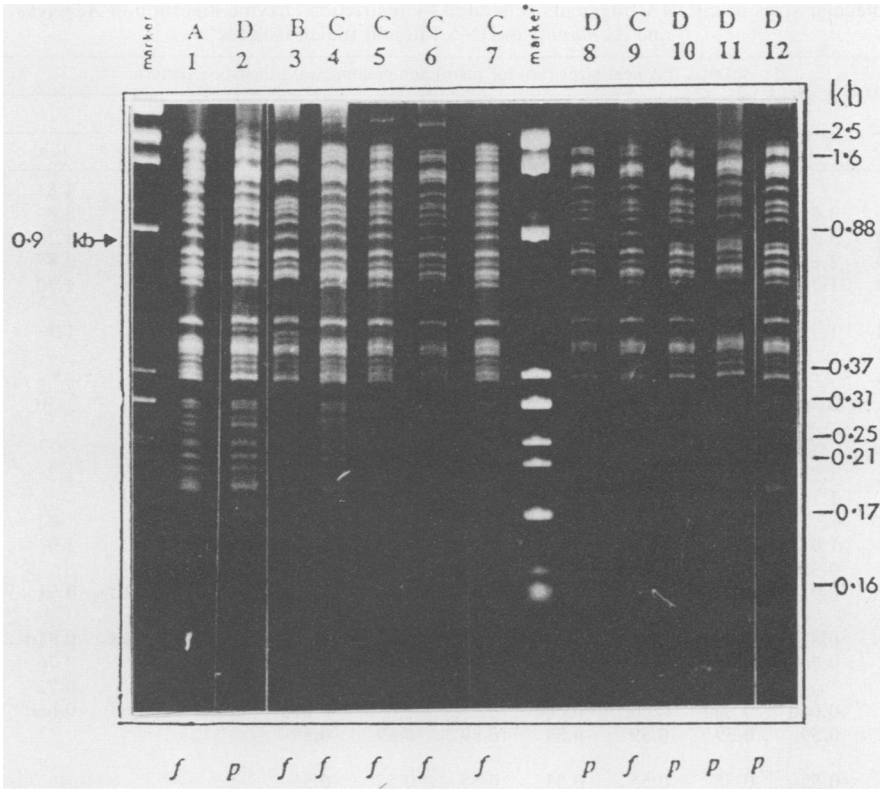

FIG. 2. Restriction fragment patterns of mtDNAs of *Aspergillus* Section *Flavi* isolates *A. flavus* (*f*) and *A. parasiticus* (*p*), digested with *Rsa*I and fractionated by polyacrylamide gel electrophoresis. The isolates (Intl isolates 1 through 12, corresponding to the isolates in Table 1) represent four of the seven different RFLP patterns (A through G) that were observed among the *Rsa*I-digested Intl isolates 1 through 36 (summarized in Tables 3 and 4). The arrows indicate species-specific polymorphic bands. The 0.9-kb band is present only in *A. flavus* isolates. *Hae*III-digested phage M13mp19 DNA was used as a size marker either alone (lane marker*) or together with *Hin*dIII-digested phage λ DNA (lane marker). From top to bottom the sizes of the phage M13mp19 bands are 2.5, 1.6, 0.88, 0.37, 0.31 (doublet), 0.25, 0.21, 0.17, 0.16, 0.12, 0.11, and 0.10 kb. Sizes of marker fragments are indicated at the right of the figure.

shared by any two strains and is estimated from RFLP data, $\hat{F} = 2n_{xy}/(n_x + n_y)$, where n_{xy} is the number of fragments in common between two isolates and n_x and n_y are the total numbers of fragments displayed by each isolate (30).

For each restriction endonuclease, F values were determined for each pair of fungal isolates. F values were allocated into one of two groups according to the number of base pairs recognized by the restriction endonucleases that were used to digest the DNA (i.e., $r = 4$ or $r = 6$). Individual p values were calculated from each of these F values. Individual p values were weighted according to the number of mtDNA fragments generated by each restriction endonuclease, and a mean p value was determined for each pair of isolates. This mean p value is probably related to the time since two organisms shared a common ancestor; a smaller p value indicates a shorter elapsed time (15). Dendrograms were constructed from the genetic distance data by unweighted pair group method arithmetic average clustering (UPGMA) analysis with the NT-SYS program (38).

RESULTS

Identification of mtDNA RFLPs. mtDNAs from Intl isolates 1 through 12 were digested with 11 restriction endonucleases. RFLPs were detected among these isolates when mtDNAs were digested with *Hin*fI (Fig. 1), *Rsa*I (Fig. 2), *Ase*I, and *Dra*I. No RFLPs were generated among these isolates when the mtDNA fractions were digested with *Cla*I, *Eco*RI, *Hin*dIII, *Nsi*I, *Pvu*II, *Xba*I, or *Xho*I.

Fragment sizes generated by *Hin*fI, *Rsa*I, *Ase*I, and *Dra*I were determined for each isolate, and a limited number of restriction enzyme fragmentation patterns were identified among these isolates. The fragment sizes which define the observed patterns are recorded in Table 3, and the isolates associated with these patterns are recorded in Table 4. Isolates were clearly differentiated into *A. flavus* and *A. parasiticus* groupings on the basis of their DNA fragmentation patterns when digested with these enzymes.

The ability of *Hin*fI and *Rsa*I to consistently differentiate *A. flavus*, *A. parasiticus*, and *A. nomius* was tested by using a more extensive range of isolates: Intl isolates 13 through 36. *A. flavus* and *A. parasiticus* isolates consistently generated RFLPs that distinguished between these fungi (Tables 3 and 4). *A. nomius* Intl isolate 16 generated a unique fragmentation pattern when digested with these enzymes (Tables 3 and 4). A further selection of isolates, Indian isolates 1 through 25, were also digested with *Hin*fI. All the Indian *A. flavus* isolates exhibited RFLPs peculiar to this species (Tables 3 and 4). *A. nomius* Indian isolate 3 generated a unique fragmentation pattern (Tables 3 and 4).

Isolates that produced identical fragmentation patterns were evaluated for biochemical and provenance similarities. None of the pattern groupings correlated with the ability of isolates to produce mycotoxin. RFLPs generated by *Hin*fI

TABLE 3. Molecular sizes of mtDNA fragments generated by restriction enzyme digestion of *A. flavus*, *A. parasiticus*, and *A. nomius* mtDNA Intl and Indian isolates[a]

mtDNA fragment sizes (kb) for restriction endonuclease digestion pattern:														
*Eco*I			*Hin*fI											
A (*f*, *p*)[b]	B (*n*)[b]	C (*p*)	A (*f*)	B (*f*)	C (*f*)	D (*f*)	E (*f*)	F (*f*)	G (*f*)	H (*p*)	I (*p*)	J (*p*)	K (*p*)	L (*n*)	M (*n*)
	10			4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.8
9.5	9.5		3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8
9.0		9.0	3.2d[c]	3.2d	3.2d	3.2d	3.2d	3.2d	3.2d	3.2d	3.2d	3.2d	3.2d	3.2d	3.2d
7.0	7.0	7.0	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8
		7.2	1.7d	1.7d	1.7d	1.7d	1.7d	1.7d	1.7d	1,7d	1.7d	1.7d	1.7d	1.7d	1.7d
	2.95														1.6
2.8		2.8	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
1.8	1.8	1.8												1.45	1.45
1.3	1.3	1.3					1.32	1.32		1.32			1.32		
			1.3d	1.3d	1.3d	1.3d	1.3	1.3	1.3d	1.3	1.3d	1.3d	1.3	1.3	1.3d
								1.23						1.23	1.23
														1.12	
			1.2	1.2	1.2	1.2	1.2		1.2	1.2	1.2	1.2	1.2		1.2
			1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1				1.1
												1.02	1.02	1.02d	
			1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0		
			0.95					0.95	0.95				0.95		0.95
			0.91	0.91d	0.91	0.91d	0.91d	0.91	0.91	0.91d	0.91d	0.91		0.91d	0.91d
					0.87								0.87		
			0.81d	0.81d	0.81d	0.81d	0.81d	0.81	0.81d	0.81d	0.81d	0.81d		0.81d	
			0.76	0.76	0.76	0.76	0.76	0.76	0.76	0.76	0.76	0.76	0.76	0.76d	0.76
												0.72			
			0.66d	0.66d	0.66d	0.66d	0.66d	0.66d	0.66d	0.66d	0.66d	0.66d	0.66d	0.66d	
			0.59	**0.59**	**0.59**	**0.59**	**0.59**	**0.59**	**0.59**					**0.59**	**0.59**
															0.58
			0.55	0.55	0.55	0.55	0.55	0.55	0.55					0.55	0.55
			0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54
										0.537	**0.537**	**0.537**	**0.537**		
														0.49	0.49
														0.48	0.48
			0.47	0.47	0.47	0.47	0.47	0.47	0.47	0.47	0.47	0.47	0.47		
			0.43	0.43	0.43	0.43	0.43	0.43	0.43	0.43	0.43	0.43	0.43		
			0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41
														0.39	0.39
			0.38d	0.38d	0.38d	0.38d	0.38d	0.38d	0.38d	0.38d	0.38d	0.38d	0.38d	0.38	0.38
			0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37		
										0.35	0.35				
			0.33d	0.33d	0.33d	0.33d	0.33d	0.33d	0.33d	0.33d	0.33d	0.33d	0.33d		
														0.31	0.31
			0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32		
														0.29	0.29
														0.27	0.27
			0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24		
			0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22		
			0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15		
			0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13		

[a] Patterns as recorded in Table 4.
[b] Abbreviations: *f*, *A. flavus*; *p*, *A. parasiticus*; *n*, *A. nomius*.
[c] d indicates a doublet (two bands of indistinguishable size).
[d] Boldface type indicates species-specific fragments.

digestion showed some associations with geographical origin of the *A. flavus* isolates. Patterns A, B, D, and E (Tables 3 and 4) were not found in Indian isolates. Patterns C and G were found predominantly in Indian isolates and represent 8 and 13 of the 23 Indian isolates, respectively (Table 5). These differences are highly significant ($\chi^2_{df = 6} = 24.42$; $P < 0.005$).

To examine the possibility that these mtDNA RFLPs were generated as a consequence of size variation, we digested mtDNA of these isolates with *Eco*RI (Fig. 3). *Eco*RI cuts the mitochondrial genome of these fungi into a small number of fragments. The *Eco*RI patterns were identical for all *A. flavus* and *A. parasiticus* isolates examined, with the single exception of *A. parasiticus* Intl isolate 27. However, *A. nomius* Intl isolate 16 and Indian isolate 3 generated identical restriction patterns, which were different from those of the other isolates.

The fragmentation pattern for Intl isolate 16 and Indian isolate 3 is consistent with a 1-kb insertion into the 9-kb *Eco*RI fragment and a 0.15-kb insertion into the 2.8-kb *Eco*RI fragment. The number of *Eco*RI sites, however, has been conserved. The size of the *A. nomius* mitochondrial genome was therefore estimated to be 33 kb.

Assessing genetic relationships. The proportion of frag-

TABLE 3—*Continued*

*Rsa*I							*Dra*I						*Ase*I			
A (*f*)	B (*f*)	C (*f*)	D (*p*)	E (*p*)	F (*p*)	G (*n*)	A (*f*)	B (*f*)	C (*f*)	D (*f*)	E (*p*)	F (*p*)	A (*f*)	B (*f*)	C (*f*)	D (*p*)
	2.5	2.5					2.4	2.4	2.4	2.4	2.4	2.4		1.48	1.48	1.48
2.4	2.4	2.4	2.4			2.4						2.0			1.23	
2.3	2.3	2.3	2.3	2.3	2.3	2.3	1.66	1.66	1.66	1.66	1.66	1.66				**1.19**[d]
1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.62	1.62	1.62	1.62	1.62		1.12d	1.12	1.12	**1.12d**
1.6	1.6	1.6	1.6	1.6	1.6	1.6						1.51	1.07			
1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.45	1.45	1.45	1.45	1.45	1.45	1.02	**1.02d**	1.02	1.02
						1.43	1.32	1.32	1.32	1.32	1.32		**0.98**	**0.98**	**0.98**	0.98
1.4	1.4	1.4	1.4	1.4	1.4	1.4						1.29	**0.93**		**0.93**	
	1.3						1.15	1.15	1.15	1.15	1.15	1.15	0.85	0.85	0.85	0.85
1.2		1.2	1.2	1.2	1.2	1.2				1.12			0.79	0.79	0.79	0.79
	1.16						1.10	1.10	1.10	1.10	1.10		0.71	0.71	0.71	0.71
1.12	1.12	1.12	1.12	1.12	1.12						**1.02**	**1.02**	0.68	0.68	0.68	0.68
1.08	1.08	1.08	1.08	1.08	1.08	1.08	0.95	0.95	0.95	0.95	0.95		0.59	0.59	0.59	0.59
0.9	**0.9**	**0.9**									0.92		0.56	0.56	0.56	0.56
			0.83	**0.83**	**0.83**	**0.83**	0.89	0.89	0.89	0.89	0.89	0.89	0.53	0.53	0.53	0.53
				0.82			0.78			0.78		0.78		0.50	0.50	0.50
0.79d	0.79d	0.79d	0.79d		0.79d	0.79d	0.77	0.77	0.77	0.77	0.77	0.77	0.49	0.49	0.49	0.49
				0.76			0.76	0.76	0.76	0.76	0.76	0.76	0.48	0.48	0.48	0.48
0.75	0.75	0.75	0.75	0.75	0.75	0.75		0.74	0.74				0.45	0.45	0.45	0.45
0.71d	0.71d	0.71d	0.71d	0.71d	0.71d	0.71	0.62	0.62	0.62	0.62	0.62d	0.62	0.42	0.42	0.42	0.42
0.66	0.66	0.66	0.66	0.66	0.66	0.66	0.6d	0.6d	0.6d	0.6d	0.6	0.6	0.37	0.37	0.37	0.37
0.62		0.62	0.62	0.62	0.62		0.56	0.56	0.56	0.56	0.56d	0.56d	0.36	0.36	0.36	0.36
0.49d	0.49d	0.49d	0.49d	0.49d	0.49d	0.49d	0.52	0.52	0.52	0.52	0.52	0.52	0.34	0.34	0.34	0.34
0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.48	0.48	0.48	0.48	0.48	0.48	0.32	0.32	0.32	0.32
0.44d	0.44d	0.44d	0.44d	0.44d	0.44d	0.44d	0.46	0.46	0.46	0.46		0.46	0.31	0.31	0.31	0.31
0.43d	0.43d	0.43d	0.43d	0.43d	0.43d	0.43d	0.43	0.43	0.43	0.43	0.43	0.43	0.26	0.26	0.26	0.26
0.39	0.39	0.39	0.39	0.39	0.39	0.39		0.39			0.39		0.23	0.23	0.23	0.23
0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.40		0.40	0.40		0.40	0.25	0.25	0.25	0.25
0.37d	0.37d	0.37d	0.37d	0.37d	0.37d	0.37d	0.38		0.38	0.38			0.22	0.22	0.22	0.22
0.33	0.33	0.33	0.33	0.33	0.33	0.33					0.34	0.34	0.21	0.21	0.21	0.21
0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.33	0.33	0.33	0.33	0.33	0.33	0.209	0.209	0.209	0.209
0.31	0.31	0.31	0.31	0.31	0.31	0.31	0.30	0.30	0.30	0.30	0.30	0.30	0.20	0.20	0.20	0.20
0.29d	0.29d	0.29d	0.29d	0.29d	0.29d	0.29d	0.28	0.28	0.28	0.28	0.28	0.28	0.17	0.17	0.17	0.17
0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.26	0.26	0.26	0.26	0.26	0.26	0.165	0.165	0.165	0.165
0.27d	0.27d	0.27d	0.27d	0.27d	0.27d	0.27d	0.25	0.25	0.25	0.25	0.25	0.25	0.162	0.162	0.162	0.162
0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.21	0.21	0.21	0.21	0.21	0.21	0.15	0.15	0.15	0.15
0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.19	0.19	0.19	0.19	0.19	0.19	0.14	0.14	0.14	0.14
0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.17	0.17	0.17	0.17	0.17	0.17	0.13	0.13	0.13	0.13
0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.16	0.16	0.16	0.16	0.16	0.16	0.12	0.12	0.12	0.12
0.19	0.19	0.19	0.19	0.19	0.19	0.19							0.11	0.11	0.11	0.11
0.18	0.18	0.18	0.18	0.18	0.18	0.18							0.10	0.10	0.10	0.10
													0.09	0.09	0.09	0.09
													0.08	0.08	0.08	0.08
													0.07	0.07	0.07	0.07

ments shared between each pair of Intl isolates 1 to 12 and the corresponding sequence divergence (*p*) values are presented in Table 6. A dendrogram representing the relationships between these isolates was calculated from the *p* values and is presented in Fig. 4. *A. parasiticus* Intl isolates 8 and 10; *A. parasiticus* Intl isolates 2 and 12; and *A. flavus* Intl isolates 4 and 7 were not differentiated by using these endonucleases. This analysis was not applied to the entire collection because of the considerable labour involved in isolating sufficiently large amounts of mtDNA, uncontaminated by nuclear DNA.

Location of restriction endonuclease cleavage sites in mtDNA. To examine the size, structure, and organization of the mitochondrial genomes of *A. flavus* Intl isolate 1 and *A. parasiticus* Intl isolate 10, the positions of the recognition sites for the restriction endonucleases *Cla*I, *Eco*RI, *Pvu*II, and *Xho*I were determined from single and double digests. These restriction enzymes were chosen because they cleave the mitochondrial genome into a moderate number of fragments (Fig. 5). These two isolates generated identical restriction fragment patterns when digested with these enzymes. The total genome size was calculated by summing the *Pvu*II-generated mtDNA fragments and found to be approximately 32 kb. A restriction endonuclease site map was constructed for these four enzymes. The genome was found to be circular. A linear restriction endonuclease site map was constructed with the origin shown at the *Xho*I site between the genes coding for apocytochrome *b* (*cobA*) and the large ribosomal RNA (LrRNA) (see Fig. 6). This linear map has the 5′→3′ direction of the LrRNA sequence reading from left

TABLE 4. RFLP patterns detected for fungal isolates[a] of *A. flavus*, *A. parasiticus*, and *A. nomius* Intl and Indian isolates

Pattern for *A. flavus* Intl isolate:

Enzyme	1	3	4	5	6	7	9	13	14	15	17	19	20	22	23	24
*Eco*RI	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
*Hin*fI	A	B	C	D	G	C	D	E	E	E	E	E	F	E	E	F
*Rsa*I	A	B	C	C	C	C	C	A	A	A	A	A	A	A	A	A
*Dra*I	A	B	A	A	C	A	D									
*Ase*I	A	B	C	C	C	C	C									

Pattern for *A. flavus* Indian isolate:

Enzyme	1	2	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	21	22	23	24	25
*Eco*RI	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
*Hin*fI	G	G	C	G	F	G	C	F	G	C	C	C	C	C	C	G	G	G	G	G	G	G	G
*Rsa*I																							
*Dra*I																							
*Ase*I																							

Pattern for *A. parasiticus* Intl isolate, *A. nomius* Intl isolate 16 and *A. nomius* Indian isolate 3:

Enzyme	2	8	10	11	12	25	26	27	28	29	30	31	32	33	34	36	*A. nomius* Intl isolate 16	*A. nomius* Indian isolate 3
*Eco*RI	A	A	A	A	A	A	A	C	A	A	A	A	A	A	A	A	B	B
*Hin*fI	H	I	I	I	H	J	I	I	K	I	I	I	I	I	I	I	L	M
*Rsa*I	D	D	D	D	E	F	D	D	D	D	D	D	D	D	D	D	G	
*Dra*I	E	E	E	F	E													
*Ase*I	D	D	D	D	D													

[a] Isolate numbers correspond to those in Table 1.

TABLE 5. Association of *Hin*fI RFLPs with geographical location among *A. flavus* isolates[a]

Country of origin	No. of isolates with pattern[b]: A	B	C	D	E	F	G
India	0	0	8	0	0	2	13
Other countries	1	1	2	2	7	2	1

[a] Data are summarized from Tables 1 and 4. Significance of differences tested by using chi-square analysis (40a) is as follows: $\chi^2_{df=6} = 24.42$, i.e. highly significant.

[b] Patterns as defined in Table 4 and Fig. 1.

to right. The precise orientation to two *Eco*RI sites could not be confirmed by these digestion. The sites represented by dotted lined in Fig. 6 indicate one of the two possible arrangements.

Localization of mitochondrial genes. Seven mitochondrial genes of *A. parasiticus* Intl isolate 10 were localized to mtDNA fragments by Southern blot hybridization with mtDNA fragments from petite mutants of *S. cerevisiae*. Autoradiograms of Southern blot hybridization of two genes coding for cytochrome *c* oxidase subunits I and II (*oxiA* and *oxiB*, respectively) localized within the mitochondrial genome are presented in Fig. 5. The positions of four genes coding for LrDNA, apocytochrome *b* (*cobA*, also localized by Southern blot hybridization), and cytochrome *c* oxidase subunits I and II are presented on the mtDNA map in Fig. 6. A further three genes coding for the ATPase subunit 6 (*oliI*), the small rRNA (SrRNA), and cytochrome *c* oxidase subunit III (*oxiC*) were localized on the three *Eco*RI fragments arranged consecutively between the *cobA* and LrRNA genes in the 5'→3' direction. The failure of any of these restriction enzymes to cut these *Eco*RI fragments, however, prevented unequivocal determination of the order of these fragments (and hence the genes encoded) within the mitochondrial genome.

DISCUSSION

All *A. flavus* and *A. parasiticus* mitochondrial genomes were identical in size and were estimated to be 32 kb. No insertions were identified in the *A. flavus* and *A. parasiticus* mitochondrial genomes. In contrast to *A. flavus* and *A. parasiticus*, *A. nomius* has a slightly larger mitochondrial genome with insertions at two sites.

The size of the mitochondrial genome of members of *A. flavus*, *A. parasiticus*, and *A. nomius* is similar to that of *A. nidulans*, which is 33 to 37 kb (4). These fungal genomes are smaller than most other mtDNAs. Fungal mitochondrial genomes vary in size between 18.9 kb in *Torulopsis glabrata* (7) and 176 kb in *Agaricus bitorquis* (18). These variations are apparently due to deletions and insertions which are collectively termed length mutations. In the yeasts *Dekkera* spp. and *Brettanomyces* spp. (19), and between *A. nidulans* and *A. nidulans* var. *echinulatus* (sexually reproducing fungi [11]), insertion of untranslated sequences comprising introns or spacer regions between genes have been have been identified as the source of mitochondrial genome size variation.

The mitochondrial gene orders of *A. flavus* Intl isolate 1 and *A. parasiticus* Intl isolate 10 appeared to be identical, because identical fragment patterns were generated when mtDNAs of these isolates were digested with either single or double digests of *Cla*I, *Eco*RI, *Pvu*II, and *Xho*I. In the mitochondrial genome of *A. parasiticus* Intl isolate 10, four

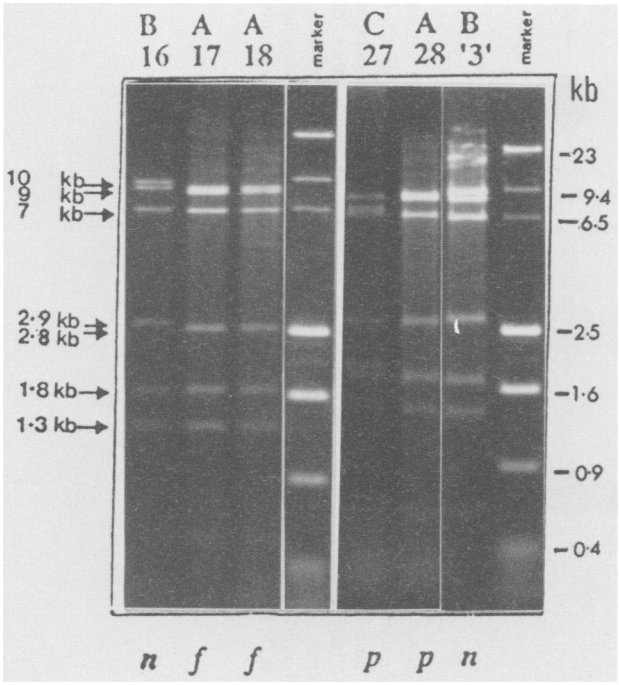

FIG. 3. Restriction fragment patterns of mtDNAs of *Aspergillus* Section *Flavi* isolates *A. flavus* (*f*), *A. parasiticus* (*p*), and *A. nomius* (*n*), digested with *Eco*RI and fractionated by polyacrylamide gel electrophoresis. The isolates shown (Intl isolates 16, 17, 18, 27, 28, and Indian isolate 3, corresponding to the isolates in Table 1) represent all the different RFLP patterns (A through C) that were observed among the *Eco*RI-digested Intl isolates 1 through 36 and Indian isolates 1 through 25 (summarized in Table 4). *Hae*III-digested phage M13mp19 DNA was used as a size marker, together with *Hin*dIII-digested phage λ DNA (lane marker). From top to bottom the sizes of the marker bands that are evident are 23, 9.4, 6.5, 4.3, 2.5, 2.3, 2.0, 1.6, and 0.88 kb. Sizes of marker fragments are indicated at the right of the figure.

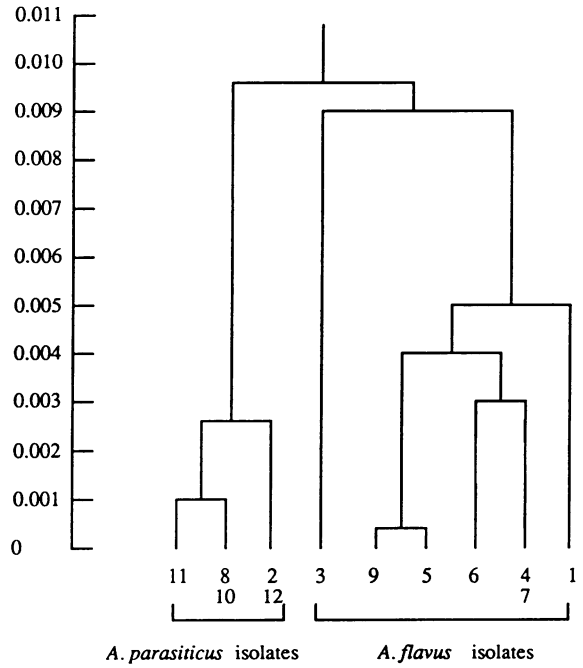

FIG. 4. UPGMA cluster dendrogram of nucleotide sequence divergence (*p*) values calculated from mtDNA RFLP data for *A. flavus* and *A. parasiticus* Intl isolates 1 through 12. Isolate numbers correspond to those in Table 1. The extent of nucleotide sequence diversity is indicated by a scale at the left of the diagram which ranges from 0, when all individuals exhibit the same nucleotide sequence, to 1, when each individual in unique.

genes examined (LrRNA, *oxiA*, *oxiB*, and *cobA*) were arranged in the same order as in the mitochondrial genome of *A. nidulans* (4). This conservation of gene order between these genomes of less closely related *Aspergillus* species suggests that sequence diversity among the mtDNA of these isolates was not generated by rearrangement of DNA sequences involving inversions and translocations. Similarity in mitochondrial genome size and similar gene arrangement (among the limited number of genes able to be precisely located) between *A. flavus*, *A. parasiticus*, and *A. nidulans* suggest that there may be extensive conservation in the general composition of mtDNA of the genus *Aspergillus*, with sequence variation (identified by Kozlowski and Stepien [24]) limited entirely to the spacers and introns.

Polymorphism in the mitochondrial genomes of *A. flavus* and *A. parasiticus* was identified in this study to predominate in the A+T-rich regions, presumably of the intergenic spacers or introns. The disproportionate identification of RFLPs generated by restriction endonucleases that recognize AT sequences is consistent with the extremely A+T-

TABLE 6. RFLPS and nucleotide sequence divergence estimates for *A. flavus* and *A. parasiticus* Intl isolates 1 through 12

Isolate no.	Proportion of common fragments[a] or *p* value[b] for isolate:											
	1	2	3	4	5	6	7	8	9	10	11	12
1		161/342	162/339	165/337	166/340	164/338	165/337	162/339	166/339	162/339	162/340	161/342
2	0.0101		164/343	162/341	164/342	164/342	162/341	170/343	164/343	170/343	170/345	171/342
3	0.0093	0.0103		166/344	167/342	166/345	166/344	165/344	162/346	165/344	166/346	164/343
4	0.0047	0.01081	0.0074		169/341	168/341	170/340	163/342	169/342	163/342	163/343	162/341
5	0.0050	0.0085	0.0066	0.0022		168/342	169/341	165/343	171/343	160/344	165/344	164/342
6	0.0065	0.0085	0.0081	0.0032	0.0039		168/341	163/343	167/343	163/343	163/345	164/342
7	0.0047	0.0108	0.0074	0.0000	0.0022	0.0032		163/342	166/342	163/343	163/343	162/341
8	0.0093	0.0022	0.0095	0.0100	0.0077	0.0107	0.0100		165/344	172/344	172/346	170/343
9	0.0045	0.0089	0.0148	0.0027	0.0005	0.0054	0.0072	0.0082		165/344	167/346	164/343
10	0.0093	0.0022	0.0095	0.0100	0.0082	0.0107	0.0105	0.0000	0.0082		172/346	170/343
11	0.0098	0.0032	0.0095	0.0105	0.0082	0.0012	0.0105	0.0010	0.0071	0.0010		170/345
12	0.0101	0.0000	0.0103	0.0108	0.0085	0.0085	0.0033	0.0022	0.0089	0.0022	0.0032	

[a] Above the diagonal: number of common fragments/total number of fragments.
[b] Below the diagonal: nucleotide sequence divergence (*p*) values.

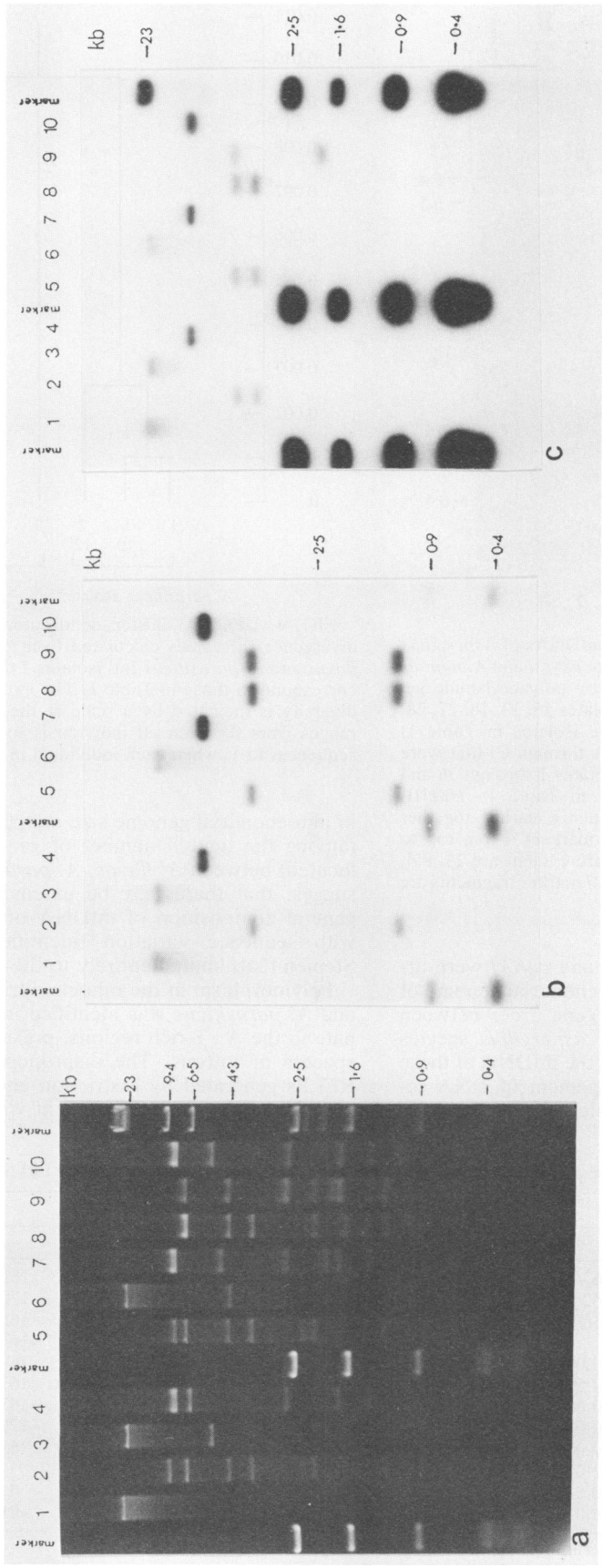

FIG. 5. Localization of genes on the mtDNA fragments of *A. parasiticus* IntI isolate 10 generated by digestion with *Cla*I (lane 1), *Pvu*II (lane 2), *Xho*I (lane 3), *Eco*RI (lane 4), *Cla*I and *Pvu*II (lane 5), *Cla*I and *Xho*I (lane 6), *Cla*I and *Eco*RI (lane 7), *Pvu*II and *Xho*I (lane 8), *Pvu*II and *Eco*RI (lane 9), and *Xho*I and *Eco*RI (lane 10). (a) Restriction fragment pattern of mtDNA fractionated on a 0.8% agarose gel and stained with ethidium bromide. (b) Autoradiogram of Southern blot-hybridized ^{32}P-labeled DS6/A422, containing a 5.3-kb fragment of *S. cerevisiae* cytochrome *c* oxidase I (*oxi4*) gene. (c) Autoradiogram of Southern blot-hybridized ^{32}P-labelled pScm5, containing a 704-bp fragment of *S. cerevisiae* cytochrome *c* oxidase II (*oxiB*) gene. *Hae*III-digested phage M13mp19 DNA was used as a size marker either alone (marker in left and middle lanes), or together with *Hin*dIII-digested phage λ DNA (marker in right lane). From top to bottom the sizes of the phage M13mp19 bands are 2.5, 1.6, 0.88, 0.37, 0.31 (doublet), 0.25, 0.21, 0.17, 0.16, 0.12, 0.11 and 0.10 kb. Sizes of marker fragments are indicated at the right of the figure.

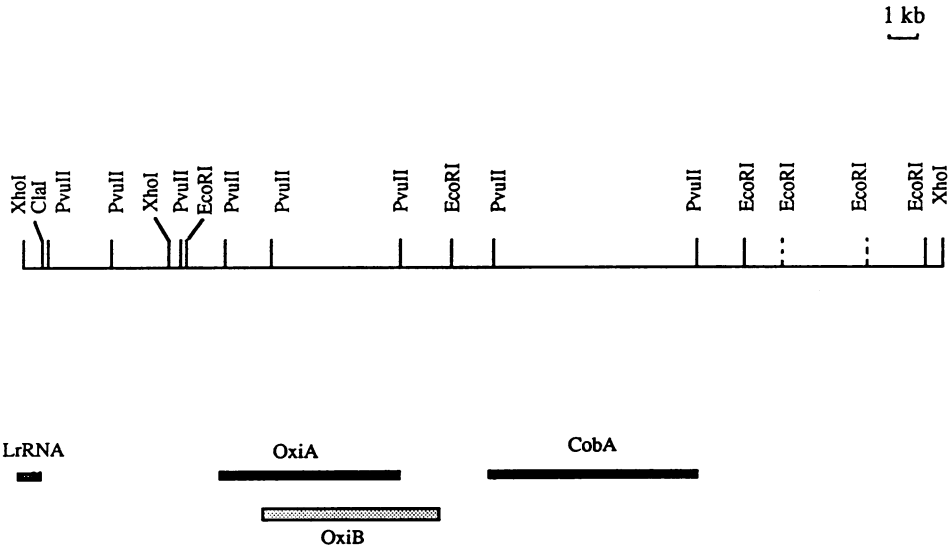

FIG. 6. Restriction map of the mitochondrial genome of *A. parasiticus* Intl isolate 10. The circular mitochondrial genome has been linearized at the *Xho*I site between the *cobA* and the LrRNA genes in the 5'-to-3' direction. The upper part of the figure shows the recognition sites for the four restriction-mapped enzymes. *Eco*RI sites represented by dotted lines show one of two possible arrangements within the mitochondrial genome. The positions of hybridizable gene probes are shown as boxes. Gene abbreviations are those described in the text.

rich nature of most fungal mitochondrial genomes. Identification of fewer polymorphisms in G+C-rich regions of the genome is consistent with the observation of Ayala and Kiger (2) that different DNA sequences in the same genome vary in sequence divergence. The conservation of the G+C-rich regions of the mitochondrial genome of these fungi may reflect the necessity of gene conservation for function of the encoded product.

RFLPs identified by *Hin*fI, *Rsa*I, *Ase*I, and *Dra*I digestion unambiguously distinguished *A. flavus* and *A. parasiticus* isolates. RFLPs identified by *Hin*fI and *Rsa*I digestion were also able to distinguish *A. nomius* isolates. Cloning of the variable fragments should provide appropriate probes for differentiating *A. flavus*, *A. parasiticus*, and *A. nomius* by hybridization to total DNA. RFLPs generated by *Hin*fI digestion of mtDNAs showed associations with geographic location. No RFLPs were found which correlated with mycotoxin production capabilities or source material. This contrasts with an RFLP analysis of *Cochliobolus* mtDNA, which clearly distinguished between producers of a host-specific toxin (16). An alternative means of analyzing the diversity of the *A. flavus* group would be to examine the sequence diversity of the nuclear genome of these fungi (29). Although mtDNA is reported to evolve at a rate 10 to 100 times faster than nuclear DNA in animals (5), the relative rates of evolution in filamentous fungi are unknown.

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