

Combined Molecular and Biochemical Approach Identifies *Aspergillus japonicus* and *Aspergillus aculeatus* as Two Species

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We examined nine *Aspergillus japonicus* isolates and 10 *Aspergillus aculeatus* isolates by using molecular and biochemical markers, including DNA sequences of the ITS1-5.8S rRNA gene-ITS2 region, restriction fragment length polymorphisms (RFLP), and secondary-metabolite profiles. The DNA sequence of the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene could not be used to distinguish between *A. japonicus* and *A. aculeatus* but did show that these two taxa are more closely related to each other than to other species of black aspergilli. *Aspergillus niger* pyruvate kinase (*pkIA*) and pectin lyase A (*pelA*) and *Agaricus bisporus* 28S rRNA genes, which were used as probes in the RFLP analysis, revealed clear polymorphism between these two taxa. The *A. niger* *pkIA* and *pelA* probes placed six strains in an *A. japonicus* group and 12 isolates in an *A. aculeatus* group, which exhibited intraspecific variation when they were probed with the *pelA* gene. The secondary-metabolite profiles supported division of the isolates into the two species and differed from those of other black aspergilli. The strains classified as *A. japonicus* produced indole alkaloids and a polar metabolite, while the *A. aculeatus* isolates produced neoxaline, okaramins, paraherquamidlike compounds, and secalonic acid. *A. aculeatus* CBS 114.80 showed specific RFLP patterns for all loci examined. The secondary-metabolite profile of strain CBS 114.80 also differed from those of *A. japonicus* and *A. aculeatus*. Therefore, this strain probably represents a third taxon. This study provides unambiguous criteria for establishing the taxonomic positions of isolates of black aspergilli, which are important in relation to industrial use and legal protection of these organisms.

Representatives of the black aspergilli are commonly used by the fermentation industry to produce extracellular enzymes and metabolites such as citric acid. It is important to clearly establish the taxonomic positions of such *Aspergillus* isolates due to the use of their products in the food and feed industry and for legal protection purposes. Traditionally, morphological criteria like color, shape, size, and ornamentation of conidia have been used to classify such strains (1, 17, 23, 27, 31, 32).

However, the black aspergilli also vary significantly in their morphological and physiological characteristics. Therefore, unambiguous identification of an isolate requires molecular and biochemical identification techniques. The major taxonomic efforts with the black aspergilli have focused on the *Aspergillus niger* aggregate (20, 21, 26, 34–36). Using mitochondrial DNA (mtDNA) and nuclear DNA restriction fragment length polymorphisms (RFLPs) and randomly amplified polymorphic DNA patterns, workers have proposed that the strains of the *A. niger* aggregate (1) should be divided into four taxa: *A. niger*, *Aspergillus tubingensis* (20), *Aspergillus brasiliensis* (35), and *Aspergillus foetidus* (26). Similar studies have been carried out with isolates of *Aspergillus carbonarius* (15, 25) and with isolates of the uniseriate (i.e., metulae are not present) species,

Aspergillus japonicus and *Aspergillus aculeatus*, as these species cannot be identified reliably on the basis of morphological features (9). A high degree of mtDNA polymorphism was found among the *A. aculeatus* and *A. japonicus* isolates, and this polymorphism correlated with randomly amplified polymorphic DNA patterns. Therefore, it also is difficult to use mtDNA polymorphisms to discriminate between these two taxa.

The objectives of this study were (i) to establish reliable methods for discriminating between *A. japonicus* and *A. aculeatus* isolates, (ii) to compare the results obtained with those obtained with well-characterized representatives of the other black aspergilli, and (iii) to provide guidelines for identification of all black aspergilli. We compared 19 *A. japonicus* and *A. aculeatus* strains by using three different approaches; we compared sequences of the ITS1-5.8S rRNA gene-ITS2 region, RFLPs of nuclear DNA, and secondary-metabolite profiles. *A. aculeatus* has been reported to produce the secondary metabolites emodin, secalonic acids D and F (2, 18), aculeasins (22, 28), and okaramins A, B, H, and I (13), while in *A. japonicus* festuclavine and cycloclavine (8), E-64 (10, 11), and neoxaline (14, 16) have been identified. By combining RFLP analysis of nuclear DNA, DNA sequencing, and secondary-metabolite profile analysis we could discriminate between isolates of these two taxa and improved the method by which strains of black aspergilli are identified. The accuracy obtained is important for characterizing and protecting industrial strains, for studying fungal biodiversity, and for providing a rationale for selecting

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TABLE 1. Final grouping of the *A. japonicus* and *A. aculeatus* isolates after RFLP analysis of nuclear DNA and ITS1-5.8S rRNA gene-ITS2 sequencing

Group	Strain	EMBL accession no. ^a	Pattern based on:						
			rDNA (<i>Sma</i> I)	<i>pkiA</i> (<i>Kpn</i> I- <i>Xho</i> I)	26S rRNA (<i>Kpn</i> I- <i>Xho</i> I)	26S rRNA (<i>Pst</i> I- <i>Sal</i> I)	<i>pelA</i> (<i>Pst</i> I- <i>Sal</i> I)	ITS1-5.8S rRNA gene-ITS2	
<i>A. japonicus</i>	<i>A. japonicus</i> CBS 568.65	AJ279991	D	F	A	L	H	I	
	<i>A. japonicus</i> CBS 522.89	AJ279990	D	F	A	L	H	I	
	<i>A. japonicus</i> CBS 101.14 ^b	AJ279983	D	F	A	M	H	I	
	<i>A. atroviolaceus</i> CBS 113.48 ^{Tc}	AJ279984	D	F	A	M	H	I	
	<i>A. japonicus</i> CBS 114.51 ^T	AJ279985	D	F	A	N	H	I	
	<i>A. aculeatus</i> CBS 611.78	AJ279992	D	F	A	N	I	I	
<i>A. aculeatus</i>	<i>A. japonicus</i> CBS 114.34	AJ279996	D	G	B	I	L	II	
	<i>A. lucknowensis</i> CBS 119.49	AJ279986	D	H	B	I	M	I	
	<i>A. luchuensis</i> CBS 116.80	AJ279997	D	G	B	P	L	II	
	<i>A. luchuensis</i> CBS 101.43	AJ279995	D	H	B	I	M	II	
	<i>A. japonicus</i> CBS 620.78	AJ280002	D	G	B	H	J	II	
	<i>A. japonicus</i> CBS 312.80	AJ279989	D	I	B	F	K	I	
	<i>A. aculeatus</i> CBS 172.66	AJ279988	D	J	B	H	N	I	
	<i>A. aculeatus</i> CBS 610.78	AJ280001	D	G	B	G	O	II	
	<i>A. aculeatus</i> CBS 186.67	AJ279998	D	G	B	J	P	II	
	<i>A. aculeatus</i> CBS 308.80	AJ279999	D	G	B	K	Q	II	
	<i>A. aculeatus</i> CBS 313.89	AJ280000	D	G	B	P	R	II	
	<i>A. bruno-violaceus</i> CBS 621.78 ^T	AJ280003	D	G	B	O	T	II	
	?	<i>A. aculeatus</i> CBS 114.80	AJ280005	D	K	C	Q	S	III
	<i>A. niger</i>	<i>A. niger</i> CBS 120.49	AJ280006	B	B I	B	B	B	
	<i>A. tubingensis</i>	<i>A. tubingensis</i> CBS 643.92	AJ280008	C	B II	A	C	D	
	<i>A. tubingensis</i>	<i>A. tubingensis</i> CBS 127.49	AJ280007	C	ND ^d	A	C	D	
<i>A. foetidus</i>	<i>A. foetidus</i> var. <i>acidus</i> CBS 564.65 ^T	AJ280009	C	B II	A	D	C		
<i>A. brasiliensis</i>	<i>A. brasiliensis</i> IMI 381727	AJ280010	F	E	D	B	G		
<i>A. carbonarius</i>	<i>A. carbonarius</i> CBS 111.26 ^{Tc}	AJ280011	A	A	C	A	A		
<i>A. heteromorphus</i>	<i>A. heteromorphus</i> CBS 117.55 ^T	AJ280013	E	D	A	E	F		
<i>A. ellipticus</i>	<i>A. ellipticus</i> CBS 707.79 ^T	AJ280014	E	C	A	E	E		

^a EMBL accession number for the ITS1-5.8S rRNA gene-ITS2 region sequence.

^b Reclassified by Pařenicová et al. (25).

^c T = type strain.

^d ND, not determined.

^e Neotype strain.

fungal strains when screening for specific metabolites or novel enzymes.

MATERIALS AND METHODS

Strains and plasmids. The strains used (Table 1) were obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. Plasmids carrying the pyruvate kinase-encoding *pkiA* gene of *A. niger* (pGW1100) (4), the pectin lyase A-encoding *pelA* gene of *A. niger* (pGW820) (12), a 0.9-kb *Eco*RI fragment of the 28S rRNA of *Agaricus bisporus* (pIM2131) (29), and the whole *A. bisporus* ribosomal DNA (rDNA) unit (pIM2132) (P. J. Schaap, unpublished results) were propagated in *Escherichia coli* DH5 α (38).

Isolation of genomic DNA, digestion, and gel electrophoresis. Genomic DNA isolation, digestion with the *Sma*I, *Xho*I-*Kpn*I, and *Pst*I-*Sal*I restriction enzymes, and agarose gel electrophoresis were carried out essentially as described previously (26). Instead of 0.7% (wt/vol) agarose gels, a 0.8% (wt/vol) agarose gel was used.

Southern blotting and hybridization. Transfer of DNA from agarose gels has been described previously (26). Radioactive labeling of the probes was carried out as described by Kusters-van Someren et al. (19). To identify DNA polymorphisms, the following probes were used for Southern blot hybridization: a 3.5-kb *Bam*HI-*Hind*III fragment of the *A. niger pkiA* gene comprising the entire coding region and the 5' and 3' noncoding regions; a 10-kb *Bam*HI fragment carrying the whole rDNA repeat of *A. bisporus*; a 0.9-kb *Eco*RI fragment containing the 3' end of the 28S rRNA of *A. bisporus* and a downstream sequence; and a 1.6-kb *Cla*I fragment of the *A. niger pelA* gene comprising part of the coding region and some of the adjacent 3' noncoding sequence (Fig. 1).

Hybridization and washing were carried out as described previously (26). After probing with the *pelA* probe, the blots carrying *Pst*I-*Sal*I-digested chromosomal DNA were stripped at 100°C in a 0.1% (wt/vol) sodium dodecyl sulfate solution and subsequently used for reprobing with the 28S rRNA probe.

PCR amplification. The ribosomal ITS1-5.8S rRNA gene-ITS2 region (length, approximately 600 bp) was amplified by using primers ITS4 and ITS5 (37). PCR amplification was performed in 50- μ l reaction mixtures containing 5 μ l (5 to 10 ng) of genomic DNA template, 2.5 U of *Ampli*Taq DNA polymerase (PE Corporation, Norwalk, Conn.), 1 μ M primer ITS4, 1 μ M primer ITS5, each deoxynucleoside triphosphate at a concentration of 50 μ M, 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 0.1 mg of bovine serum albumin per ml, 3 mM MgCl₂, 0.25% (vol/vol) Tween 20, and 10% (vol/vol) dimethyl sulfoxide. Amplification was performed with a GeneAmp 2400 PCR system (PE Corporation) by using the following temperature profile: initial denaturation at 94°C for 1 min, followed by 40 cycles of 15 s at 94°C, 1 min at 53°C, and 1 min at 72°C. Following amplification, PCR products were cleaned by using MicroSpin S-400 HR columns (Amersham Pharmacia Biotech, Uppsala, Sweden).

DNA sequencing and analysis. PCR products were sequenced by using primers ITS1, ITS2, ITS3, and ITS4 (37), a Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech, Little Chalfort, United Kingdom), and an automated sequencer (A.L.F. Express; Amersham Pharmacia Biotech) according to the manufacturer's instructions. Sequences were generated from both strands, and they were edited and initially aligned by using the CLUSTAL W multiple-sequence alignment program, version 1.6 (33). Manual corrections were included to improve the alignment by using the MacClade program (version 3.05; Sinauer Associates, Sunderland, Mass.).

The data matrix consisted of 541 aligned nucleotide characteristics, some of which were scored as deletions or unknowns for one or more taxa. Phylogenetic analysis was performed with the PAUP software (version 4.0b2; Sinauer Associates, Sunderland, Mass.) by using neighbor joining, maximum-likelihood distances, and bootstrapping as described previously (30).

Isolation of secondary metabolites and identification of these metabolites. The *Aspergillus* strains were cultured on the following media optimized for production of secondary metabolites: Czapek yeast autolysate agar and yeast extract sucrose agar (6). All cultures were incubated for 10 days in the dark at 30°C. For

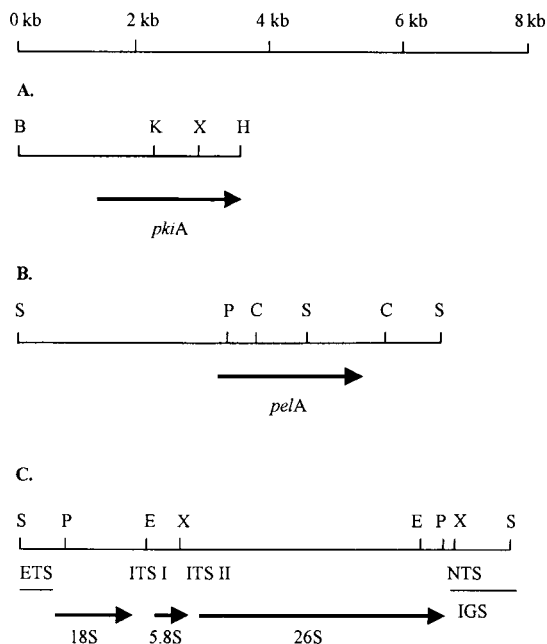


FIG. 1. Restriction endonuclease maps of the *Bam*HI-*Hind*III fragment of the *A. niger pkiA* gene (A), the *Sal*I fragment of the *A. niger pelA* gene (B), and the rDNA repeat from *A. niger* as described by O'Connell et al. (24) (C). NTS, nontranscribed spacer; ETS, external transcribed spacer; ITS, internal transcribed spacer; IGS, intergenic spacer region consisting of nontranscribed spacer and external transcribed spacer. Restriction endonuclease sites: S, *Sal*I; P, *Pst*I; E, *Eco*RI; X, *Xho*I; H, *Hind*III; K, *Kpn*I; B, *Bam*HI; C, *Cla*I.

metabolite analysis the content of each plate was extracted as previously described (5) and analyzed by high-performance liquid chromatography with diode array detection (7). The metabolites found were compared to a spectral UV library made from authentic standards examined under the same conditions, and the retention indices were compared with those of standards.

Nucleotide sequence accession numbers. The ITS1-5.8S rRNA gene-ITS2 sequences of the *A. japonicus* and *A. aculeatus* strains reported in this paper have been deposited in the EMBL Nucleotide Sequence Database under the accession numbers shown in Table 1. The EMBL accession numbers for the other black aspergilli that we used are as follows: *A. niger* CBS 120.49, AJ280006; *A. tubingensis* CBS 127.49, AJ280007; *A. tubingensis* CBS 643.92, AJ280008; *A. foetidus* var. *acidus* CBS 564.65^T, AJ280009; *A. brasiliensis* IMI 381727, AJ280010; *A. carbonarius* CBS 111.26^T, AJ280011; *Aspergillus heteromorphus* 117.55^T, AJ280013; and *Aspergillus ellipticus* CBS 707.79^T, AJ280014. The following other species were included in the study: *Aspergillus fumigatus* (accession no. AFO078889), *Aspergillus flavus* (AF138287), *Petromyces albertensis* (AJ005673), *Petromyces muricatus* (AJ005674), and *Neosartorya fischeri* (U18355).

RESULTS

Sequence analysis of the ITS1-5.8S rRNA gene-ITS2 region.

The ITS1-5.8S rRNA gene-ITS2 sequences from the 19 *A. japonicus* and *A. aculeatus* strains differed at only three nucleotide positions. Based on the ITS sequence differences, we divided the 19 *A. japonicus* and *A. aculeatus* strains into three distinct sequence types. Sequence type I comprised nine strains, sequence type II contained nine strains, and the third sequence type consisted of only one strain, *A. aculeatus* CBS 114.80. All sequence type I strains differed from the sequence type II strains by having a T instead of a C at positions 211 and 229. (The numbers refer to positions in the ITS1-5.8S rRNA gene-ITS2 sequence of sequence type I strain *A. japonicus* CBS

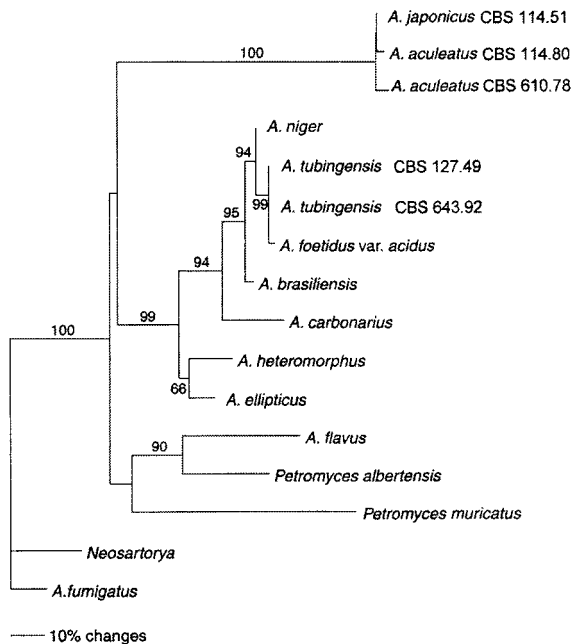


FIG. 2. Neighbor-joining tree based on phylogenetic analysis of the ITS1-5.8S rRNA gene-ITS2 sequences. The numbers at branch points are the percentages of 1,000 bootstrapped data sets that supported the specific internal branches. Bootstrap values less than 50% are not shown. For the phylogenetic analysis, *A. japonicus* CBS 114.51^T was used as a representative of sequence type I, *A. aculeatus* CBS 610.78 was used as a representative of sequence type II, and *A. aculeatus* CBS 114.80 was the sequence type III strain. Bar = 10% estimated sequence divergence.

114.51^T, which starts at the 5' end of conserved primer ITS5 [37].) The ITS sequence of *A. aculeatus* CBS 114.80 (sequence type III) was identical to the sequences of the type I strains except for a single nucleotide (C instead of T) at position 189. Except for three strains (*A. japonicus* CBS 312.80, *Aspergillus lucknowensis* CBS 119.49, and *A. aculeatus* CBS 172.66) which had a type I sequence but which on the basis of RFLP and secondary-metabolite analysis data belonged to *A. aculeatus*, the sequence type I strains were *A. japonicus* isolates. Sequence type II strains were found only in the *A. aculeatus* taxon, not in the *A. japonicus* taxon. Since the region sequenced is so highly conserved, sequence data for the ITS1-5.8S rRNA gene-ITS2 region are indicative but not definitive.

We identified a significant number of differences when the ITS1-5.8S rRNA gene-ITS2 sequences of the 19 *A. japonicus* and *A. aculeatus* strains were compared with the corresponding sequences of other black aspergilli (Table 1). The highest degree of ITS sequence dissimilarity (approximately 19%) was found when we compared the *A. japonicus* and *A. aculeatus* strains with the strains belonging to the *A. niger* aggregate (1). We inferred phylogenetic relationships (Fig. 2) among the black aspergilli based on a neighbor-joining analysis of the aligned ITS sequences. The closely related species *A. fumigatus* and *N. fischeri* were designated outgroups based on comparisons of 18S rRNA gene sequences with ARB (<http://www.mikro.biologie.tu-muenchen.de/pub/ARB/documentation/arb.ps>). *A. flavus* and two *Petromyces* species were included in the

TABLE 2. Band patterns of nuclear rDNA obtained after *Sma*I digestion

Pattern	Species	Sizes of UV-visualized and hybridizing bands (kb)
A	<i>A. carbonarius</i>	4.1 , 2.3 ^a
B	<i>A. niger</i>	3.2 , 2.3 , 1.8
C	<i>A. tubingensis</i> , <i>A. foetidus</i> var. <i>acidus</i>	2.6 , 1.9 , 1.8
D	<i>A. japonicus</i> , <i>A. aculeatus</i>	1.9 , 1.8 , 0.9
E	<i>A. ellipticus</i> , <i>A. heteromorphus</i>	1.9 , 1.8 , 1.6
F	<i>A. brasiliensis</i>	2.1 , 2.1 , 1.8

^a Boldface type indicates bands that gave a hybridization signal in the Southern blot analysis.

analysis based on comparisons of morphology and secondary-metabolite profiles. The analysis showed that the *A. japonicus* group and *A. aculeatus* CBS 114.80 form a well-supported clade along with the *A. aculeatus* group. This clade represents the uniseriate black aspergilli and was clearly separated from the biseriata black aspergilli; i.e., high bootstrap support (99%) was found for the branch leading to the cluster including the *A. niger* aggregate strains, *A. brasiliensis*, *A. carbonarius*, and the *A. heteromorphus*-*A. ellipticus* clade.

RFLP analysis: *Sma*I digestion and rDNA polymorphism. The rDNA repeat (Fig. 1C) is present at a level of 100 to 300 copies per haploid fungal genome (3). We previously reported (26) that an *Sma*I digest of nuclear DNA yielded five distinct rDNA band patterns. That analysis did not include *A. brasiliensis*. We used the 10-kb rDNA repeat of *A. bisporus* in a Southern blot analysis of *Sma*I-digested nuclear DNA to further characterize these patterns and identified six band patterns (Table 2). All the *A. japonicus* and *A. aculeatus* isolates produced the same pattern (pattern D). The *Sma*I-generated rDNA band pattern of *A. brasiliensis* (Table 2, pattern F) (35), is also clearly different from those established previously (26).

Analysis of the 26S rRNA locus. We also used the 3' portion of the *A. bisporus* 28S rRNA gene that included a downstream sequence of the nontranscribed spacer region as a probe. When we digested the DNA with *Xho*I and *Kpn*I and probed the preparation with the *A. bisporus* 0.9-kb 28S rRNA *Eco*RI fragment, the *A. japonicus* and *A. aculeatus* isolates were separated into two groups (Table 1). We detected four patterns (patterns A to D), each containing a single hybridizing band (7.5, 7, 5, or 4 kb) (Fig. 3). Three *A. japonicus* isolates, one *Aspergillus atroviolaceus* isolate, and one *A. aculeatus* isolate produced pattern A, as did CBS 101.14. Since type strain *A. japonicus* CBS 114.51 also produced pattern A, we concluded that these strains represent the *A. japonicus* taxon. The remaining 12 strains, (five *A. aculeatus* strains, three *A. japonicus* strains, two *Aspergillus luchuensis* strains, one *A. lucknowensis* strain, and one *Aspergillus bruneo-violaceus* strain) all produced pattern B. These strains were grouped into the *A. aculeatus* taxon. One *A. aculeatus* isolate, CBS 114.80, produced a third pattern, pattern C. If we used the same 0.9-kb *Eco*RI 28S rRNA probe to identify the RFLP patterns in *Pst*I-*Sal*II digests of nuclear DNA, then more variation was detected (Fig. 4 and Table 1). A 6.0-kb hybridizing band was found in each of the 11 patterns typical of the *A. japonicus* and *A. aculeatus* isolates (patterns F to P), and this band distinguished the uniseriate taxa from the other black aspergilli (Fig. 4, lanes A to E). The

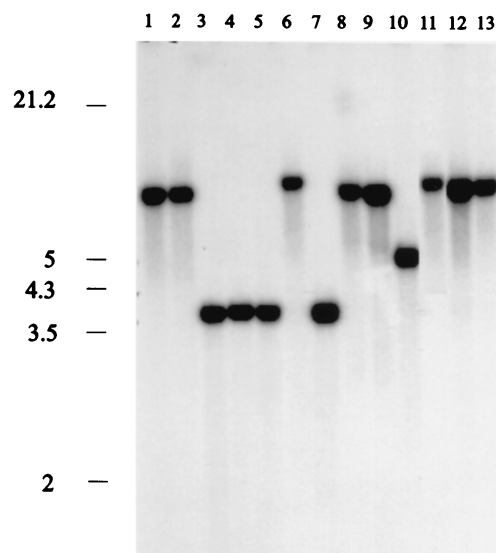


FIG. 3. RFLP patterns obtained after digestion of nuclear DNA with *Kpn*I and *Xho*I and probing with the 0.9-kb 28S rRNA *Eco*RI fragment of *A. bisporus*. The sizes (in kilobases) of λ DNA fragments digested with *Eco*RI and *Hind*III are indicated on the left. The hybridizing bands correspond to the following isolates: lane 1, *A. japonicus* CBS 114.51^T; lane 2, *A. aculeatus* CBS 611.78; lane 3, *A. luchuensis* CBS 101.43; lane 4, *A. aculeatus* CBS 308.80; lane 5, *A. bruneo-violaceus* CBS 621.78^T; lane 6, *A. aculeatus* CBS 114.80; lane 7, *A. niger* CBS 120.49; lane 8, *A. tubingensis* CBS 643.92; lane 9, *A. foetidus* var. *acidus* CBS 564.65^T; lane 10, *A. brasiliensis* IMI 381727; lane 11, *A. carbonarius* CBS 111.26^T; lane 12, *A. heteromorphus* CBS 117.55^T; and lane 13, *A. ellipticus* CBS 707.79^T. The patterns in Table 1 correspond to the following sizes of hybridizing bands: pattern A, 7.0 kb; pattern B, 4.0 kb; pattern C, 7.5 kb; and pattern D, 5.0 kb.

small hybridizing bands detected in the different patterns resulted from DNA sequence variation in the nontranscribed spacer of the rDNA repeat (Fig. 1C). Thus, digestion with *Pst*I plus *Sal*II highlighted the intragroup variations among the isolates of *A. japonicus* and *A. aculeatus*. It is obvious that the nontranscribed spacer region of the *A. japonicus* and *A. aculeatus* isolates frequently undergoes DNA rearrangements, including introduction of small insertions or deletions, given the numerous small size variations in the patterns. *A. aculeatus* CBS 114.80 produced only one hybridizing band at 8.0 kb (Fig. 4, lane Q).

Analysis of the *pkiA* locus. The *A. japonicus* and *A. aculeatus* isolates produced patterns distinct from those of the other black aspergilli (Table 1) when they were probed with the 3.5-kb *Bam*HI-*Hind*III fragment (Fig. 1A) containing the entire coding region of the *A. niger pkiA* gene and the 5' and 3' noncoding sequences. Isolates identified as *A. japonicus* based on the 26S rRNA RFLPs produced a unique pattern, pattern F (5.3, 4.8, and 0.5 kb) (Table 1). All of the RFLP patterns for the remaining uniseriate strains (Table 1) contained two (7.5 and 0.4 kb) of three hybridizing bands (Table 1). The 0.4-kb hybridizing fragment probably corresponded to a conserved internal *Kpn*I-*Xho*I fragment (Fig. 1A) of the *pkiA* gene equivalent, so the differences among the patterns were probably due to changes in one of the restriction sites in the 5' or 3' noncoding regions. The *A. brasiliensis pkiA* pattern also was different, although it had the 7.5- and 0.4-kb hybridizing bands. *A.*

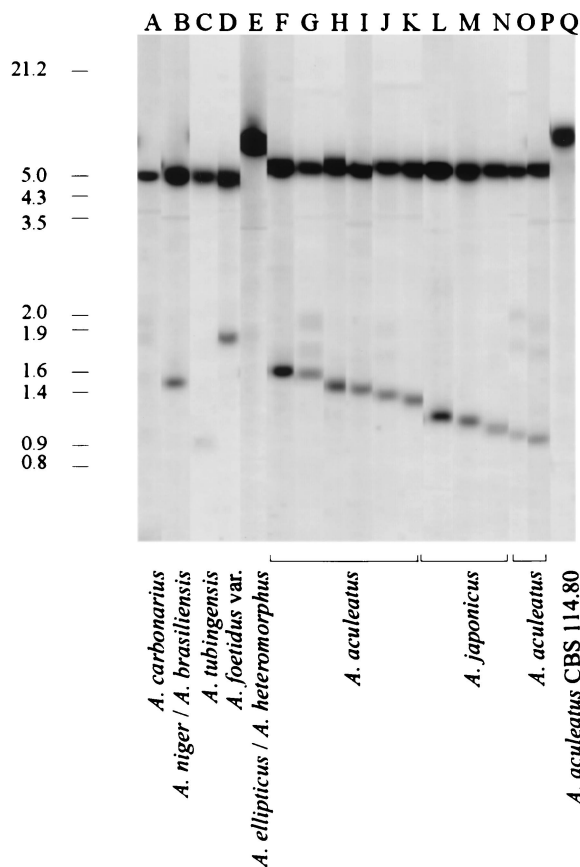


FIG. 4. RFLP patterns for different black aspergilli (lanes A to E) and *A. japonicus* and *A. aculeatus* isolates (lanes F to Q) after digestion of nuclear DNA with *Pst*I plus *Sal*I and probing with the 0.9-kb 28S rRNA *Eco*RI fragment of *A. bisporus*. The sizes (in kilobases) of bands corresponding to bands of λ DNA digested with *Hind*III and *Eco*RI are indicated on the left. The estimated sizes of the small hybridizing bands in *A. aculeatus* and *A. japonicus* band patterns F to P in Table 1 are as follows: pattern F, 1.65 kb; pattern G, 1.60 kb; pattern H, 1.55 kb; pattern I, 1.50 kb; pattern J, 1.45 kb; pattern K, 1.40 kb; pattern L, 1.35 kb; pattern M, 1.30 kb; pattern N, 1.20 kb; pattern O, 1.10 kb; and pattern P, 1.05 kb. The black aspergilli that produce patterns A to P (Table 1) are shown at the bottom.

aculeatus CBS 114.80 produced a pattern that was different from the *A. japonicus* and *A. aculeatus* patterns and contained three hybridizing *pki* bands (6.7, 1.2, and 0.5 kb) (Table 1, pattern K).

DNA polymorphism at the *pelA* locus. We used the 1.6-kb *Cla*I fragment of the *A. niger pelA* gene in Southern blot hybridization of *Pst*I-*Sal*I-digested nuclear DNA. The *A. japonicus* and *A. aculeatus* isolates produced 13 different patterns. Five of the strains considered to belong to *A. japonicus* (Table 1) produced pattern H, which shared five of six hybridizing bands with pattern I produced by *A. aculeatus* CBS 611.78. In the final grouping (Table 1) this strain was classified as an *A. japonicus* isolate. The remaining *pelA* patterns were produced by isolates classified as *A. aculeatus*. They all contained a 0.8-kb hybridizing band.

Profiles of secondary metabolites. The strains grouped into the *A. japonicus* taxon, all produce several unknown secondary metabolites (Table 3). All of the strains except CBS 114.51^T

produce several polar metabolites with end absorption near 200 nm. One of these metabolites may be the thiol protease inhibitor E-64 previously described by Hanada et al. (10, 11). This result should be confirmed by comparison to an authentic standard. Three strains of *A. japonicus* produce festuclavine (Table 3), as reported previously for another strain of this species (8). The remaining strains produce other indole alkaloids, but we could not determine whether these metabolites included cycloclavin (8). *A. japonicus* CBS 114.51^T and CBS 568.65, *A. japonicus* CBS 522.89, and *A. aculeatus* CBS 611.78 all produce a secondary metabolite with the same characteristic chromophore.

The strains in the *A. aculeatus* group also produce several unknown secondary metabolites, but they have the same secondary-metabolite profile, which allows them to be grouped together. The profiles of these strains are characterized by the presence of nitrogen-containing secondary metabolites (e.g., neoxaline, okaramins, and paraherquamidelike compounds) and secalonic acid (2). Three of the isolates, *A. japonicus* CBS 620.78, *A. japonicus* CBS 312.80, and *A. aculeatus* CBS 172.66, do not produce any nitrogenous secondary metabolites, but they all produce, large quantities of an unknown secondary metabolite with a characteristic chromophore. In contrast to the report of Hirano et al. (14), neoxaline is produced by *A. aculeatus* but not by *A. japonicus*.

The grouping of the strains based on the secondary-metabolite analysis is the same as that based on the RFLP analyses. *A. aculeatus* CBS 114.80 produces some of the same secondary metabolites as both *A. aculeatus* and *A. japonicus*, but it also produces a series of secondary metabolites with unique chromophores. Taking all of the data into account, we think that this strain probably represents a new species.

DISCUSSION

Using a combination of restriction enzymes (viz., *Sma*I, *Kpn*I-*Xho*I, and *Pst*I-*Sal*I) and the rDNA repeat, 28S rRNA, and the *pkiA* and *pelA* genes as probes, we distinguished 10 different taxa among the black aspergilli (Table 1). The number of hybridizing bands which we observed when the rDNA repeat of the basidiomycete *A. bisporus* was used as a probe differed from the numbers of bands previously reported for the black aspergilli (9, 21). We think that the difference was due to the origin of the rDNA probe, since the two other groups of workers used the *Aspergillus nidulans* rDNA repeat in their hybridization experiments. The *A. brasiliensis* IMI 381727 RFLP patterns are clearly distinct from those of *A. niger*, *A. tubingensis*, and representatives of the *A. foetidus* varieties and thus support previous suggestions (35) that isolate IMI 381727 should be classified as a new species. Furthermore, the uniseriate *A. japonicus* and *A. aculeatus* isolates can be clearly separated into two distinct taxa. However, this division does not support the classification of individual isolates based on morphological criteria. Based on the sequence data for the ITS1-5.8S rRNA gene-ITS2 region and the data from the RFLP and secondary-metabolite analyses, we identified a third uniseriate taxon among the black aspergilli, and this taxon was represented by CBS 114.80. The 0.8-kb band hybridizing with the *pelA* probe probably corresponds to the *pelA* equivalent in *A. aculeatus* (Fig. 1B). The remaining hybridizing bands probably

TABLE 3. Classification of isolates of *A. aculeatus* and *A. japonicus* taxa based on production of known secondary metabolites^a

Strain(s)	Secondary metabolite(s)
<i>A. japonicus</i> taxon	
<i>A. japonicus</i> CBS 101.14.....	Unknown indole alkaloid ^b
<i>A. atroviolaceus</i> CBS 113.48 ^T	Festuclavin
<i>A. japonicus</i> CBS 114.51 ^T	Unknown indole alkaloid ^b
<i>A. japonicus</i> CBS 568.65.....	No known secondary metabolites ^c
<i>A. aculeatus</i> CBS 611.78.....	Festuclavin
<i>A. japonicus</i> CBS 522.89.....	Unknown indole alkaloid ^b
<i>A. aculeatus</i> taxon	
<i>A. bruneo-violaceus</i> CBS 621.78.....	Neoxaline, okaramins ^d
<i>A. lucknowensis</i> CBS 119.49.....	Neoxaline, secalonic acid D, okaramins, paraherquamidelike compounds
<i>A. luchuensis</i> CBS 116.80.....	Neoxaline, secalonic acid D, okaramins, paraherquamidelike compounds
<i>A. luchuensis</i> CBS 101.43.....	Neoxaline, secalonic acid D, okaramins, paraherquamidelike compounds
<i>A. japonicus</i> CBS 620.78.....	Secalonic acid D
<i>A. japonicus</i> CBS 312.80.....	No known secondary metabolites ^c
<i>A. japonicus</i> CBS 114.34.....	No known secondary metabolites ^c
<i>A. aculeatus</i> CBS 610.78.....	Neoxaline, secalonic acid D, paraherquamidelike compounds
<i>A. aculeatus</i> CBS 186.67.....	Neoxaline, secalonic acid D, paraherquamidelike compounds
<i>A. aculeatus</i> CBS 308.80.....	Neoxaline, secalonic acid D
<i>A. aculeatus</i> CBS 313.89.....	Secalonic acid D, paraherquamidelike compounds
New species	
<i>A. aculeatus</i> CBS 114.80.....	Secalonic acid D, okaramins A, B, H, and I ^e
Other taxa in section <i>Nigri</i>	
<i>A. niger</i> CBS 120.49.....	Tetracyclic compounds, naphtho-4-pyrones
<i>A. niger</i> CBS 554.65.....	Tetracyclic compounds, naphtho-4-pyrones, orlandin
<i>A. niger</i> CBS 618.78 and CBS 139.52.....	Tetracyclic compounds, naphtho-4-pyrones, orlandin, ochratoxin A
<i>A. tubingensis</i> CBS 643.92.....	Tetracyclic compounds, naphtho-4-pyrones, nigragillin, orlandin
<i>A. foetidus</i> var. <i>acidus</i> CBS 564.65 ^T	No known secondary metabolites ^c
<i>A. brasiliensis</i> IMI 381727.....	Tetracyclic compounds
<i>A. carbonarius</i> CBS 111.26 ^T	Tetracyclic compounds, naphtho-4-pyrones, ochratoxin A
<i>A. heteromorphus</i> CBS 117.55 ^T	No known secondary metabolites ^c
<i>A. ellipticus</i> CBS 707.79 ^T	Austdiol, terphenyllins, candidusin

^a All isolates produce several additional unknown secondary metabolites.

^b Production of cycloclavine is uncertain.

^c The isolate produces members of more than three different chromophore families of unknown secondary metabolites.

^d The okaramins produced by *A. aculeatus* also appear to be okaramins A, B, H, and I (13), but the new species represented by CBS 114.80 is a particularly good producer of these secondary metabolites.

^e CBS 114.80 and CBS 117.55^T each produce members of several chromophore families of secondary metabolites that are unique to the isolate.

reflect DNA polymorphism in the 5' and 3' *pelA* noncoding regions and in other pectin lyase-encoding genes that also hybridize (19, 20). Of all the black aspergilli, the *A. aculeatus* taxon has the highest degree of variation in the pectin lyase-encoding genes. This taxon is well known for its ability to produce a variety of pectinases.

The other common taxa in section *Nigri* typically produce one or several of the following secondary metabolites: tetracyclic compounds, naphtho-4-pyrones, orlandin, nigragillin, and ochratoxin A. None of these compounds have been found in *A. aculeatus*, *A. japonicus*, or strain CBS 114.80 (Table 3). *A. foetidus* var. *acidus* CBS 564.65 and *A. niger* produce identical unknown compounds, whereas *A. heteromorphus* and *A. ellipticus* have a unique position in section *Nigri*; each of the latter species has only one secondary metabolite biosynthetic family in common with *A. niger* and/or related species and *A. aculeatus* or related species. For example, *A. heteromorphus* produces members of seven chromophore families of secondary metabolites; one of these is also produced by *A. ellipticus*, CBS 114.80, and *A. japonicus*, and another is also produced by *A. niger* and *A. foetidus* var. *acidus*. It is important to note that even though several additional isolates of each taxon were examined by high-performance liquid chromatography, the carcinogenic nephrotoxin ochratoxin A was detected only in isolates of *A. niger* and *A. carbonarius*.

In addition to *A. niger*, other black aspergilli also are important in biotechnological processes. During screening and commercialization of new *Aspergillus* isolates as metabolite and enzyme producers, their taxonomic positions must be firmly established. Morphological criteria are unreliable and inconsistent for identifying isolates of closely related species. This study demonstrates the power of combined molecular and biochemical methods and provides criteria for precise identification for this economically important group of fungi.

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