Use of Nuclear DNA Restriction Fragment Length Polymorphisms To Analyze the Diversity of the *Aspergillus flavus* Group: *A. flavus*, *A. parasiticus*, and *A. nomius*

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Recombinant DNA clones carrying high-copy or low-copy sequences from *Aspergillus nidulans* and *Neurospora crassa* were used to identify restriction fragment length polymorphisms (RFLPs) diagnostic for members of the *A. flavus* group: *A. flavus*, *A. parasiticus*, and *A. nomius*. These fungi were resolved into three distinct categories when they were grouped according to RFLP patterns. Subgroups within these categories were also evident. This limited RFLP analysis of nuclear DNA of members of the *A. flavus* group did not identify any RFLPs that differentiate these isolates on the basis of toxin production, but limited correlation with geographic location was observed.

Aspergillus flavus, *A. parasiticus*, and *A. nomius* are members of the economically important *Aspergillus* Section *Flavi* (6) commonly referred to as the *A. flavus* group (8). The ability of many isolates of these fungi (11) to produce the carcinogenic secondary metabolites, the aflatoxins, on cereal grain and peanuts (*Arachis hypogaea* L.) has had a significant impact on agriculture worldwide.

Aflatoxigenic fungi are morphologically and biochemically diverse. *A. flavus*, *A. parasiticus*, and *A. nomius* can be distinguished by morphology by using methods that often requires 2 to 3 weeks to complete (8, 10). *A. flavus* isolates are distinguishable from *A. parasiticus* isolates by the degree of conidial roughening (8). The diameter of colonies of *A. flavus* and *A. nomius* grown at elevated temperatures provides a conclusive means of separation (10). A number of other biochemical and morphological criteria are useful for identifying most, but not all, isolates (2, 10).

Differentiation of *A. flavus* and *A. parasiticus* as separate species or as subspecies of the species *A. flavus* is controversial (7, 11). *A. flavus*, *A. parasiticus*, and *A. nomius* may be distinguished by nuclear DNA hybridization analysis (10, 11). A single isolate of *A. flavus* and a single species of *A. parasiticus* showed 79% DNA sequence relatedness (11). An *A. nomius* isolate and the *A. flavus* isolate showed 34% relatedness, and the *A. nomius* isolate and the *A. parasiticus* isolate showed 39% relatedness (10). By extrapolating from heterothallic yeasts, Kurtzman et al. (10) suggested that the extent of DNA sequence divergence between these fungi was insufficient to warrant individual species classification. They suggested that *A. flavus* and *A. parasiticus* should be reclassified as subspecies of *A. flavus*, i.e., *A. flavus* var. *flavus* and *A. flavus* var. *parasiticus*, and that the less closely related *A. nomius* should be considered a new species (10). Klich and Mullaney (7) dispute this reclassification, suggesting that taxonomic modifications on the basis of one isolate

of each species involved are inappropriate, especially when there are no guidelines for the use of such a character in the taxonomy of the filamentous fungi.

Variation in DNA sequence can be detected by restriction fragment length polymorphism (RFLP) analysis. RFLPs result from specific differences in DNA sequences which alter the fragment sizes that are generated by digestion with type II restriction endonucleases (12). RFLPs can detect minor nucleotide variations that may not be expressed at the

TABLE 1. Plasmids used in RFLP analysis of nuclear DNA

Plasmid	Description and origin
pRRH/X	pBR322 plasmid that contains the 1.75-kb HindIII-XbaI fragment of *N. crassa* rRNA gene containing the 5′ nontranscribed and external transcribed spacer of the 17S gene and 160 bp of the 5′ end of the 17S rRNA gene (27)
pKD2	pBR322 plasmid that contains the 4.8-kb HindIII-HindIII fragment of *N. crassa* rRNA gene containing the 5′ nontranscribed and external transcribed spacer of the 17S gene, the 17S gene, the 5.8S gene, and 550 bp of the 26S gene (5)
pRRH/R	pBR322 plasmid that contains the 1.47-kb HindIII-EcoRI fragment of *N. crassa* rRNA gene containing the 5′ nontranscribed spacer and external transcribed spacer of the 17S gene (27)
pGC1-CS	pUC18 plasmid that contains a 2.6-kb SalI fragment of coding, plus downstream sequence of *N. crassa* ribosomal protein gene crp-1 (9)
pBT6	pUC12 plasmid that contains a 3.1-kb HindIII containing all the β-tubulin gene and flanking sequences (16)
pBENA	pUC19 plasmid that contains the 4-kb NcoI-XbaI fragment of *A. nidulans* β-tubulin gene (constructed by K. Jung, Ohio State University, and kindly provided by Michael Hynes) (21)
pNCH3H4	pBR322 plasmid that contains the 6.9-kb SalI fragment of *N. crassa* histone 3 and histone 4 gene (28)
pHY201	pBR322 plasmid that contains the 4.1-kb XhoI fragment of *A. nidulans* trpC gene (29)
pDB(NDA2)2	pDB248 plasmid that contains the 4.2-kb HindIII fragment of *S. pombe* α-tubulin gene (26)

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TABLE 2. Molecular sizes of restriction fragments associated with the RFLP patterns for *A. nomius*, *A. parasiticus* and *A nomius* Intl and Indian isolates

| Nuclear DNA fragment sizes (kb) for restriction endonuclease digestion pattern and probe: | | | | | | | | | | | | | | | | | | | | | | | |
| rDNA, TaqI | | | rDNA, NcoI | | | r-Protein, HaeIII | | | | | | | | | | | | | β-Tubulin, XbaI | | | | |
A	B	C	A	B	C	A	B	C	D	E	F	G	H	I	J	K	L	M	A	B	C	D	E
		1.5			5.0										2.0	2.0				19			
	1.2	1.2	3.6	3.6	3.6												1.9		15				15
1.0					0.24			1.8	1.8	1.8	1.8		1.8				1.8	1.8			13		
			1.7														1.6				12		12
								1.4					1.4	1.4									10
							1.2	1.2	1.2	1.2				1.2			1.2		9	9	9		
																		1.0					
						0.9	0.9			0.9	0.9	0.9			0.9	0.9	0.9						6
						0.8		0.8	0.8	0.8	0.8	0.8	0.8	0.8		0.8					5.5		
							0.7									0.7			5	5	5	5	5
																	0.75				4	4	4
						0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6				3	

protein level and can detect changes in noncoding regions of DNA (4). RFLPs that correlate with particular morphological and biochemical characters may be identified and used to fingerprint individuals (25).

The major aim of this study was to identify nuclear DNA RFLP markers that correlate with morphological and biochemical characters of *A. flavus*, *A. parasiticus*, and *A. nomius*. A second purpose was to use nuclear DNA sequence divergence (identified by using nuclear DNA probes and restriction endonuclease combinations) to examine relationships among these fungi. We investigated nuclear DNA RFLPs from 18 *A. flavus* isolates, 16 *A. parasiticus* isolates, and 1 *A. nomius* isolate collected from 14 countries as well as 23 *A. flavus* isolates and 1 *A. nomius* isolate that were collected exclusively from southern India.

MATERIALS AND METHODS

Fungal isolates and growth conditions. The *A. flavus*, *A. parasiticus*, and *A. nomius* isolates used in these studies are listed in Table 1 of the accompanying paper (13).

Plasmids. Plasmids used for hybridization analysis are recorded in Table 1.

Nucleic acid isolation from *Aspergillus* species. Nuclear DNA was extracted by using procedures modified from those of Raedar and Broda (17) as described in the accompanying paper (13).

Restriction enzyme digestion. Restriction endonucleases used were *Hinf*I (GANTC), *Rsa*I (GTAC), *Taq*I (TCGA), *Bgl*II (AGATCT), *Cla*I (ATCGAT), *Dra*I (TTTAAA), *Eco*RI (GAATTC), *Eco*RV (GATATC), *Hae*III (GGCC), *Hind*III (AAGCTT), *Nco*I (CCATGG), *Nde*I (CATATG), *Nsi*I (ATGCAT), and *Xba*I (TCTAGA). DNA was incubated with a threefold excess of enzyme (3 U/μg of DNA) in the buffer recommended by the manufacturers at 37°C for 16 h (65°C for *Taq*I), to ensure complete digestion. Reactions were terminated by adding 5× loading buffer (0.1% sodium dodecyl sulfate, 75 mM EDTA, 50% glycerol, 0.1% bromophenol blue) or EDTA (to give a final concentration of 20 mM), ethanol precipitating the DNA, and suspending it in 1× loading buffer.

Agarose gel electrophoresis. Agarose gel electrophoresis was used to resolve restriction fragments ranging in size from 400 base pairs (bp) to 40 kilobase pairs (kb). DNA samples which had been digested with restriction endonucleases were fractionated by electrophoresis in 0.8% agarose gels in Tris-acetate buffer at 80 mA for 3 to 4 h. Bacteriophage lambda DNA digested with *Hind*III 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.

Hybridization probes. Radioactively labeled hybridization probes were prepared by random-primed synthesis of DNA, using *Escherichia coli* DNA polymerase I (Klenow fragment) (3).

Hybridization conditions. Nuclear DNA fractionated on agarose gels was transferred onto PAL Biodyne nylon membrane, and the DNA was fixed by baking as specified by the

TABLE 3. RFLP patterns detected with three recombinant DNA probes for 59 isolates[a] of *A. flavus*, *A. parasiticus*, and *A. nomius*

Probes, enzyme	Pattern for																												
	A. flavus Intl isolate:																		*A. flavus* Indian isolate:										
	1	3	4	5	6	7	9	13	14	15	17	18	19	20	21	22	23	24	1	2	4	5	6	7	8	9	10	11	12
rDNA, *Taq*I	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
rDNA, *Nco*I	A	A	A	A	A	A	A	A	B	A	A	A	A	B	A	B	A	A	A	A	A	A	A	A	B	A	B	A	A
r-Protein, *Hae*III	A	B	C	C	D	E	F	E	E	G	D	E	C	D	E	C	D	D	H	H	D	C	E	C	H	H	C	H	E
β-Tubulin, *Eco*RI	A	A	A	A	A	A	A	A	A	B	A	A	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
β-Tubulin, *Eco*RV	A	B	A	A	A	A	A	A	A	C	A	A	A	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
β-Tubulin, *Xba*I	A	B	C	A	C	A	A	C	A	D	C	A	C	B	A	C	A	C	A	E	F	A	A	E	F	E	A	A	A

[a] Isolate numbers correspond to those in reference 13, Table 1.

TABLE 2—Continued

Nuclear DNA fragment sizes (kb) for restriction endonuclease digestion pattern and probe:

β-Tubulin, XbaI									β-tubulin, EcoRI									β-tubulin, EcoRV									
F	G	H	I	J	K	L	M	N	A	B	C	D	E	F	G	H	I	A	B	C	D	E	F	G	H	I	J
								19																			
15					15	15			13		13	17	17	17	17	17	17	21						30	30		
										7				7	7				15	15						21	
12	12		12		12				6	6	6	6	6	6	6	6	6									8.5	8.5
10		10							5	5	5																
	9		9			9		9	4	4	4	4			4	4		8	8		8	8	8	8	8	8	
		8		8					3.5	3.5	3.5				3.5						7			7		7	7
		6	6									3		3	3			6	6	6	6			6	6		
												2.5				2.5	2.5				4	4	4	4	4	4	4
5					5	5	5		2		2																
4							4	4					1.7	1.7	1.7		1.7										
								3					1.5			1.5											
									0.8		0.8																

manufacturer. Filters 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% sodium dodecyl sulfate, 0.05 g of skim milk powder per liter) (modified Reed and Mann [18]). Membrane-bound nuclear DNA fragments were hybridized to denatured probe (boiled for 5 min) at 57°C for 24 h in a minimum volume of hybridization solution in sealed plastic bags. After hybridization, membranes were washed in preheated (65°C) 2× SSC to 0.2% sodium dodecyl sulfate three times for 30 min each. Following rinsing, the filters 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.

Identification of RFLP markers. International (Intl) isolates 1 through 12 were screened for RFLPs. This collection comprised seven A. flavus isolates (of which four produced aflatoxin B, one produced aflatoxin G, and two did not produce aflatoxin) and five A. parasiticus isolates (of which four produced aflatoxins B and G and one did not produce aflatoxin). DNA was digested with a range of enzymes and hybridized with 10 cloned gene fragments from Neurospora crassa and A. nidulans. A total of 39 enzyme-probe combinations were applied to these DNA samples. Enzyme and probe combinations which detected RFLPs among this small collection of isolates were then applied to a more extensive selection of A. flavus, A. parasiticus, and A. nomius isolates, Intl isolates 13 through 36, to allow a more comprehensive evaluation of the sequence diversity among the A. flavus group. Indian isolates 1 through 25 were examined to assess the genetic variation existing among isolates from a restricted region.

Analysis of data. The DNA relatedness between isolates were assessed by the method of Nei and Li (14). Enzyme-digested nuclear DNA samples were separated side by side on gels, and conserved and polymorphic fragments were identified from autoradiograms following Southern blot hybridization analysis (24) to selected cloned DNA sequences. 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 (15), and F is the proportion of DNA fragments 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 number of fragments displayed by each isolate (14).

For each restriction endonuclease and probe combination, 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 nuclear DNA fragments generated by each restriction endonuclease, and a mean p value was determined for each pair of isolates. This mean p value is presumed to relate to the time since two organisms shared a common ancestor; a smaller p value indicates a shorter elapsed time (4). Dendrograms were constructed from the

TABLE 3—Continued

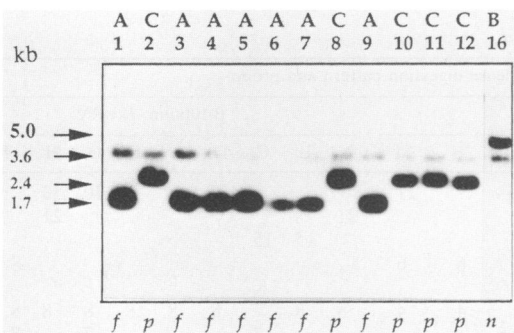| Pattern for																																			
A. flavus Indian isolate:												A. parasiticus Intl isolate:																A. nomius Intl isolate 16	A. nomius Indian isolate 3
13	14	15	16	17	18	19	21	22	23	24	25	2	8	10	11	12	25	26	27	28	29	31	32	33	34	35	36		
A	A	A	A	A	A	A	A	A	A	A	A	C	A	C	A	A	B	B	B	B	B	A	B	B	B	B	B	B	B
A	B	B	A	A	A	A	A	B	A	A	A	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	B	B
D	D	I	C	H	D	H	H	H	C	E	H	J	J	J	J	J	J	J	K	J	J	J	J	J	J	J	J	L	M
A	A	A	A	A	A	A	A	A	A	A	A	D	E	D	E	E	E	E	F	G	E	E	E	H	E	I	E	A	A
A	A	A	A	A	A	A	A	A	A	A	A	D	E	D	E	E	D	D	F	D	D	E	D	G	D	H	D	I	J
A	A	A	E	A	E	F	E	E	F	E	E	G	H	I	H	H	G	G	J	J	G	H	G	K	G	L	G	M	N

FIG. 1. Southern blot hybridization analysis of NcoI-digested nuclear DNAs of A. flavus (f), A. parasiticus (p), and A. nomius (n) isolates, with plasmid pRRH/X (rRNA; Table 1). The isolates shown (IntI isolates 1 through 12 and 16; corresponding to the isolates in reference 13, Table 1) represent all the different RFLP patterns (A through C) that were observed among the NcoI-digested and pRRH/X-probed IntI isolates 1 through 36 and Indian isolates 1 through 25 (summarized in Table 3).

genetic distance data by unweighted pair group mathematical average clustering (UPGMA) analysis using the NT-SYS program (19).

RESULTS

Identification of nuclear DNA RFLPs. Recombinant plasmids carrying DNA from N. crassa (rDNA, r-protein, β-tubulin, and histone H3 and H4 genes) and A. nidulans (β-tubulin and trpC genes) were hybridized to DNA from IntI isolates 1 through 12. Three of these probes (N. crassa rDNA, r-protein, and β-tubulin) were used to identify RFLPs among all the isolates of A. flavus, A. parasiticus, and A. nomius. The RFLP patterns observed among these isolates by using specific enzyme-probe combinations are defined according to fragment molecular weight in Table 2. The isolates corresponding to each of these patterns are recorded in Table 3.

Repetitive ribosomal DNA (rDNA). Southern blot hybridization analysis indicated that considerable homology exists between the rRNA genes from N. crassa and A. flavus and related fungi. RFLPs were detected in the rDNA of isolates when the DNA was digested with NcoI and TaqI and hybridized with rDNA clone pRRH/X from N. crassa (22, 27) (Table 1). Figure 1 shows the pattern types observed with NcoI for IntI isolates 1 through 36 and Indian isolates 1 through 25. This figure is an example of a Southern blot hybridization analysis from which the pattern types recorded in Tables 2 and 3 were defined. When the pRRH/X probe was used, only three pattern types (with a maximum of three bands) were observed among these isolates for each enzyme.

TABLE 4. Association of β-tubulin XbaI RFLPs with geographical location among A. flavus isolates[a]

Country of origin	No. of isolates with pattern[b]:		
	A	B, C, and D	E and F
India	10	0	13
Other countries	8	10	0

[a] Data are summarized from Table 3 and reference 13, Table 1. Significance of differences tested by using chi-square analysis (23) was as follows: $\chi^2_{df = 2}$ = 19.0, $P \ll 0.005$, i.e., highly significant.
[b] Patterns as defined in Table 2 and Fig. 2.

A. parasiticus isolates grouped independently from A. flavus and A. nomius isolates with NcoI. In contrast, A. flavus, A. parasiticus, and A. nomius isolates did not differentiate according to taxonomic groupings when their DNA was digested with TaqI (Table 3). Two A. parasiticus isolates from the Americas, digested with TaqI, grouped independently of other A. flavus and A. parasiticus isolates from the Americas. However, no consistent correlation to morphology, biochemistry, or geographical distribution could be derived from the RFLP patterns of isolates digested with TaqI.

No polymorphism was detected when nuclear DNA was digested with ClaI, EcoRI, EcoRV, HaeIII, HinfI, HindIII, NsiI, or XbaI or when it was double digested with XbaI plus BglII or DraI and hybridized with rDNA clone pRRH/X (data not shown). No polymorphism was detected when the nuclear DNAs of the representative 12 isolates were digested with BglII, ClaI, DraI, EcoRV, or NsiI and hybridized with rDNA clone pKD2 from N. crassa (5) (Table 1). No polymorphism was detected when nuclear DNA was double digested with XbaI plus BglII or DraI and hybridized with the rDNA clone pRRH/R from N. crassa (27) (Table 1). Enzyme probe combinations that failed to generate RFLPs when assessed by using the selected sample of IntI isolates 1 through 12 were not used in further analyses.

Low-copy-number nuclear genes. The plasmid pGC1-CS containing the single-copy r-protein gene crp-1 of N. crassa (9) (Table 1) detected many RFLPs when hybridized to nuclear DNA of isolates which had been digested with HaeIII (Tables 2 and 3) or HindIII (data not shown). A. flavus, A. parasiticus, and A. nomius were differentiated with these enzyme-probe combinations. A. flavus isolates showed a greater diversity of RFLP pattern types than did A. parasiticus isolates (Table 3). Grouping with respect to RFLP pattern did not appear to correlate with isolate morphology, biochemistry, or origin.

The pBT6 plasmid containing the N. crassa β-tubulin gene (16) (Table 1) and the plasmid pBENA containing the A. nidulans β-tubulin gene probe (21) (Table 1) detected RFLPs in the nuclear genome of A. flavus, A. parasiticus, and A.

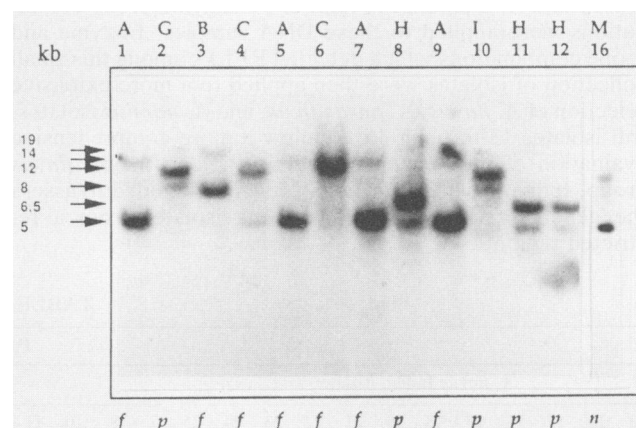FIG. 2. Southern blot hybridization analysis of XbaI-digested nuclear DNAs of A. flavus (f), A. parasiticus (p), and A. nomius (n) isolates, with plasmid pBT6 (β-tubulin; Table 1). The isolates shown (IntI isolates 1 through 12 and 16; corresponding to the isolates in reference 13, Table 1) represent 7 of the 14 different RFLP patterns (A through C, G, H, I, and M) that were observed among the XbaI-digested and pBT6-probed IntI isolates 1 through 36 and Indian isolates 1 through 25 (summarized in Table 3).
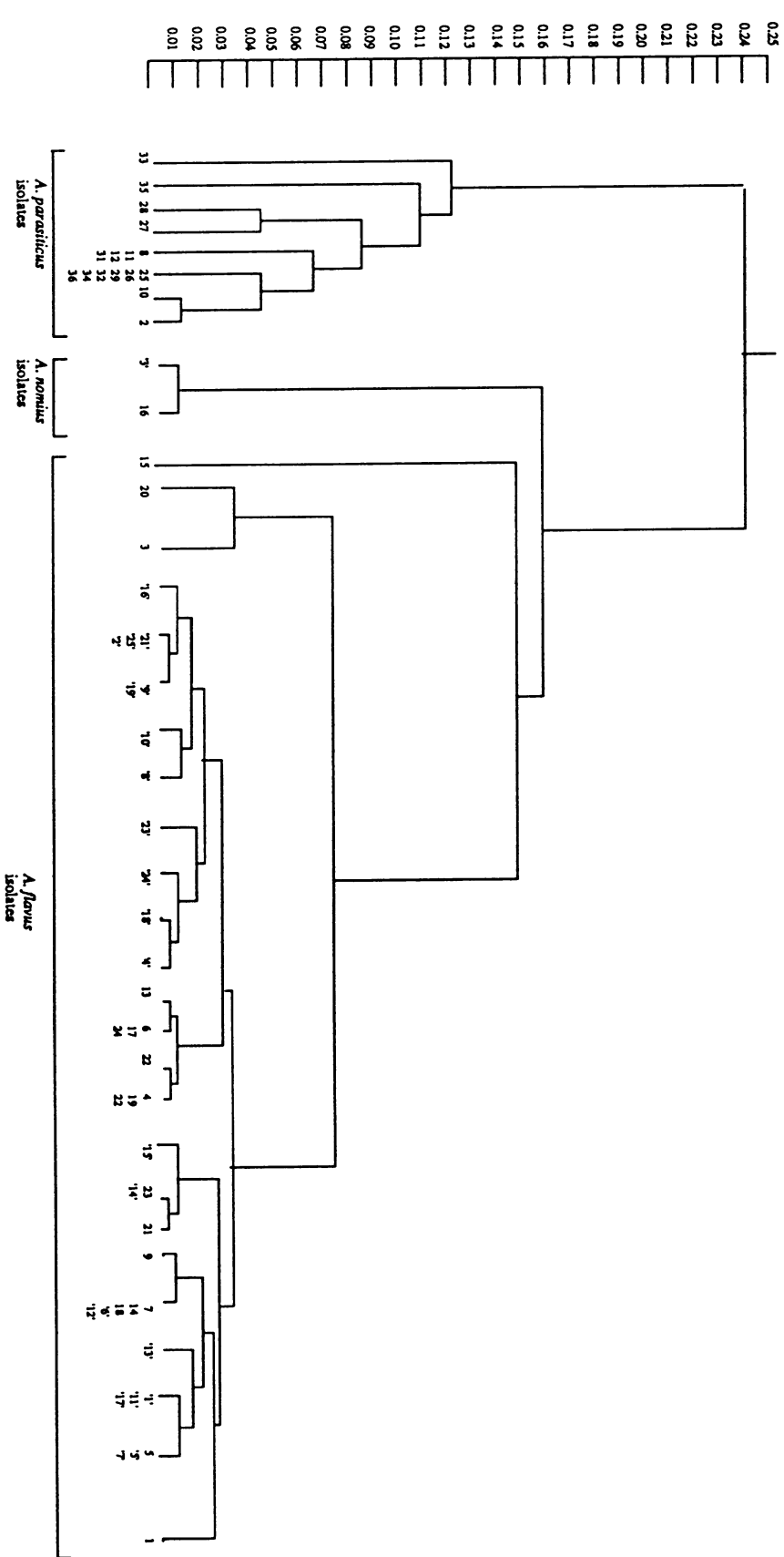
FIG. 3. UPGMA cluster dendrogram of genetic distance data *p* values calculated from nuclear DNA RFLP data of *A. flavus*, *A. parasiticus*, and *A. nomius* Intl isolates 1 through 36 and Indian isolates '1' through '25.' Isolate numbers correspond to those in reference 13, Table 1.

TABLE 5. RFLPs and nucleotide sequence divergence estimates for nuclear DNAs of *A. flavus*, *A. parasiticus*, and *A. nomius* isolates[a]

Isolate no.	Proportion of common fragments[b] or p value[c] for Intl isolate:															
	1	2	3	4	5	6	7	8	9	10	13	15	16	20	21	23
Intl																
1		6/39	14/39	16/41	18/40	16/40	19/41	5/37	19/39	7/41	17/42	10/38	10/39	14/40	18/41	17/39
2	0.2374		7/40	7/42	7/41	7/41	7/41	11/38	6/40	20/42	7/42	8/40	7/41	7/41	7/41	7/40
3	0.0649	0.178		13/41	14/41	13/40	15/41	8/38	14/40	8/40	14/41	10/40	12/41	18/41	14/41	13/40
4	0.0555	0.2075	0.0899		19/43	21/43	18/43	6/40	17/42	8/44	21/44	9/41	9/40	15/42	17/43	17/42
5	0.0340	0.2033	0.0783	0.0214		18/42	20/42	6/39	18/41	9/43	18/43	10/40	10/39	16/42	19/42	19/41
6	0.0485	0.2017	0.0836	0.0063	0.0278		18/42	6/39	17/41	8/43	21/43	9/40	9/41	15/41	17/42	17/41
7	0.0212	0.2033	0.0591	0.0336	0.0121	0.0278		6/39	20/41	8/43	19/43	10/40	11/41	16/42	20/43	19/41
8	0.2423	0.1014	0.1679	0.2329	0.2286	0.2266	0.2286		4/38	13/40	5/38	6/37	6/38	8/39	6/39	6/39
9	0.0078	0.2438	0.0719	0.0427	0.0296	0.0363	0.0063	0.2989		7/42	18/42	10/39	11/40	15/41	19/41	18/40
10	0.2233	0.0084	0.1705	0.1931	0.1704	0.1873	0.1892	0.0795	0.2296		8/44	7/41	7/42	8/41	8/43	8/42
13	0.0427	0.2075	0.0707	0.0121	0.0336	0.0063	0.0214	0.2737	0.0278	0.1931		8/41	10/42	15/42	18/43	17/42
15	0.1123	0.2004	0.1349	0.1500	0.1272	0.1430	0.1272	0.2071	0.1201	0.2233	0.1723		10/39	12/41	11/40	11/39
16	0.1337	0.2033	0.1170	0.1542	0.1337	0.1606	0.1189	0.2350	0.1131	0.2075	0.1393	0.1656		12/42	12/41	11/40
20	0.0689	0.2017	0.0380	0.0599	0.0482	0.0535	0.0482	0.1641	0.0567	0.1794	0.0599	0.0973	0.1210		15/42	15/41
21	0.0296	0.2033	0.0707	0.0434	0.0205	0.0376	0.0124	0.2286	0.0147	0.1892	0.0312	0.1108	0.1045	0.0599		20/41
23	0.0354	0.1975	0.0836	0.0376	0.0147	0.0312	0.0147	0.2286	0.0233	0.1834	0.0376	0.1038	0.1257	0.0535	0.0063	
25	0.2304	0.0358	0.1914	0.2017	0.1975	0.1953	0.2600	0.0515	0.2374	0.0442	0.2017	0.2304	0.1932	0.2226	0.1975	0.1912
27	0.2071	0.1054	0.1641	0.2248	0.2205	0.2184	0.1935	0.1097	0.2142	0.1290	0.1978	0.2534	0.2242	0.1953	0.1978	0.1242
28	0.2304	0.0747	0.1914	0.2017	0.1975	0.1953	0.1975	0.0788	0.2374	0.0831	0.2017	0.2081	0.1932	0.2226	0.1975	0.1912
33	0.1978	0.0873	0.1746	0.1612	0.1844	0.1554	0.1695	0.1314	0.1786	0.0841	0.1464	0.1730	0.1528	0.1372	0.1695	0.1786
35	0.2261	0.0932	0.2266	0.2248	0.1932	0.2184	0.1932	0.0905	0.2374	0.1141	0.2248	0.2885	0.2161	0.2226	0.1932	0.1912
Indian																
1	0.0254	0.2400	0.0962	0.0279	0.0059	0.0359	0.0200	0.2497	0.0140	0.2255	0.0346	0.1205	0.1377	0.0553	0.0211	0.0211
2	0.0552	0.2522	0.1081	0.0302	0.0264	0.0382	0.0405	0.2265	0.0345	0.2178	0.0370	0.1327	0.1500	0.0675	0.0424	0.0424
3	0.1531	0.2047	0.1598	0.2141	0.1789	0.2081	0.1789	0.2660	0.1729	0.2125	0.2075	0.1493	0.0855	0.1591	0.1553	0.0689
4	0.0421	0.2047	0.0788	0.0264	0.0226	0.0204	0.0226	0.2329	0.0306	0.1898	0.0211	0.2054	0.1461	0.0647	0.0266	0.0266
8	0.0459	0.2522	0.1200	0.0394	0.0357	0.0474	0.0497	0.2265	0.0437	0.2178	0.0481	0.1160	0.1353	0.0799	0.0335	0.0335
9	0.0513	0.2483	0.1943	0.0264	0.0226	0.0345	0.0366	0.2583	0.0306	0.2333	0.0341	0.1289	0.1461	0.0647	0.0396	0.0396
10	0.0529	0.2141	0.1005	0.3340	0.0297	0.0394	0.0412	0.2070	0.0497	0.1797	0.0411	0.1230	0.1332	0.0729	0.0265	0.0265
13	0.0340	0.1964	0.0708	0.0279	0.0059	0.0219	0.0059	0.2242	0.0140	0.1820	0.0226	0.1205	0.1377	0.0433	0.0091	0.0091
15	0.0254	0.1964	0.0565	0.0640	0.0407	0.0459	0.0286	0.2242	0.0407	0.1820	0.0455	0.1038	0.1230	0.0675	0.0120	0.0120
16	0.0526	0.2141	0.0886	0.0242	0.0204	0.0302	0.0319	0.2070	0.0405	0.1797	0.0310	0.1397	0.1440	0.0615	0.0364	0.0346
18	0.0459	0.2087	0.0826	0.0302	0.0264	0.0242	0.0264	0.2070	0.0345	0.1742	0.0250	0.1327	0.1500	0.0555	0.0304	0.0304
23	0.0487	0.2102	0.0848	0.0204	0.0166	0.0264	0.0281	0.2388	0.0366	0.1952	0.0247	0.1359	0.1401	0.0577	0.0326	0.0326
24	0.0405	0.2141	0.0706	0.0356	0.0319	0.0302	0.0204	0.2070	0.0264	0.1797	0.0310	0.1397	0.1300	0.0615	0.0364	0.0364

[a] When RFLP patterns were identical for two isolates, the p values for only a single representative was listed. The following isolates were identified with the same RFLP patterns: Intl 4, 19 and 22; Intl 6, 17 and 24; Intl 7, 14, and 18; Intl 8, 11, 12, and 31; Intl 25, 26 29, 32, 34 and 36; Intl 5, Indian 5, and Indian 7; Intl 7, Indian 6, and Indian 12; Intl 23 and Indian 14; Indian 1, 11, and 17; Indian 2, 25, and 21; and Indian 9 and 19 (see Table 3).

[b] Above the diagonal: number of common fragments/total number of fragments.

[c] Below the diagonal: nucleotide sequence divergence (p) values.

nomius when nuclear DNA of Intl isolates 1 through 12 was digested with *Bgl*II, *Cla*I, *Dra*I (data not shown), *Eco*RI, *Eco*RV (Tables 2 and 3), and *Xba*I (Fig. 2; Tables 2 and 3). *Eco*RI resolved *A. flavus* and *A. parasiticus* isolates. However, *A. nomius* isolates grouped with *A. flavus* isolates. All the Indian *A. flavus* isolates resolved into the same subgrouping. *Eco*RV differentiated *A. flavus*, *A. parasiticus*, and *A. nomius* independently. All Indian *A. flavus* isolates resolved into a single subgroup. *Xba*I differentiated *A. flavus*, *A. parasiticus*, and *A. nomius* separately. The Indian *A. flavus* isolates were divided into three subgroups with *Xba*I. A number of isolates consistently generated restriction fragments patterns that were unique among the sample collection. These isolates, however, did not appear to possess any distinguishing phenotypic characters.

The β-tubulin RFLPs identified with *Xba*I showed some associations with geographical location among the *A. flavus* isolates. For example patterns E and F, which together appeared among 13 of the 23 Indian isolates, did not appear among any of the Intl isolates (Table 4). Conversely, patterns B, C, and D, which appeared among 10 of the 18 Intl isolates, did not appear at all among the Indian isolates

(Table 4). These differences are highly statistically significant ($\chi^2_{df = 2} = 19.0$; $P \ll 0.005$).

Nucleotide sequence divergence determination. Nucleotide sequence divergence values (p values) were determined for all the isolates and are recorded in Table 5. These values were subjected to UPGMA analysis (see Fig. 3). *A. flavus*, *A. parasiticus*, and *A. nomius* isolates clearly separated into three discrete groups. Groupings derived from these analyses did not correlate with geographical origin or toxigenicity. *A. nomius* isolates were estimated at a shorter distance from *A. flavus* than from *A. parasiticus* isolates. By using these limited RFLP markers, a number of isolates could not be independently differentiated. Isolates sampled from those from southern India were closely related.

DISCUSSION

We have used highly conserved cloned genes from related fungi to identify RFLPs among *A. flavus*, *A. parasiticus*, and *A. nomius* and hence to identify potential diagnostic species markers and to estimate the genetic relatedness of these fungi. The use of conserved heterologous probes enables

TABLE 5—Continued

					Proportion of common fragments[b] and p value[c] for Indigo isolate:												
25	27	28	33	35	1	2	3	4	8	9	10	13	15	16	18	23	24
6/38	6/37	6/38	7/40	6/37	18/39	16/42	11/41	17/41	17/42	16/41	17/43	17/39	18/39	17/43	17/42	17/42	18/44
16/39	11/38	13/39	13/40	12/38	6/40	6/43	7/42	7/42	6/43	6/42	7/49	7/41	7/41	7/44	7/43	7/43	7/44
7/39	8/39	7/39	8/41	6/39	13/40	13/43	11/42	14/42	12/43	13/42	13/44	14/40	15/41	14/44	14/43	14/43	15/44
7/41	6/40	7/41	9/42	6/40	18/42	19/45	8/44	19/44	18/45	19/44	19/46	18/42	15/41	20/46	19/45	20/45	19/46
7/40	6/39	7/40	8/41	7/39	20/41	19/44	10/43	19/43	18/44	19/43	19/45	20/41	17/40	20/45	19/44	20/44	19/45
7/40	6/39	7/30	9/41	6/39	19/41	18/44	8/43	19/43	17/44	18/43	18/45	18/41	16/41	19/45	19/44	19/44	19/45
5/40	7/39	7/40	9/41	7/39	19/41	18/44	10/43	19/43	17/44	18/43	18/45	20/41	18/41	19/45	19/44	19/44	20/45
14/37	10/36	12/37	10/38	11/36	5/38	6/41	5/40	6/40	6/41	5/40	7/42	6/38	6/38	7/42	7/42	6/41	7/42
6/39	6/38	6/39	8/40	6/39	9/40	18/43	10/42	18/42	17/43	18/42	17/44	19/40	17/40	18/44	18/43	18/43	19/44
16/41	10/40	13/41	14/42	11/40	7/42	8/45	7/44	8/44	8/45	7/44	7/46	8/42	8/42	9/46	9/45	8/45	9/46
7/41	7/40	7/41	10/42	6/40	17/41	18/44	8/43	19/43	17/44	28/43	18/45	18/41	16/41	19/45	19/44	19/44	19/45
6/38	5/37	7/38	8/40	4/37	10/39	10/39	11/41	11/21	11/42	10/41	11/43	10/39	11/39	10/43	10/41	10/42	10/43
7/39	6/38	7/39	9/40	6/38	10/40	10/43	15/42	10/42	11/43	10/42	11/43	10/40	11/40	10/42	10/43	10/41	11/44
6/40	7/40	6/40	10/42	6/40	15/41	15/44	11/43	16/43	14/44	15/43	15/45	16/41	14/41	16/45	16/44	16/44	16/45
7/40	7/40	7/40	9/41	7/39	18/40	17/43	11/42	18/42	18/43	17/42	19/44	19/40	19/40	18/44	18/43	18/43	18/44
7/39	6/38	7/39	8/40	7/39	18/40	17/43	11/42	18/42	18/43	17/42	19/43	19/40	19/40	18/44	18/43	18/43	18/44
	12/37	15/38	11/39	13/38	6/39	6/42	8/41	7/41	6/42	6/41	7/43	7/39	7/39	7/43	7/42	7/42	7/43
0.0788		15/37	11/38	9/36	5/38	6/41	5/40	7/40	6/41	6/40	6/42	6/38	6/38	7/42	7/41	7/41	8/42
0.0411	0.0399		11/39	13/38	4/39	7/42	7/41	8/41	7/42	7/41	8/43	7/39	7/39	8/43	8/42	8/42	8/43
0.1088	0.1014	0.1251		8/38	8/39	9/42	5/41	10/41	9/42	9/41	10/43	9/39	8/39	10/43	10/42	10/42	10/43
0.0683	0.1316	0.0990	0.1665		6/38	6/41	7/40	7/40	6/41	6/40	7/42	7/38	7/38	7/42	7/41	7/41	7/42
0.2305	0.2549	0.2959	0.1754	0.2267		19/43	10/42	18/42	18/43	19/42	18/44	19/40	18/40	19/44	18/43	19/43	18/44
0.2434	0.2389	0.2206	0.1594	0.2396	0.0204		10/35	17/44	22/46	18/44	18/47	17/43	17/43	22/47	21/46	18/45	21/48
0.1979	0.2587	0.1979	0.2678	0.1941	0.1729	0.1421		11/44	10/45	11/44	10/46	11/42	10/42	11/46	11/45	11/45	11/46
0.1979	0.1941	0.1751	0.1436	0.1941	0.0306	0.0474	0.1673		20/46	21/45	21/46	19/42	17/41	22/46	22/45	22/45	22/46
0.2434	0.2389	0.2206	0.1604	0.2396	0.0297	0.0071	0.1841	0.0312		18/44	23/47	17/43	18/43	22/47	22/46	18/45	21/47
0.2389	0.1487	0.2161	0.1566	0.2351	0.0166	0.0334	0.1673	0.0200	0.0334		21/46	18/42	17/42	22/46	21/45	22/45	21/46
0.2087	0.2328	0.1856	0.1534	0.2046	0.0357	0.0465	0.1901	0.0171	0.0059	0.0171		18/44	17/43	23/48	22/47	18/46	22/48
0.1895	0.2138	0.1895	0.1634	0.1857	0.0140	0.0437	0.1598	0.0166	0.0437	0.0306	0.0357		18/39	19/44	19/43	14/43	19/44
0.1895	0.2138	0.1895	0.1754	0.1857	0.0226	0.0437	0.1729	0.0333	0.0345	0.0399	0.0204	0.0160		16/43	17/43	16/42	17/44
0.2084	0.2039	0.1856	0.1534	0.2046	0.0264	0.0131	0.1769	0.0096	0.0131	0.0096	0.0017	0.0264	0.0618		23/47	14/36	23/48
0.2024	0.1979	0.1796	0.1474	0.1986	0.0345	0.0211	0.1709	0.0036	0.0211	0.0176	0.0131	0.0204	0.0437	0.0059		19/45	23/47
0.2039	0.2000	0.1811	0.1496	0.2000	0.0226	0.0394	0.1733	0.0059	0.0394	0.0059	0.0449	0.0226	0.0580	0.0242	0.0302		22/47
0.2084	0.2039	0.1856	0.1534	0.2046	0.0405	0.0271	0.1769	0.0096	0.0271	0.0236	0.0185	0.0264	0.0497	0.0114	0.0059	0.0150	

RFLPs to be identified in poorly characterized fungi (or other organisms) without the need to clone and screen new probe sequences from the fungus. We used as probes rRNA, ribosomal protein crp-1, β-tubulin, and histone H3 and H4 genes from N. crassa, β-tubulin, trpC gene from A. nidulans, and an α-tubulin gene from Schizosaccaromyces pombe, of which all but the last hybridized to A. flavus and A. parasiticus sequences. Of the probes which hybridized, all the low-copy-number protein-coding genes readily detected RFLPs with a variety of enzymes. In contrast, the 17S rRNA probe detected no RFLPs with 5 enzymes, whereas the rRNA spacer probe detected RFLPs with only 3 of 13 enzymes. We used the N. crassa rRNA (5, 27), β-tubulin gene (tub-2 [16]), and ribosomal protein (crp-1 [9]) genes as probes to characterize in detail the genetic relatedness of 59 A. flavus, A. parasiticus, and A. nomius isolates that had different biochemical and morphological characteristics and were obtained from different countries and substrates.

In higher eucaryotes, rDNA repeat units consist of highly conserved rRNA coding sequences (17S-5.8-25S) separated by highly polymorphic, repetitive spacer regions. The spacer sequences of N. crassa and A. flavus and related species were insufficiently conserved to hybridize directly. We used a probe containing the highly conserved 160-bp 5' end of the N. crassa 17S rRNA gene to detect restriction fragments extending into the adjacent spacer region. Surprisingly, very little polymorphism was detected in the spacer region among these A. flavus, A. parasiticus, and A. nomius isolates. In contrast, substantial variation occurs in the rDNA spacer both between and within different Neurospora species (20). These observations, and the small amount of sequence variation in the mitochondrial DNAs of A. flavus, A. parasiticus, and A. nomius, (13) suggest that these species have diverged quite recently.

The β-tubulin probe, which was a 650-bp AvaI fragment containing the most highly conserved region of the gene, detected one to seven bands in each of three different restriction digests. Most of the hybridizing fragments were in the size range 2 to 20 kb. The large number and large size of the fragments make it unlikely that multiple internal fragments of a single gene were being detected. It is more likely that multiple tubulin genes or tubulin-related genes were being detected. A. nidulans contains two α-tubulin genes and two β-tubulin genes. Additional bands could have been generated by internal cleavage within some of the genes. Some patterns, such as XbaI D, H, J, L and M (Fig. 2), which had only one or two bands, may have resulted from some fragments being too large or small to be detected. The ribosomal protein gene, crp-1, detected small HaeIII fragments. Many of these were probably small internal fragments of the gene(s). The number of crp-1-type ribosomal protein genes in A. nidulans is unknown. There is one such

gene in *N. crassa* containing seven introns (B. M. Tyler unpublished data) and two such genes in *Saccharomyces cerevisiae* (1).

The RFLPs detected by the β-tubulin probe in combination with *XbaI* digestion of the nuclear DNAs showed statistically significant associations with geographical location, although the associations were not sufficiently strict to have predictive value. Two patterns (E and F) were found exclusively among the collection of 23 Indian *A. flavus* isolates, whereas three patterns (B, C, and D) were found only outside India. One pattern (A) was found among both Indian and Intl isolates. One could speculate that pattern A isolates represent recent introduction to (or exports from) India.

The strongest correlations with RFLP patterns were taxonomic ones. Hybridization of the β-tubulin probe to *Eco*RI digests unambiguously distinguished *A. flavus* and *A. nomius* isolates (patterns A, B, and C) from *A. parasiticus* (D through I), whereas β-tubulin hybridization to *XbaI* and *Eco*RV digests or r-protein hybridization to *Hae*III digests unambiguously distinguished each of the three species. The fact that each species displayed multiple patterns with these probe-enzyme combinations may limit the ability of this approach to identify new isolates which have patterns different from previously identified isolates. Hybridization of the rDNA probe to *NcoI* digests may be more useful for species identification, either alone or in combination with other probe-enzyme combinations: pattern C was observed for all 17 *A. parasiticus* isolates, pattern B was observed for the 2 *A. nomius* isolates, and pattern A was observed for 33 of the 41 *A. flavus* isolates. Seven *A. flavus* isolates showed a B pattern, characteristic of *A. nomius*, although they resembled *A. flavus* in their morphology, β-tubulin, and r-protein RFLPs and in their mitochondrial DNA digestion patterns (13).

The strong correlations with taxonomic groupings are also reflected in the calculations of genetic relatedness of all the isolates (Fig. 3). The UPGMA analysis indicated that there is greater similarity between *A. flavus* and *A. nomius* isolates than between *A. flavus* and *A. parasiticus* isolates. This result contrasts with the finding of Kurtzman et al. (10) that an *A. nomius* isolate showed considerable divergence from single *A. flavus* and *A. parasiticus* isolates. It also contrasts with our findings (13) that 39 *A. flavus* and 17 *A. parasiticus* isolates had identical-length mitochondrial DNAs and mitochondrial gene order, whereas the 2 *A. nomius* isolates examined had similarly sized insertions into two places in their mitochondrial genomes. Accurate estimation of the level of relatedness of *A. nomius* to *A. flavus* and *A. parasiticus* requires examination of larger numbers of *A. nomius* isolates.

Our RFLP data presented are not able to resolve the taxonomic question of whether *A. flavus* and *A. parasiticus* should be considered distinct species or subspecies. We have shown that both nuclear and mitochondrial DNA polymorphisms and quantitative measures of genetic relatedness can clearly differentiate *A. flavus*, *A. parasiticus*, and *A. nomius* into distinct entities. Nevertheless, the actual amount of variation found in the rDNA spacer regions and the mitochondrial DNAs of *A. flavus*, *A. parasiticus*, and *A. nomius* isolates is relatively small, indicating that the three groups are closely related. Kurtzman et al. (10) used correlation between DNA sequence complementarity and mating among heterothallic yeasts as a guide for defining *Aspergillus* species on the basis of DNA complementarity. However, such correlations have not been authenticated for any other

fungi. The classification of *A. flavus* and *A. parasiticus* fungi into different species rather than subspecies of *A. flavus* is consistent with morphological differences and is a convenient definition. RFLP analysis provides a simple means for distinguishing these fungi which is consistent with conventional morphological and biochemical methods, and it provides additional information on the relatedness and possible origins of individual species.

ACKNOWLEDGMENTS

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