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Aspergillus vadensis, a new species of the group of black Aspergilli

Ronald P. de Vries[1,2,*], Jens C. Frisvad[3], Peter J.I. van de Vondervoort[1,5], Kim Burgers[1,6], Angelina F.A. Kuijpers[4], Robert A. Samson[4] and Jaap Visser[1,7]

*[1]Molecular Genetics of Industrial Microorganisms, Wageningen University, Dreijenlaan 2, 6703 HA Wageningen, The Netherlands; [2]Microbiology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands; [3]Mycology Group, Technical University of Denmark, Soltofts Plads, DK-2800 Lyngby, Denmark; [4]Centraalbureau voor Schimmelcultures, P.O. Box 85167, 3508 AD Utrecht, The Netherlands; [5]Current address: Laboratory of Phytopathology, Wageningen University, P.O. Box 8025, 6700 EE Wageningen, The Netherlands; [6]Current address: CatchMabs, P.O. Box 134, 6700 AC, Wageningen, The Netherlands; [7]Current address: Fungal Genetics and Technology Consultancy, P.O. Box 396, 6700 AJ Wageningen, The Netherlands; *Author for correspondence (e-mail: r.p.devries@bio.uu.nl.; phone: +31 302533016; fax: +31 302513655)*

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Abstract

A strain from the group of black Aspergilli was analysed in detail to determine the species to which it belongs. A detailed analysis of morphology, RFLP patterns and metabolite profiles was carried out. In addition, a phylogenetic tree was constructed for the black Aspergilli using the ITS and the β-tubulin sequences of the individual strains. The new species differs by its poor growth on glycerol and galacturonate and its unique extrolite profile consisting of aurasperone B, nigragillin, asperazine and kotanins. RFLP analysis using three genes as probes also resulted in a unique pattern. These data indicate that the strain was closely related but not identical to *Aspergillus foetidus*, *Aspergillus niger* and *Aspergillus tubingensis*. It was therefore designated as a novel species and named *Aspergillus vadensis*.

Introduction

The black Aspergilli (section *Nigri*) form a subgroup of the genus *Aspergillus*. Traditionally, fungal strains are assigned to a particular species based on their morphological characteristics such as, color, shape, size, ornamentation of the conidia and the length of the conidiophore. Using these techniques, the black Aspergilli were divided into 12 (Raper and Fennell 1965) and later into seven species (Al-Musallam 1980). The phenotype of a fungal strain can vary significantly depending on the growth conditions (Samson 1994), indicating that errors may occur when assigning strains by morphological methods. The development of molecular techniques (RFLP, RAPD, PCR, DNA fingerprinting and nucleotide sequencing) for the identification of fungal strains has resulted in a reclassification of black Aspergilli (Kusters-van Someren et al. 1991; Varga et al. 1993; Varga et al. 1994; Accensi et al. 1999; Parenicová et al. 1997; Parenicová et al. 2001; Abarca et al. 2004). The strength of RFLP analysis and other molecular

and biochemical techniques in the classification of the black Aspergilli was demonstrated by two recent studies involving strains of all known species of the black Aspergilli (Parenicová et al. 1997; Parenicová et al. 2001). These studies resulted in the identification of 8 species in the group of black Aspergilli: *A. niger*, *A. tubingensis*, *A. foetidus*, *A. carbonarius*, *A. aculeatus*, *A. japonicus*, *A. heteromorphus*, and *A. ellipticus* (Parenicová et al. 1997).

In this paper we describe the identification of a new species of the section *Nigri*, designated *A. vadensis*, using a combination of the methodologies described above. This species is closely related to *A. foetidus*, *A. tubingensis* and *A. niger* but is different on all characteristics examined. This species was recently studied with respect to its potential for homologous and heterologous protein production (de Vries et al. 2002; de Vries et al. 2004).

Materials and methods

Strains, media and culture conditions

The strains used in this study are listed in Table 1. Minimal medium (MM) contained (per liter): 6.0 g of $NaNO_3$, 1.5 g of KH_2PO_4, 0.5 g of KCl, 0.5 g of $MgSO_4$, 200 μl trace elements (10 g/l EDTA, 4.4 g/l $ZnSO_4{\cdot}7H_2O$, 1.01 g/l $MnCl_2{\cdot}\ 4H_2O$, 0.32 g/l $CoCl_2{\cdot}6H_2O$, 0.315 g/l $CuSO_4{\cdot}\ 5H_2O$, 0.22 g/l $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 1.47 g/l $CaCl_2{\cdot}2H_2O$, 1.0 g/l $FeSO_4{\cdot}7H_2O$, modified from Vishniac and Santer (1957)), and 1% (w/v) glucose as a carbon source unless otherwise indicated. Liquid cultures were inoculated with 10^6 spores/ml and incubated at 30 °C in an orbital shaker at 250 rpm. Agar was added at 1.5% (w/v) for solid medium. For the growth comparison on different carbon sources, strains were grown on solid MM. Carbon source concentrations were: vanillic acid, 0.1% (w/v); serine, tyrosine, galactitol, 0.2% (w/v), all other carbon sources, 1% (w/v). Plates were analysed after 2 days of growth at 30 °C for D-glucose and D-xylose and after 3 days of growth for the other carbon sources.

For the morphological studies the strains were grown on Czapek Yeast agar and 2% (w/v) malt extract agar and incubated at 25 °C for 7 days.

Molecular biology methods and sequencing

Standard methods were used for DNA manipulations, such as Southern analysis, subcloning, DNA digestions and DNA isolations (Sambrook et al. 1989). Chromosomal DNA was isolated using the FastDNA®Kit (Bio101, Carlsbad, USA). Amplification of beta-tubulin gene was performed using the primers Bt2a and Bt2b (Glass and Donaldson 1995). Amplification of the two internal transcribed spacers (ITS 1 and ITS 2) including the 5.8 S rDNA was performed using the primers LS266 (Masclaux et al. 1995) and V9G as proposed by de Hoog and Gerrits-van den Ende (1998). PCR was performed in a 50 μl reaction mixture using 10–20 ng genomic DNA in a GeneAmp PCR system 9700 (AB Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Excess primers and dNTP's were removed using a PCR DNA Purification kit (Amersham Bioscience, Roosendaal, The Netherlands). The PCR fragments were directly sequenced in both directions with the primers Bt2a and Bt2b for the beta-tubulin gene and primers ITS 1 and ITS 4 for the ITS region using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Bioscience, Roosendaal, The Netherlands) on an ABI Prism 3700 Genetic Analyzer (AB Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands). A concensus was computed using Seqman and Editseq from the lasergene package (DNAStar Inc., Madison, WI). The alignments of the partial beta-tubulin gene and ITS sequence data were performed using BioNumerics (Applied Maths) and manual adjustments for improvement were made by eye where necessary. The phylogenetic analyses of sequence data were done using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2000). Alignment gaps were treated as fifth character state, missing data were identified by '?' and all characters were unordered at equal weight. Maximum parsimony analysis was performed with beta-tubulin and ITS datasets and a combination of the datasets using the heuristic search option. All the generated sequences for this study were deposited at GenBank; only the sequence from *A. flavus* was retrieved from Genbank http://www.ncbi.nlm.nih.gov. Accession numbers for the sequences are listed in Table 1.

Table 1. Strains used in this study. TUB = β-tubulin sequence, ITS = internally transcribed spacer sequence, T = type strain

Species	Strain number	Alternative number/ description	Accession number		Reference
			TUB	ITS	
A. aculeatus	CBS 101.43				(Parenicová et al. 1997)
A. aculeatus	CBS 620.78		AY585538	AY585556	(Parenicová et al. 2001)
A. aculeatus	CBS 172.66 T		AY585540	AY585558	(Parenicová et al. 2001)
A. aculeatus	CBS 119.49		AY585541	AY585559	(Parenicová et al. 2001)
A. aculeatus	CBS 114.80		AY585539	AY585557	(Parenicová et al. 2001)
A. carbonarius	CBS 111.26 T	ATCC 1025, IMI 016136	AY585532	AY585550	(Parenicová et al. 1997)
A. ellipticus	CBS 707.79 T	IMI 278384	AY585530	AY585548	(Parenicová et al. 1997)
A. flavus	NRRL 1957		AY017536	AF027863	(Peterson et al. 2001)
A. foetidus	CBS 564.65 T		AY585533	AY585551	(Parenicová et al. 1997)
A. foetidus	CBS 565.65	ATCC 16884, IMI 175963, WB 4797, IBT 3644, IBT 4670	AY585534	AY585552	(Parenicová et al. 1997)
A. foetidus	CBS 115.52				(Parenicová et al. 1997)
A. heteromorphus	CBS 117.55 T	ATCC 12064, IMI 172288	AY585529	AY585547	(Parenicová et al. 1997)
A. japonicus	CBS 114.51		AY585542	AY585560	(Parenicová et al. 1997)
A. japonicus	CBS 101.14 T		AY585543	AY585561	(Parenicová et al. 2001)
A. japonicus	CBS 611.78		AY585544	AY585562	(Parenicová et al. 2001)
A. niger	CBS 120.49 T	N400, ATCC 9029, IMI 041876, NRRL 3	AY585535	AY585553	(Parenicová et al. 1997)
A. niger	CBS 554.65		AY585536	AY585554	(Kusters-van Someren et al. 1991)
A. niger	CBS 101699		AY585537	AY585555	This study
A. niger	N402	*cspA1*			(Bos et al. 1988)
A. tubingensis	CBS 114.37	IMI 313493, WB 4856, IBT 4944			(Parenicová et al. 1997)
A. tubingensis	CBS 161.79		AY585527	AY585545	This study
A. tubingensis	CBS 126.52 T		AY585528	AY585546	(Parenicová et al. 1997)
A. vadensis	CBS 113365 T	CBS 102787, IMI 142717, IBT 24658	AY585531	AY585549	(de Vries et al. 2002; de Vries et al. 2004)

RFLP analysis of fungal strains

Chromosomal DNA was digested with *Sal*I and *Pst*I and separated on a 0.7% agarose gel. Southern blots of the gels were hybridised using the following fragments as probes: a 0.8 kb *Sal*I fragment of the *A. nidulans pkiA* gene; a 0.9 kb *Eco*RI fragment comprising the 3′-end of the 28 S ribosomal DNA of *Agaricus bisporus*; a 1.6 kb *Cla*I fragment containing the 3′-end and some flanking region of the *A. niger pelA* gene. Hybridisation was performed over night at 60 °C after which the blots were washed twice for 30 min with 2 × SSC, 0.5% SDS at 60 °C. Hybridisation patterns of *A. vadensis* were compared to those described previously (Parenicová et al. 1997).

Metabolite analysis

CBS 113365 was cultured on media that result in the most optimal conditions for the production of secondary metabolites: Czapek yeast autolysate agar (CYA), Blakeslee malt agar (MEA), yeast extract sucrose agar (YES), and oatmeal agar (OA) (Frisvad et al. 1989). All cultures were incubated for 7 days in the dark at 25 °C. For metabolite analysis three agar plugs were extracted as described previously (Frisvad 1987) and analysed by high-performance liquid chromatography with diode array detection (Frisvad and Thrane 1987) as modified by Smedsgaard (Smedsgaard 1997). 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 the standards.

Results

Growth of several Aspergilli on different carbon sources

The growth of several *Aspergillus* strains was compared on solid medium using different carbon sources. Clear differences can be identified between all the strains tested (Table 2), whereas no differences were observed between different strains of the same species (data not shown). *A. vadensis* grows poorly on glycerol, D-galacturonate and acetate compared to the other strains, while *A. tubingensis* has reduced growth on citrate. *A. foetidus* grew significantly poorer on D-xylose and xylitol. *A. japonicus* was the only species tested that grew on D-galactose.

RFLP analysis of A. vadensis *and other black Aspergilli strains*

RFLP analysis was performed on chromosomal DNA of *A. vadensis* and representatives of the 8 black Aspergilli. Using the *A. niger pkiA* probe, hybridising fragments of 3.4, 2.65 and 0.55 kb were detected for *A. vadensis* (Table 3) while for *A. niger*, *A. tubingensis* and *A. foetidus* only one fragment of 3.4 kb hybridised with this probe. *A. carbonarius* resulted in a fragment of 6.0 kb, but other strains of this species have been reported to contain a hybridising fragment of 0.9 or 4.8 kb (Parenicová et al. 1996). None of the other black Aspergilli had hybridising fragments of the same size as *A. vadensis*. Two hybridising fragments of 5.0 and 1.4 kb were detected for *A. vadensis* using the 28 S probe. A 5.0 kb fragment was also detected for *A. niger*, *A. tubingensis*, *A. foetidus* and *A. carbonarius*, but no other black Aspergilli contained a hybridising 1.4 kb fragment. For *A. niger* and *A. foetidus* a

Table 2. Growth comparison of several Aspergilli on different carbon sources. The symbols indicate no growth (−), poor growth (+), moderate growth (++), good growth (+++) and very good growth (++++). For each species except for *A. vadensis*, three strains were tested and were shown to have identical growth profiles. Results for one strain per species are indicated in the table. All strains showed identical growth on duplicate plates. The results are the average of three individual experiments

	A. vadensis	*A. niger*	*A. tubingensis*	*A. foetidus*	*A. japonicus*
	CBS 113365	N402	CBS 114.37	CBS 115.52	CBS 114.51
D-glucose	++++	+++	++++	++++	++
D-xylose	+++	+++	+++	++	+++
glycerol	+	+++	+++	++	++
xylitol	++	+++	+++	+	+
D-galacturonate	+	+++	+++	++	++
acetate	+	++	++	++	++
citrate	++	++	+	++	++
No C-source	−	−	−	−	−

Table 3. RFLP analysis of black Aspergilli. The values in the *pkiA*, 28S, and *pelA* columns correspond to the size of the fragments hybridising with the probes (in kb) while the letters correspond to the group assignment published previously (Parenicová et al. 1997)

Species	CBS	*pkiA*		28S		*pelA*	
A. vadensis	113365	3.4, 2.65, 0.55	F	5.0, 1.4	H	5.1, 2.0, 1.1	L
A. foetidus	565.65	3.4	B	5.0, 1.5	D	4.8, 2.8, 2.2, 1.5	C
A. tubingensis	114.37	3.4	B	5.0, 0.9	B	5.0, 1.2	B
A. niger	120.49	3.4	B	5.0, 1.5	C	4.5, 1.2, 1.0	E
A. carbonarius	111.26	6.0	A	5.0, 0.3	A	2.9, 1.2	J
A. aculeatus	101.43	3.0	C	6.0, 1.6	F	3.6, 1.8, 0.8	H
A. japonicus	114.51	3.0	C	6.0, 1.2	E	3.2, 2.4, 1.7, 0.8	I
A. heteromorphus	117.55	2.8	D	7.5, < 0.5	G	2.4	K
A. ellipticus	707.79	2.8	D	7.5, < 0.5	G	3.8, 2.0	G

1.5 kb fragment was detected, which was clearly distinguishable from the 1.4 kb fragment of *A. vadensis*. Using the *pelA* probe, three hybridising fragments were detected for *A. vadensis* of 5.1, 2.0 and 1.1 kb, respectively. None of these fragments were identical in size to any of the fragments detected for the other black Aspergilli.

Metabolite analysis of A. vadensis *and other black Aspergilli strains*

An analysis of the production of secondary metabolites was performed on *A. vadensis* and several other strains of the black Aspergilli as described in Materials and methods. Clear differences in metabolite profiles were observed between the species (Table 4) but only to a small extend between strains of the same species (data not shown). *A. vadensis* produced asperazine, nigragillin, aurasperone B and a polar kotanin-like compound (Table 4). Asperazine is also produced by *A. foetidus* and *A. tubingensis*, but not by any of the other black Aspergilli. Low levels of nigragillin were also produced by *A. tubingensis*, but not by any of the other black Aspergilli. *A. foetidus*, *A. niger*, *A. tubingensis* and *A. carbonarius* also produced aurasperone B. Kotanins are specific for *A. niger*, but the kotanin-like compound produced by *A. vadensis* is more polar and is specifically produced by this strain. *A. vadensis* neither produced pyranopyrrol commonly secreted by acid producing species (Hiort 2003), including *A. tubingensis*, *A. niger*, *A. foetidus* and *A. carbonarius* (Frisvad unpublished data). *A. vadensis* also did not produce ochratoxin A, which is produced by *A. niger* and *A. carbonarius*.

Construction of a phylogenetic tree for the black Aspergilli base on the ITS and the β*-tubulin sequence*

Sequence analysis of the beta-tubulin gene and the ITS region was performed on a number of black Aspergilli to construct a phylogenetic tree. The identities of the strains are listed in Table 1. For the taxa, the names as presently listed in the CBS catalogue (http:/www.cbs.knaw.nl) were used. Maximum parsimony analysis was performed to construct phylogenetic trees. For the ITS dataset 4 similar maximum parsimony trees were produced (TL = 74 steps, CI = 0.811, RI = 0.946, RC = 0.767), while for the Beta-Tubulin 18 similar maximum parsimony trees were produced (TL = 538 steps, CI = 0.755, RI = 0.831, RC = 0.627). A representative tree for each group is shown in Figure 1. Both analyses were rooted to *A. flavus* NRRL 1957 as an out-group. Bootstrap support from 1000 replicates is shown at the

Table 4. Metabolite analysis of black Aspergilli. OTA: ochratoxin A, AUB: aurasperone B, NIG: nigragillin, ASP: asperazine; KOT: kotanins (orlandin, desmethylkotanin and kotanin), PKO: a polar kotanin-like compound (more polar than orlandin), PYR: pyranopyrrol, a compound produced by all strong acid producing species in *Aspergillus* section *Nigri* and *Penicillium glabrum* (Hiort 2003), ANT: antafumicin. *A. niger* CBS 120.49 did not produce ochratoxin A, but other *A. niger* strains do

Species	CBS	OTA	AUB	NIG	ASP	KOT	PKO	PYR	ANT
A. vadensis	113365	–	+	+	+	–	+	–	–
A. foetidus	565.65	–	+	–	+	–	–	+	+
A. tubingensis	114.37	–	+	+[a]	+	–	–	+	–
A. niger	120.49	+[a]	+	–	–	+[a]	–	+	–
A. carbonarius	111.26	+	+	–	–	–	–	+	–

[a] This compound is produced by some strains of the species. There is only one strain available in culture collections of *A. heteromorphus*, *A. ellipticus* and *A. vadensis*. For the other species, 13 strains of each species were analysed. No differences were observed for the *A. foetidus* strains (CBS 114.52; CBS 565.65; CBS 105.47; CBS 564.65; CBS 115.52; IBT 20295; IBT 20290; IBT 21580; IBT 24822; IBT 24831; IBT 24798; IBT 24799; IBT 24800) and the *A. carbonarius* strains (CBS 111.26; CBS 114.29; CBS 116.49; CBS 117.49; CBS 111.80; IMI 041875; NRRL 67; IMI 313489; CBS 113.80; NRRL 369; IBT 16756; IBT 21854; IBT 22445). For *A. tubingensis* (CBS 114.37; CBS 118.35; CBS 117.48; CBS 121.48; CBS 121.49; CBS 130.5; CBS 136.52; CBS 107.55; CBS 126.52; CBS 115.29; CBS 137.52; CBS 558.65; CBS 643.92), production of nigragillin was only observed for the latter five species. More differences were observed for the *A. niger* strains (CBS 618.78; CBS 126.48; CBS 139.52; CBS 126.49; CBS 117.52; CBS 131.52; CBS 554.65; CBS 557.65; CBS 117.80; CBS 112.52; CBS 120.49; CBS 616.78; CBS 113.33). The first 4 strains produced ochratoxin A, 9 strains (underlined) produced kotanins and 3 strains (in bold) produced aspergillin. All 13 isolates produced pyranopyrrol and aurasperone B. None of 5 strains of *A. aculeatus* and *A. japonicus* produced any of the metabolites listed in this table.

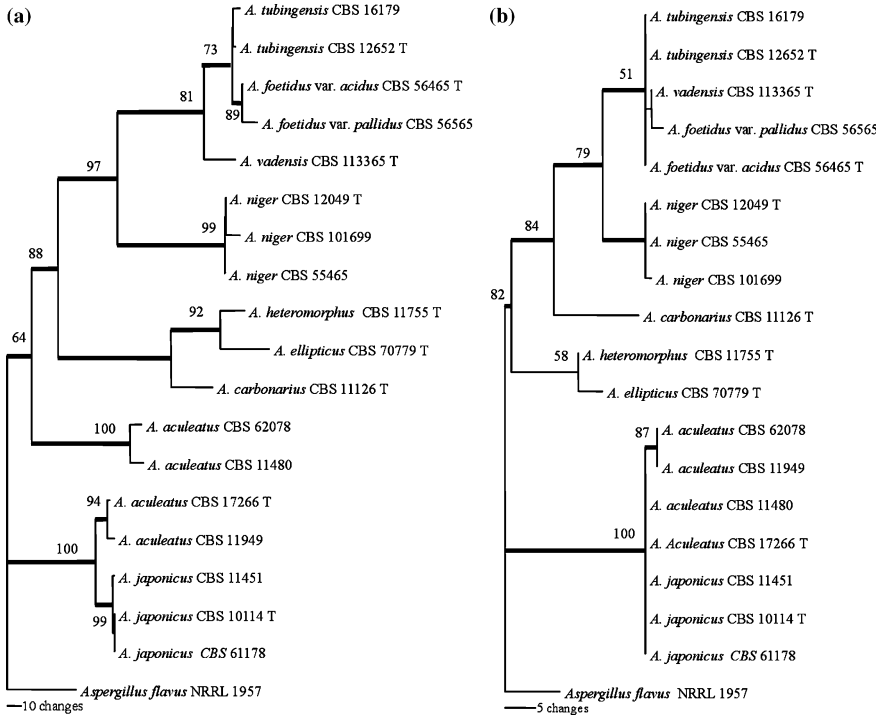

Figure 1. Phylogenetic trees of the black Aspergilli. The branches in bold are 100% in the 70% majority-rule consensus of similar parsimonious trees. The numbers represent bootstrap percentages > 50%. A. One of the 18 trees of 538 steps based on heuristic search partial beta-tubulin sequences with *A. flavus* as an out-group (CI = 0.755, RI = 0.831 RC = 0.627, HI = 0.245). B. One of the 4 trees of 74 steps based on heuristic search ITS sequences with *A. flavus* as an out-group (CI = 0.811, RI = 0.946 RC = 0.767, HI = 0.189).

nodes. The lines in bold designate branches present in the strict consensus tree (i.e. in 100%) of the maximum parsimony trees.

The β-tubulin tree demonstrates a clear separation of the black *Aspergillus* species. *A. vadensis* is most similar to *A. tubingensis* and *A. foetidus*, while *A. niger* is more distant. *A. carbonarius* groups together with *A. heteromorphus* and *A. ellipticus*, while two of the four *A. aculeatus* strains group with *A. japonicus*. The ITS dataset shows less difference between the black *Aspergillus* species. *A. vadensis* is in a group with *A. tubingensis* and *A. foetidus* but distinct from the other species and *A. niger* is a separated group. *A. aculeatus*, and *A. japonicus* are in one group but separated from two species of *A. aculeatus* (CBS 62078 and CBS 11480).

Discussion

We have analysed a strain from the group of black Aspergilli using a combination of molecular, biochemical and morphological characteristics to determine to which species the strain belongs. Species boundaries of organisms without a known sexual state are not always clear, and rely on a combination of these techniques to assign strains to the different species (Varga et al. 1993; Parenicová et al. 1997; Parenicová et al. 2001).

Growth of *A. vadensis* on various carbon sources was similar to that of *A. niger*, *A. tubingensis* and *A. foetidus*, with the exception of growth on glycerol, acteate and D-galacturonate. On these carbon sources, growth was much slower, suggesting that the metabolic pathways related to these compounds are less efficient in *A. vadensis*.

The RFLP analysis using 3 different genes as markers gives varying results. As *A. niger*, *A. foetidus*, and *A. tubingensis* have the same pattern for the *pkiA* probe, no conclusions can be drawn to which species *A. vadensis* is most closely related based on this probe. The pattern observed for *A. vadensis* is more complicated as it contains a 2.65 and 0.55 kb fragment in addition to the

3.4 kb fragment also observed for *A. niger*, *A. tubingensis*, and *A. foetidus*. One explanation for this difference is that the chromosomal DNA from *A. vadensis* is only partially digested, as the combined size of the two smaller fragments is similar to the larger fragment. However, the pattern was found in several analysis and no other partial bands were found. An alternative explanation is that gene duplication of *pkiA* has occurred in *A. vadensis* and that mutation of one of the two copies has resulted in the additional restriction site. Based on the 28 S probe, *A. vadensis* is most closely related to *A. niger* and *A. foetidus*. The pattern obtained with the *pelA* probe has no similarities with any of the other strains. Multiple bands were found for most species, most likely due to the fact that the *pelA* probe can also hybridise with *pelD* (Parenicová et al. 1997). These data indicate that *A. vadensis* is related to *A. niger*, *A. tubingensis* and *A. foetidus*, but not identical to them. A similar RFLP analysis has been performed previously on a large number of black Aspergilli strains (Parenicová et al. 1997) in which strains of one species had highly conserved RFLP profiles. Therefore, the differences in the RFLP profile observed for *A. vadensis* suggest that this strain is a separate species.

A. vadensis also produces a different set of secondary metabolites than the other black Aspergilli. Asperazine is found in *A. tubingensis* and *A. foetidus*, but not in *A. niger* (Frisvad and Smedsgaard, unpublished data). Nigragillin is often produced by older cultures of *A. tubingensis* and *A. niger*. *A. vadensis* is most closely related to *A. tubingensis* from a chemotaxonomical point of view, but it differs from that species by the production of the polar kotanin type of molecule. It also differs by the lack of pyranopyrrol (Hiort 2003) found in all isolates of *A. niger*, *A. tubingensis*, *A. foetidus* and *A. carbonarius* (Frsivad, unpublished data). This compound seems to be associated with acid production as it is also produced by all strains of *Penicillium glabrum* (Frsivad, unpublished data). All strains of these species except *A. vadensis* produce citric acid.

In the phylogenetic tree, *A. flavus* was used as an out-group. The ITS tree does not give a very clear distinction between the individual species, although the subgroups of species in the group of black Aspergilli become visible. From this tree it is clear that *A. vadensis*, *A. foetidus* and *A. tubingensis* are highly similar with respect to the ITS sequence. Most similar to these three species is *A. niger*, followed by *A. carbonarius* and *A. heteromorphus* and *A. ellipticus*. *A. japonicus* and *A. aculeatus* are highly similar as well, but clearly distant from the other black Aspergilli. The β-tubulin tree separated the species out to a larger extend and indicates that *A. tubingensis* and *A. foetidus* are highly similar with respect to the β-tubulin sequence, while *A. vadensis* can be distinguished from them. *A. niger* is again most similar to these three species. However, in this tree, *A. carbonarius*, *A. heteromorphus* and *A. ellipticus* are visible as a separate subgroup, with the latter two most closely similar to each other. Two of the four *A. aculeatus* strains form a separate group (CBS 62078 and 11480), while the other two strains group with *A. japonicus*. In a previous study, CBS 62078 was originally classified as *A. japonicus* but was recently re-assigned to the *A. aculeatus* group based on RFLP pattern and ITS sequence (Parenicová et al. 2001). CBS 11480 was described in the same study, but differed in RFLP profile and ITS sequence from the other strains. The variability among the *A. aculeatus* strains demonstrated in this study complicates the assignment of a species to novel strains of this group and requires further study. The position of *A. vadensis* in the trees indicates that it is at least as distant from *A. foetidus* and *A. tubingensis* as they are from each other.

The acceptance of eight species of black Aspergilli was based on a number of characteristics (Parenicová et al. 1997; Parenicová et al. 2001). *A. vadensis* differs on all these characteristics from the other species. Although this species is hitherto only represented by a single isolate, the combination of characters makes it unique and indicates that *A. vadensis* should be considered as the first representative of a new species of the group of black Aspergilli.

Aspergillus vadensis *Samson, de Vries, Frisvad and Visser, sp. nov.*

A simili *Aspergillo nigro* coloniis fere lente crescentibus (2.5–3.0 cm diam post 7 dies 25 °C) differt. Coloniae dilute brunneae vel olivaceo-brunneae. Conidiophora 150 × 6–15 μm. Neque sclerotia neque teleomorphosis formata. Species

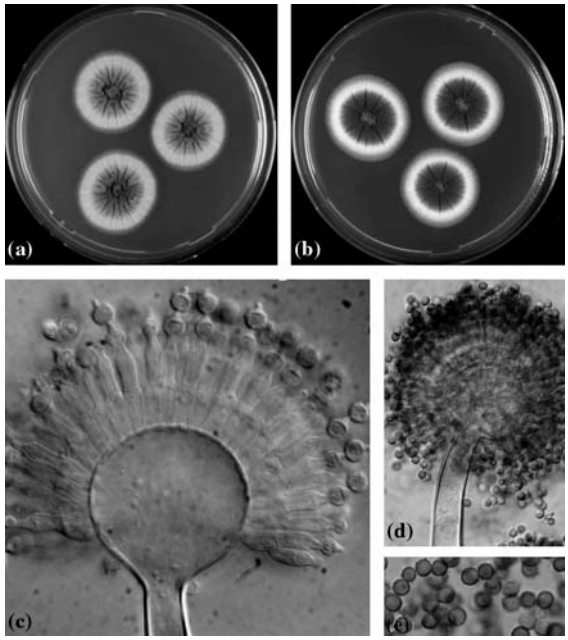

Figure 2. Morphological characterization of *A. vadensis*. Pictures a and b are *A. vadensis* colonies grown for 7 days on Czapek Yeast agar and MEA, respectively. Pictures c and d are microscopic images of conidiophores from *A. vadensis* and picture e is a microscopic image of *A. vadensis* conidia.

exigue crescens in glycerolo et galacturonato et metabolitis (aurasperonum B, nigragillinum, asperazinum, kotanina) distinguenda. RFLP analysis tribus sequentiis hybridisata patrona distincta praebuit.

Typus CBS 113365 (holotypus exsiccatus in herb. CBS), isolates ex aere in Aegypto ab A.H. Moubasher.

The species is named after Wageningen, the town where extensive work has been carried out on the biology and genetics of black Aspergilli.

Colonies of *A. vadensis* CBS 113365 grew to 2.5 and 3.00 cm in diameter within 7 days on Czapek Yeast agar and MEA, respectively, resulting in abundant sporulation giving the colonies a light brown to olive green brown colour (Figure 1). This colour is unusual as most other black Aspergilli produce brown to black conidiophores. The conidiophores were up to 150 μm high with a 6–15 μm wide stipe, and were biseriate with globose vesicles with a diameter of 25–35 μm. The conidia globose were 3–4 μm in diameter, brown, and rough walled to finely echinulate. No sclerotia or teleomorph were observed.(Figure 2)

The new species differs by its poor growth on glycerol and galacturonate and its unique extrolite profile consisting of aurasperone B, nigragillin, asperazine and kotanins (Table 4). Furthermore RFLP analysis using three genes as probes resulted in a unique pattern (Table 3).

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