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The use of RFLP analysis in classification of the black Aspergilli: reinterpretation of the *Aspergillus niger* aggregate

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Summary. By studying ribosomal banding patterns in ethidium bromide-stained gels of chromosomal digests we are able to provide a rapid and reliable classification of a number of taxonomically important isolates of the black Aspergilli. This classification is supported by Southern blots using several pectin lyase genes, isolated from *A. niger* CBS 120.49, as probes. Taxonomy on the basis of RFLP analysis leads to a classification which is completely different from, but more reliable than, the current one which is mainly based on morphological characteristics. The 23 *Aspergillus niger* isolates investigated could be divided into two distinct groups on the basis of our results. We propose that these groups represent two different species: *A. niger* and *A. tubigensis*. This is supported by preliminary results showing failure of heterokaryon formation between typical representatives of both groups.

Key words: *Aspergillus niger* – Taxonomy – RFLP analysis – Pectin lyase genes – Ribosomal DNA

Introduction

Black Aspergilli (e.g., *A. niger* and *A. awamori*) are widely used and studied for industrial purposes. Since *A. niger* holds the GRAS (Generally Regarded As Safe) status it is the preferred species for the production of organic acids or extracellular enzymes to be used in the food industry (Pariza and Foster 1983; Upshall 1986). On the other hand, black Aspergilli are common in the tropics and subtropics where they cause date-rot and onion-rot. It is, therefore, important to be able to unambiguously identify and classify these species.

Identification up to now is based on morphological characteristics, such as conidial shape, colour and size. Thom and Raper (1945) and Raper and Fennell (1965) divided the black Aspergilli into 15 and 12 species respectively. In 1980, Al-Musallam revised this classification and recognized five readily distinguishable species and an *A. niger* aggregate which she subdivided into seven varieties. However, the taxonomy remains debatable and new, more objective, criteria are needed. Therefore, ethidium bromide-stained gels were used to investigate restriction fragment length polymorphisms (RFLPs) in ribosomal DNA. Additionally, *A. niger* genes were used as probes to detect RFLPs in the chromosomal DNA of the black Aspergilli.

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Materials and methods

Strains and plasmids. All *Aspergillus* strains, except NW756, were obtained from the Centraalbureau voor Schimmelcultures in Baarn, The Netherlands, and are shown in Table 1. NW756 is an industrial strain which belongs to the *A. niger* aggregate, but has not been further classified according to the outline proposed by Al-Musallam (1980). Plasmids on which the pectin lyase-encoding genes, *pel*A, *pel*B and *pel*D, are situated have been described by Harmsen et al. (1990).

Isolation of chromosomal DNA. Cultures were grown by inoculating 10^6 spores/ml in complete medium (Pontecorvo et al. 1953) using 1% glucose as a carbon source. The mycelium was grown for 20 h in 250 ml flasks containing 50 ml medium using a rotary shaker at 30 °C. Chromosomal DNA was isolated as described by de Graaff et al. (1988).

Digestion of chromosomal DNA. Using the appropriate buffer (Gibco Europe Breda) and temperature, 2 µg chromosomal DNA was digested for 4 h in a total volume of 200 µl. Forty Units of restriction enzyme (Gibco Europe, Breda) were used per digestion.

DNA manipulations. Plasmid DNA isolation, recovery of restriction fragments from agarose gels, and Southern blotting were performed as described by Maniatis et al. (1982).

Labelling of DNA fragments. From 50 to 100 ng of the appropriate restriction fragment was heated for 3 min at 100 °C, together with 50 ng hexanucleotides (Pharmacia, Uppsala, Sweden) in a total volume of 10 µl 100 mM Tris-HCl pH 8.0, 50 mM $MgCl_2$. The mixture was cooled on ice and then dGTP, dCTP and dTTP (end concentration 3.3 mM each), 1 U Klenow polymerase (Gibco Europe, Breda) and 50 µCi α-^{32}PdATP (3000 Ci/mmol) as well as sterile bidest, were added, to a total volume of 20 µl. After incubation for 30 min at 30 °C, non-incorporated nucleotides were removed by spin-column chromatography through Sephadex G50 (Pharmacia, Uppsala, Sweden) in 10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 7.5 (Maniatis et al. 1982). Just before use, the probe was heated for 5 min at 100 °C.

Southern hybridization. After electrophoresis, DNA was transferred to nitrocellulose (Schleicher and Schuell, Hertogenbosch, Nederland) by Southern blotting. Prehybridization was for 30 min at the hybridization temperature in standard hybridization buffer (Maniatis et al. 1982) containing 6 × SSC (1 × SSC consists of 0.15 M NaCl, 0.015 M sodium citrate). After addition of the denatured probe, hybridization was continued overnight at 65 °C. Washes (30 min each) were also done at 65 °C; twice using 2 × SSC, 0.5% SDS, then twice using 0.2 × SSC, 0.5% SDS.

Heterokaryosis. Complete medium (Pontecorvo et al. 1953), containing 25 mM sucrose, 10 mM uridine and 0.0068% adenine, was inoculated with spores from both 182.22 and N828. After 24 h of growth at 30 °C a mycelium mat is formed. The mat was washed in saline (0.9% NaCl), several pieces of 4 mm^2 were cut out, placed in a petridish containing minimal medium agar and 25 mM sucrose, and grown for 7 days at 30 °C. Heterokaryosis should then be visible as a sector of sporulating mycelium growing at normal rate, carrying both fawn and black conidia.

Sequence homology. Hybridizing fragments of strains were pairwise compared, combining the data from different Southern blots. Besides the Southern blot data shown in this paper we also used data from a blot comparable to the one shown in Fig. 3a but which contained DNA from an additional number of strains not shown in Fig. 3a. The homology percentages were determined as proportions of base substitutions by comparing conserved hybridizing fragments with all hybridizing fragments of a strain pair (Nei and Li 1979). All visible hybridizing fragments were taken into consideration except those which were clearly the result of partial digestion of the DNA, and four different very weak bands in strains 15, 16, 23 and 223 (Fig. 3c) which occur only in those strains.

Results

Table 1 shows the 36 isolates used in our study, their systematic names at the time they came into possession of the Centraalbureau voor Schimmelcultures, and the way they were named by Al-Musallam (1980). She classified a large number of black Aspergilli which previously were thought to represent different species as *A. niger* or varieties thereof (Thom and Raper 1965). We have given each strain a number (see Table 1) which we will use throughout this paper for clarity.

Analysis of A. niger strains

Figure 1 shows an ethidium bromide-stained agarose gel containing chromosomal DNA of a number of *A. niger* strains digested with *Sma*I. The ribosomal banding patterns, produced by digestion of the highly repetitive DNA (100–300 times per haploid genome) encoding the ribosomal RNAs, are clearly visible. The organization of the ribosomal RNA gene cluster in *A. niger* is not known. In *A. nidulans*, however, tandem repeats of 7.8 kb contain 5.8S, 18S and 26S rDNA, whereas the 5S rDNA is located elsewhere in the genome (Borsuk et al. 1982). If the lengths of the coding regions of these rDNAs are comparable to those from yeast, they should total 5.3 kb (Rubtsev et al. 1980; Erdmann 1980; Philippsen et al. 1978), thus leaving 2.5 kb as untranscribed spacer regions. Digestion with restriction enzymes recognizing sequences within the coding regions should mostly result in species-specific restriction fragments. However, if the restriction enzymes cut in the untranscribed spacer, this may lead to RFLPs within a species.

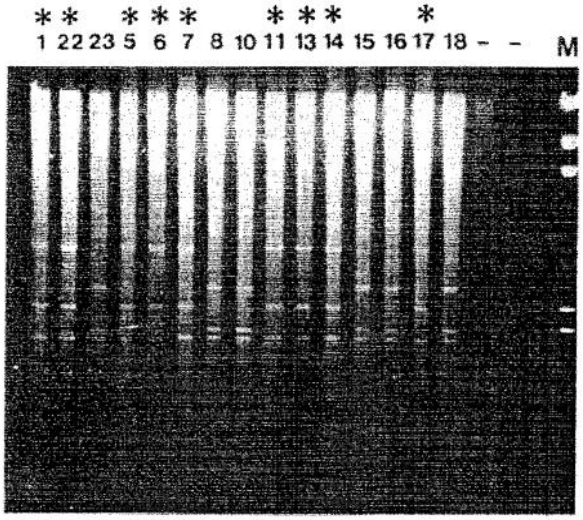

Fig. 1. Ethidium bromide-stained agarose gel with chromosomal DNA digested with *Sma*I. Strain numbers as indicated in Table 1. *, Group I strains. *M*, lambda DNA digested with *Hin*dIII

On the basis of their RFLP patterns, the *A. niger* isolates can be divided in two groups, as indicated in Table 2. *A. niger* CBS 120.49 is identical to our laboratory strain N400. All strains with the same pattern as CBS 120.49 (strain 22) are named group I strains. The others, named group II strains, also show an identical pattern, which is, however, different from that of group I (Fig. 1). A number of other restriction enzymes were also used (data not shown), some resulting in RFLPs within a group. This may be explained by assuming that the restriction sites recognized by these enzymes are situated in the non-transcribed spacer. There are also restriction enzymes with which group I and group II strains show similar patterns, showing the strong relatedness between these two groups.

A Southern blot of the gel from Fig. 1 was probed with the *A. niger* N400 (strain 22) pectin lyase gene *pel*B (Harmsen et al. 1990). A simplified restriction map of that gene is shown in Fig. 2a. If the DNA is digested with *Sma*I, four hybridizing fragments are to be expected (1.0, 0.8, 1.1 and larger than 0.9 kb) for *A. niger* N400 (strain 22, Fig. 3a). Some other strains examined have these same four bands and also belong to group I (strains 5 and 11 in Fig. 3a, strain 35 not shown). The other group I strains, however, lack the 0.8 kb and 1.1 kb bands. Instead, they show a different band of 1.9 kb, which is the added lengths of the 1.1 and 0.8 kb fragments (strains 1, 6, 7, 13, 34 and 17 in Fig. 3a). Therefore, this RFLP can be explained by a single mutation in one *Sma*I site, although there may of course be other (point) mutations. The group II strains, however, show a pattern which is completely different from those of group I. This is a good example of why a probe containing a well-characterized gene is to be preferred over a random probe. Knowing the restriction map of the *pel*B gene and its flanking sequences, it is easy to attribute a RFLP pattern to a point mutation. Digestion of chromosomal DNA with *Xho*I leads to the expected 3 kb band for *A. niger*

Table 1. Strains used and their classification

Type/ neotype	Origin	CBS number	ATCC number	Al-Musallam (1980)	Number[a]
NT	*A. niger*	554.65	16 888	*A. niger var. niger*	1
T	*A. pseudoniger*	128.48			2
T	*A. tubigensis*	134.48			3
T	*A. pseudocitricus*	127.48			4
NT	*A. awamori*	557.65	16 877	*A. niger var. awamori*	5
T	*A. foetidus*	563.65	16 878		6
	'A. phoenicis'	126.49	10 698	*A. niger var. phoenicis*	7
	'A. tubigensis'	135.48			8
T	*A. satoi-kagoshimaensis*	137.52	11 363		9
T	*A. satoi*	136.52	11 362	*A. niger var. nanus*	10
	'A. nanus'	131.52	6 275		11
	'A. fuliginosus'	117.48			12
T	*A. usamii*	139.52	11 364	*A. niger var. usamii*	13
	'A. usamii'	553.65	16 880		14
	'A. tubigensis'	559.65		*A. niger var. intermedicus*	15
	'A. niger'	117.32			16
T	*A. hennebergii*	118.35		*A. niger var. hennebergii*	17
T	*A. inuii*	125.52	11 360		18
T	*A. cinnamomeus*	103.12	1 027		19
T	*A. kawachi*	117.80			20
T	*A. schiemannii*	122.28	1 040		21
	'A. usamii'	120.49		*A. niger var. usamii* N400	22
	unknown			*A. niger* NW756	23
NT	*A. carbonarius*	111.26	1 025	*A. carbonarius*	24
	'A. carbonarius'	127.49			25
	'A. carbonarius'	420.64			26
T	*A. ellipticus*	707.79		*A. ellipticus*	27
T	*A. helicothrix*	677.79		*A. helicothrix*	28
T	*A heteromorphus*	117.55	12 064	*A. heteromorphus*	29
T	*A. japonicus*	114.51		*A. japonicus var. japonicus*	30
T	*A. brunneo-violaceus*	621.78			31
	'A. aculeatus'	172.66	1 687	*A. japonicus var. aculeatus*	32
T	*A. yezoensis*	115.80			33
	'A. luchuensis'	119.49			34
T	*A. aureus*	121.28		*A. foetidus*	35
	'A. foetidus'	618.78			36

[a] Each strain is given a number which is used throughout this paper for clarity

Table 2. Classification of the *A. niger* aggregate on the basis of RFLP analysis of rDNA

Strain number	Group I		CBS number	Strain number	Group II		CBS number
1	NT	*A. niger*	554.65	2	T	*A. pseudoniger*	128.48
4	T	*A. pseudocitricus*	127.48	3	T	*A. tubigensis*	134.48
5	NT	*A. awamori*	557.65	8		*'A. tubigensis'*	135.48
6	T	*A. foetidus*	563.65	9	T	*A. satoi-kagoshimaenis*	137.52
7		*'A. phoenicis'*	126.49	10	T	*A. satoi*	136.52
11		*'A. nanus'*	131.52	12		*'A. fuliginosus'*	117.48
13	T	*A. usamii*	139.52	15		*'A. tubigensis'*	559.65
14		*'A. usamii'*	553.65	16		*'A. niger'*	117.32
17	T	*A. hennebergii*	118.35	18	T	*A. inuii*	125.52
20	T	*A. kawachi*	117.80	19	T	*A. cinnamomeus*	103.12
22		*'A. usamii'*	120.49	21	T	*A. schiemannii*	122.28
35	T	*A. aureus*	121.28	23		unknown	
36		*'A. foetidus'*	618.78	25		*'A. carbonarius'*	127.49

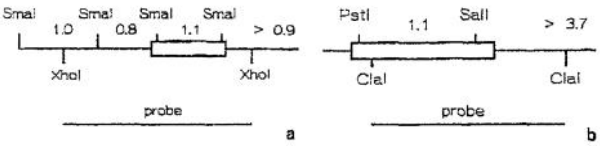

Fig. 2a, b. Simplified restriction maps of *A. niger* N400 pectin lyase genes, showing the lengths of the expected hybridized fragments if the indicated restriction enzymes and probes are used. **a** *pel*B gene; **b** *pel*A gene

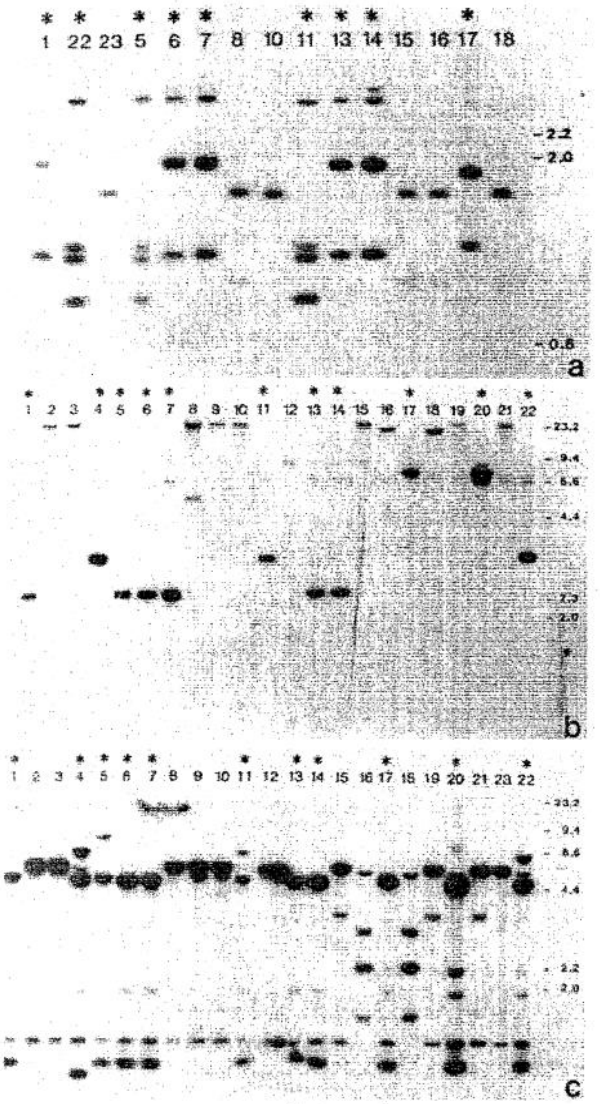

Fig. 3a–c. Southern blots of genomic DNA digests. **a** Blot from the gel shown in Fig. 1a. Genomic DNA digested with *Sma*I, probed with the *Xho*I fragment of the *A. niger* N400 *pel*B gene (Fig. 2a); **b** genomic DNA digested with *Xho*I, probed with the *Xho*I fragment of the *A. niger* N400 *pel*B gene (Fig. 2a); **c** genomic DNA digested with *Pst*I and *Sal*I, probed with the *Cla*I fragment of *pel*A (Fig. 2b). Strain numbers as indicated in Table 1. The lengths of the marker DNA fragments (lambda DNA digested with *Hin*dIII) are shown in kb. *, Group I strains

N400 on the Southern blot (Fig. 3b, strain 22). The other, weaker, band which hybridizes is the result of heterologous hybridization with the *pel*A gene, known to be related to the *pel*B gene (Kusters-van Someren et al., unpublished results). The upper bands are probably caused by partial digestion of the DNA. Again, there is variation in the lengths of the hybridizing bands in group I strains, but the distinction between group I and group II strains is still striking. All hybridizing bands of group I strains containing the *pel*B gene are more intense than any of the group II bands. There is also some variation amongst group II strains (e.g., strains 15 and 16).

If the *pel*A gene (Fig. 2b) is used as a probe, this leads to similar results. Chromosomal DNA digested with *Pst*I and *Sal*I has been probed with the *pel*A-containing *Cla*I fragment (Fig. 3c). For *A. niger* N400 (strain 22) two bands are expected, a band larger than 3.7 kb and a band of 1.1 kb. A third band which can be seen (1.4 kb) is the result of heterologous hybridization with the *A. niger pel*D gene which is highly homologous with the *pel*A gene (Kusters-van Someren et al., unpublished results). Two other weaker bands which occur, and which can also be seen in strains 17 and 20, are probably the result of hybridization with other related genes. All group I strains have the same high molecular weight band as *A. niger* N400, whereas all group II strains contain a fragment of an even higher molecular weight. Additionally, with the exception of strain 4 which has a slightly smaller band, all group I strains have the 1.1 kb *pel*A band. Except for strains 16 and 18, the conservation of the *pel*D band (1.4 kb) throughout group I and II strains is especially striking. A previous RFLP analysis of a number of black Aspergilli using the *pel*D gene as a probe, but with other restriction enzymes to digest the genomic DNA, showed similar results (Kusters-van Someren et al. 1990). Here, however, two enzymes were used both of which cut in the gene itself, whereas one of the two sites resulting in the fragment in Fig. 3c resides in the promoter of the *pel*D gene. Thus, while the *pel*A and *pel*B gene analyses show polymorphisms, even in fragments which comprise only coding DNA (plus introns, although the restriction enzymes used do not cut in any of these introns), the *pel*D gene results show a remarkable conservation even in the non-coding region approximately 315 bp upstream of the gene.

The *A. niger* pyruvate kinase gene (*pki*) is strongly conserved in different organisms such as *A. nidulans*, yeast, rat and chicken (de Graaff 1989). Yet, using this gene as a probe, group I and group II strains can still be discriminated (data not shown).

To establish the relatedness of all strains with each other, we calculated the percentage of the number of hybridizing restriction fragments shared between two strains as compared with the total number of hybridizing restriction fragments present in these two strains (Table 3). Some strains (e.g., 1, 7 and 13) cannot be distinguished by our RFLP analysis and are, therefore, grouped together. In Fig. 4 homology percentages are shown. Because of the limited number of restriction fragments taken into consideration, and the sometimes large differences in relatedness depending on the probe used [e.g., strains 1, 7

and 13 share four out of five fragments with strain 22 if the *pel*A probe is used (Fig. 3c), but only one out of six fragments when *Sma*I-digested DNA is probed with the *pel*B gene (Fig. 3a)], the homology percentages shown in Table 3 and Fig. 4 must be regarded only as rough indications of phylogenetic relatedness.

Strains classified within one group all share over 20% sequence homology, except for strains 16 and 18, which, however, are still more closely related to most other group II strains than to group I strains. Strains 19 and 21 seem to be most related to group I strains.

Heterokaryosis

We used a non-leaky *ade*F12 mutant from group I strain CBS 120.49 N400 (N828) and a non-leaky *pyr*A mutant from group II strain NW756 (182.22) to test whether heterokaryon formation between group I and group II strains is possible. After growth of a mixture of N828 and 182.22 mycelia on selective medium, both fawn (from N828) and black (from 128.22) spores can be seen on the inoculum, but there is no heterokaryon formation between the mycelia. There was some outgrowth of non-sporulating mycelium, which was transferred to complete medium containing either adenine or uridine. After 2 days, growth of both strains was apparent. This means that both strains were present and that the mycelial outgrowth arose from cross-feeding. Crosses of N828 with another N400 mutant or of 128.22 with another NW756 mutant led to normal heterokaryosis.

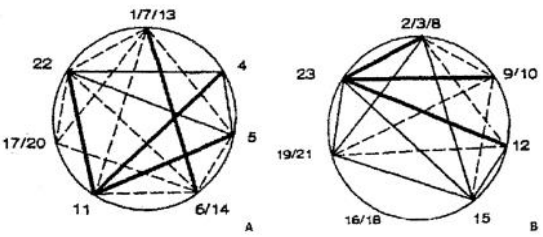

Fig. 4 A, B. Homology percentages showing phylogenetic relationships between strains. The thickness of the lines is a measure of sequence homology (*thick line* >80%; *thin line* 60–80%; *broken line* 40–60%). Only sequence homologies greater than 40% are shown. Strain numbers as indicated in Table 1. **A**, Group I strains; **B**, group II strains

Analysis of non-A. niger strains

In Fig. 5 a Southern blot of chromosomal DNA of several different non-*A. niger* strains probed with the *pel*A gene are shown, indicating that RFLP analysis is also valuable for these species. Firstly, two strains turn out to belong to group I (35 and 36), while a third is a group II strain (25). This is supported by rDNA pattern analysis (data not shown). *A. foetidus* was considered by Al-Musallam (1980) to belong to the *A. niger* aggregate, although she classified it as a separate species, *A. carbonarius* CBS 127.49. This was probably a misclassification since reexamination of this strain gives morphological indications that it belongs to the *A. niger* aggregate. Secondly, *A. helicothrix*, which was segregated by Al-Musallam (1980) from *A. ellipticus* as a separate species, must now be considered merely a mutant variant since the type strains show identical RFLP patterns, and identical rDNA patterns (data not shown). Finally, although the *A. japonicus* and *A. aculeatus* strains show little identity on the blot shown in Fig. 5, their rDNA patterns

Table 3. Sequence homology between *A. niger* strains[a]

	Group I							Group II						
Strain number[b]	1 7 13	4	5	6 14	11	17 20	22	2 3 8	9 10	12	15	16 18	19 21	23[c]
Group I														
1, 7, 13	9/9	5/14	7/12	9/10	6/13	7/12	6/14	1/15	1/15	1/16	1/16	1/17	2/15	1/12
4	36	10/10	8/12	5/15	9/11	4/16	9/12	1/16	1/16	1/17	1/17	1/18	2/16	1/13
5	58	67	10/10	7/13	9/11	5/15	9/12	1/16	1/16	1/17	1/17	1/18	2/16	1/13
6, 14	90	33	54	10/10	6/14	7/13	6/15	1/16	1/16	1/17	1/17	1/18	2/16	1/13
11	46	82	82	43	10/10	5/15	10/11	1/16	1/16	1/17	1/17	1/18	2/16	1/13
17, 20	58	25	33	54	33	10/10	6/15	1/16	1/16	1/17	1/17	0/19	1/17	1/13
22	43	75	75	40	91	40	11/11	1/17	1/17	1/18	1/18	1/19	2/17	1/14
Group II														
2, 3, 8	7	6	6	6	6	6	6	7/7	5/9	5/10	6/9	2/14	2/9	5/6
9, 10	7	6	6	6	6	6	6	56	7/1	4/11	5/10	1/15	5/10	5/6
12	6	6	6	6	6	6	6	50	36	8/8	6/10	3/14	5/11	5/6
15	6	6	6	6	6	6	6	67	50	60	8/8	2/15	7/9	5/7
16, 18	7	6	6	6	6	0	5	14	7	21	13	9/9	3/14	2/10
19, 21	13	13	13	13	13	6	12	66	50	45	78	21	8/8	5/7
23	8	8	8	8	8	8	7	83	83	83	71	20	71	6/6

[a] The fractions represent the number of conserved hybridizing fragments/total number of hybridizing fragments visible in Fig. 3. The other numbers reflect the relatedness in percentages

[b] Strain numbers as described in Table 1

[c] Strain 23, as opposed to all other strains, was only used in two Southern blots (Figs. 3a and 3c)

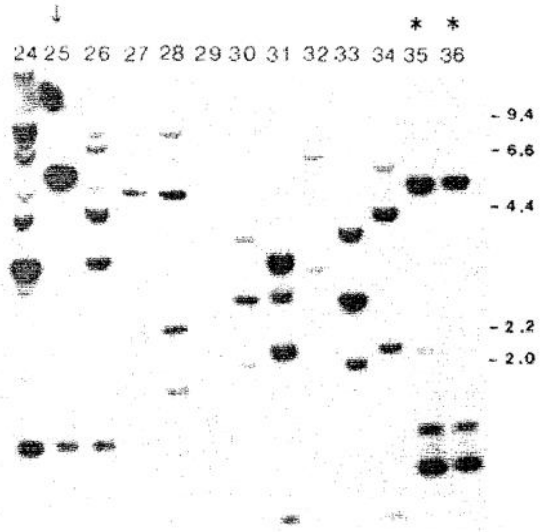

Fig. 5. Southern blot of genomic DNA of non-*A. niger* strains, digested with *PstI*/*SalI* and probed with the *pelA ClaI* fragment (Fig. 2b). Strain numbers as indicated in Table 1. *, Group I strains. The *arrow* indicates a group II strain

appear to be identical. Therefore, they are probably the same species, although a further study based on more isolates needs to be carried out.

Conclusions

The reason why the taxonomy of the black Aspergilli has been difficult, and not always reliable, is due to the facts that: (1) morphology is determined both by genetic and environmental factors, with the morphological criteria being rather subjective, and (2) distinct morphological differences may be caused by simple mutations and thus are not always reliable for establishing genetic relationships. RFLP analysis, on the other hand, is shown to be both reliable and quick, especially when looking at the rDNA banding patterns, and may, therefore, be the preferred way to achieve better criteria for fungal taxonomy. This is particularly so for difficult groups like the black Aspergilli. Kusters-van Someren et al. (1990) have already separated the investigated black Aspergilli into *A. japonicus*, *A. carbonarius*, *A. heteromorphus*, *A. ellipticus*, and a large *A. niger* aggregate. Using RFLP analysis, the *A. niger* aggregate can now be divided into two different groups. The discrimination between these groups can easily be established by the detection of RFLPs in the highly conserved DNA-encoding rRNA and by Southern blots using various probes. Calculation of homology percentages clearly shows the difference in genetic distances both within one group and between the two groups. Strains 16 and 18 are the only ones which have less than 20% sequence homology, with most strains belonging to the same group. Previous results have shown that monoclonal antibodies raised against pectin lyase II (which was purified from a commercial *A. niger* pectinolytic preparation) react with pectin lyases from all *A. niger* strains tested except strains 16 and 18 (Kusters-van Someren et al. 1990). On the basis of these results these two latter strain should perhaps be considered a subspecies. Furthermore, preliminary studies suggest that heterokaryon formation between isolates of group I and group II strains is not possible. Therefore, we propose that the two groups represent two different species. This division is not in agreement with a classification based on morphological criteria (Al-Musallam 1980), which has now been shown to be unreliable when used to identify such closely related species. Because the neotype culture of *A. niger* (CBS 554.65) belongs to group I and the type culture of *A. tubigensis* (CBS 134.48) to group II, we propose to name group I strains *A. niger*, and group II strains *A. tubigensis*.

At both species are obviously strongly related, and up to now have been considered the same species, it seems logical to include *A. tubigensis* in the list of species with a GRAS status.

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References

Al-Musallam A (1980) Revision of the black *Aspergillus* species. Thesis, Rijksuniversiteit, Utrecht

Borsuk PA, Nagiec MM, Stepien PP, Bartnik E (1982) Gene 17:147–152

Erdmann VA (1980) Nucleic Acids Res 8:r31–48

Graaff LH de (1989) Structure and expression of the pyruvate kinase gene of *Aspergillus nidulans* and *Aspergillus niger*. Thesis, Agricultural University, Wageningen

Graaff LH de, van den Broek HWJ, Visser J (1988) Curr Genet 13:315–321

Harmsen JAM, Kusters-van Someren MA, Kester HCM, Visser J (1990) Curr Genet 18:161–166

Kusters-van Someren MA, Samson RA, Visser J (1990) In: Samson RA, Pitt JI (eds) Modern concepts in *Penicillium* and *Aspergillus* classification. NATO ASI Series vol 185, Plenum Press, New York, pp 321–334

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Nei M, Li W-H (1979) Proc Natl Acad Sci USA 76:5269–5273

Pariza MW, Foster EM (1983) J Food Protect 46:453–468

Philippsen P, Thomas M, Kramer RA, Davis RW (1978) J Mol Biol 123:378–404

Pontecorvo G, Roper JA, Hemmons LJ, MacDonald KD, Bufton AWJ (1953) Adv Genet 5:141–238

Raper KB, Fennell DI (1965) In: The genus *Aspergillus*. Williams and Wilkins Company, Baltimore, pp 293–345

Rubtsev PM, Musakhanov MM, Zakharyev VM, Krayev AS, Skryabin KG, Bayev AA (1980) Nucleic Acids Res 8:5779–5794

Thom C, Raper KB (1945) In: A manual of the Aspergilli. Williams and Wilkins Company, Baltimore, pp 1–373

Upshall A (1986) Biotechniques 4:158–166

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Note added in Proof

Recently, O'Connell et al. (1990) cloned the ribosomal repeat of *A. niger*, and found that the restriction maps of the *A. niger* and *A. nidulans* ribosomal repeats are indentical. Since these species are not as closely related as the black Aspergilli, this suggests that the differences we find in RFLP patterns of the chromosomal DNA which encodes ribosomal RNA are due to species-specific differences in the untranscribed spacers, and not in the coding regions.

References

O'Connell MJ, Dowzer CEA, Kelly JM (1990) Fungal Genet Newslett 37:29–30