New species in Aspergillus *section* Fumigati *from reclamation sites in Wyoming (U.S.A.) and revision of* A. viridinutans *complex*

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New species in *Aspergillus* section *Fumigati* from reclamation sites in Wyoming (U.S.A.) and revision of *A. viridinutans* complex

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Abstract The *Aspergillus viridinutans* complex includes morphologically similar, soil-inhabiting species. Although its species boundaries have not been fully defined, many isolates from the complex have been isolated as opportunistic human and animal pathogens. In the present study, these species were dominant in spoil sites subjected to various types of reclamation management after coal mining. These species were characterised using two different PCR-fingerprinting methods, sequence data from the β-tubulin (*benA*) and calmodulin (*caM*) genes, macro- and micromorphology (optical and scanning electron microscopy), maximum growth temperatures and mating experiments. In addition, RNA polymerase II gene (*RPB2*), actin (*act1*) and ITS sequences were deposited for the ex-type isolates of newly described species. The mating experiment results, phylogenetic analyses and ascospore morphology suggested the presence of five species in the *A. viridinutans* complex. *Aspergillus aureolus* (syn. *Neosartorya aureola*) was the only homothallic species. Three species, *A. felis*, *A. udagawae* (syn. *N. udagawae*) and *A. wyomingensis* sp. nov., were heterothallic and their morphologically distinguishable teleomorph was induced by systematic mating experiments. *Aspergillus viridinutans* s. str. seems to be a very rare species and was represented only by the ex-type isolate in which the MAT1-1 locus was amplified. *Aspegillus viridinutans* and *A. aureolus* were typified in accordance with the rules of the new botanical code. Other species outside the *A. viridinutans* complex isolated from the reclamation sites were *A. fumigatiaffinis* and *A. lentulus* as well as two new sister species, *A. brevistipitatus* sp. nov. and *A. conversis* sp. nov. which were closely related each to other and to *N. papuensis*. Both new species are phylogenetically distant from all anamorphic species and resemble *A. brevipes*, *A. duricaulis* and *A. unilateralis* in micromorphology and are distinguishable from each other by the slower growth of *A. conversis* on all tested media. Interestingly, no isolate from the reclamation sites represented *A. fumigatus* s. str. which is usually reported as the dominant species from the section *Fumigati* in soil.

Keywords *Aspergillus fumigatus* · Heterothallic species · MAT locus · *Neosartorya udagawae* · PCR fingerprinting · Soil fungi

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Introduction

Aspergillus section *Fumigati* includes species that have an overall economic impact. Some of these species are causal agents of human and animal infections or important decomposers of organic matter in soil, and they are isolated as foodstuff contaminants (Samson 1989; Tournas 1994; Balajee et al. 2005, 2009; Katz et al. 2005; Yaguchi et al. 2007, 2012; Hubka et al. 2012). Section *Fumigati* members produce many different compounds, including mycotoxins which are used or have potential to be used in pharmacology and biotechnology (Wong et al. 1993; Tomoda et al. 1994; Larsen et al. 2007; Samson et al. 2007a). Soil is the most important reservoir of section *Fumigati* members and *A. fumigatus* is usually reported as the most common species worldwide (Klich 2002; Domsch et al. 2007).

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Morphological differentiation of heterothallic and anamorphic species is limited because of their overlapping features on the anamorph, and molecular methods are routinely used to identify them. Four new anamorphic species were recently described based on molecular data from a group considered in the past to represent *A. fumigatus* (Balajee et al. 2005; Hong et al. 2005, 2008). In contrast to anamorph micromorphology, ascospore features belong to the most informative phenotypic characteristics in section *Fumigati* taxonomy. Using systematic crossing of opposite mating type isolates verified by molecular methods, the teleomorph was discovered in *A. fumigatus* (O'Gorman et al. 2009) which was previously treated as a strictly anamorphic species. This systematic approach replaced previous teleomorph discovery attempts based on randomly crossing phenotypically similar isolates which were successful in several heterothallic species (Kwon-Chung and Kim 1974; Takada and Udagawa 1985; Horie et al. 1995; Takada et al. 2001).

During the course of a survey of soil microorganisms in spoil sites subjected to various types of reclamation management after coal mining in Wyoming (U.S.A.), *Aspergillus* section *Fumigati* members were among the most frequently isolated organisms. These isolates were characterised using PCR-fingerprinting methods, sequence data, phenotypic analysis and mating experiments. This approach resulted in the discovery of three new species which are described in this study.

Material and methods

Description of studied sites and sampling and isolation methods

Soil samples were collected from two coal mine reclamation sites in the Powder River Basin, Converse County, Wyoming, U.S.A. One site was the Belle Ayr Mine, a functional coal mine located just south of Gillette. This site was dominated by cool season grass. The average annual precipitation in this area is 376 mm and the average air temperature is 6.7 °C (Wick et al. 2007). Samples were collected from this site in May 2008 and 2011. The other site was the Rolling Hills Wind Plant (formerly the Dave Johnson Coal Mine), located to the north of Glenrock. The mine was closed down in 2002 and the whole mine is currently being reclaimed. This research site was dominated by sagebrush grassland (Rana et al. 2007). The average annual precipitation in this area is 266 mm and the average air temperature is 7.4 °C (Ganjegunte et al. 2009). Samples were collected from this site in September 2010. All soil samples from both sites were collected from a depth of 0–5 cm by trowel and placed into sterile bags.

The soil dilution plate method and soil washing technique (Garrett 1981; Kreisel and Schauer 1987) and dichloran rose bengal chloramphenicol agar (DRBC), Sabouraud glucose agar (SGA) and beer-wort agar (BWA) (Atlas 2010) were used to isolate microscopic fungi. The method of heating of soil samples at 75 °C for 30 min according to Samson et al. (1996) and Marvanová (1999) was used to isolate thermoresistant microfungi. Keratin and cellulose bait techniques were also used for the isolation of soil microfungi in damp chambers in laboratory conditions. Pronghorn antelope and jackrabbit dungs collected at the studied sites were cultivated on filtrate paper or soil samples in damp chambers at room temperature to isolate coprophilic microfungi.

Phenotypic studies

The strains were grown on malt extract agar (MEA; malt extract−Fluka Chemie GmbH, Switzerland), Czapek Yeast Autolysate Agar (CYA; yeast extract−Fluka Chemie GmbH, Switzerland), yeast extract sucrose agar (YES) and Czapek-Dox agar (CZA) plates at 25 °C. Agar media were formulated following the methods of Frisvad and Samson (2004) and Atlas (2010). Micromorphology was observed on MEA. Colour determination was performed according to the ISCC-NBS Centroid Colour Charts (Kelly 1964). Scanning electron microscopy (SEM) was performed using a JEOL JSM-6380 LV scanning electron microscope (JEOL Ltd, Tokyo, Japan). Pieces of colonies growing on OA or MEA (5×5 mm) with ascomata were fixed and observed using the settings described in Hubka et al. (2013a).

Growth at 42, 45 and 47 °C was tested on MEA plates sealed with Parafilm. The production of cyclopiazonic acid or related alkaloids was tested using the Ehrlich test and the production of acid compounds in the agar medium was tested on creatine sucrose agar (CREA) following the methods of Samson et al. (2007b).

Molecular studies and phylogenetic analysis

DNA was extracted from seven-day-old colonies using the Microbial DNA Isolation Kit (Mo-Bio Laboratories). PCR fingerprinting, using the phage *M13-core* sequence as an oligonucleotide primer (5′-GAGGGTGGCGGTTCT) and the primer *834t* [$(AG)_8CG$], was performed and evaluated as described previously (Hubka and Kolařík 2012; Nováková et al. 2012). Partial *benA* and *caM* sequences were amplified from at least one isolate with a unique combination of fingerprinting patterns. The PCR conditions for amplification of ITS, *benA* and *caM* loci were as described by Hubka et al. (2012). The annealing temperature for amplification of *act1* gene was 60 °C. The *RPB2* gene was amplified using Touchdown cycling conditions, as described by Hubka and Kolařík (2012). The partial *benA* gene (encoding *β*-tubulin) was amplified with primers *T10* (5′- ACGATAGGTTC ACCTCCAGAC) or *Bt2a* (5′- GGTAACCAAATCGGTG

CTGCTTTC) and *Bt2b* (5′- ACCCTCAGTGTAGTGACC CTTGGC); the partial *caM* gene (encoding calmodulin), with *CF1M* (5′- AGGCCGAYTCTYTGACYGA) and *CF4* (5′- TTTYTGCATCATRAGYTGGAC); the partial *act1* gene (encoding actin), with *ACT-512 F* (5′-ATGTGCAAGGCCG GTTTCGC) and *ACT-783R* (5′-TACGAGTCCTTCTGG CCCAT); the partial *RPB2* gene (encoding RNA polymerase II), with *fRPB2-5F* (5′-GAYGAYMGWGATCAYTTYGG) and *fRPB2-7cR* (5′-CCCATRGCTTGYTTRCCCAT); and the ITS region with *ITS1F* (5′- CTTGGTCATTTAGAGG AAGTAA) and *NL4* (5′- GGTCCGTGTTTCAAGACGG).

Sequences were inspected and assembled using Bioedit v. 7.0.0 (www.mbio.ncsu.edu/BioEdit/bioedit.html). Alignments of the regions were performed using the FFT-NSi strategy, as implemented in MAFFT v. 6.861b (Katoh et al. 2005). The *benA* and *caM* loci were combined and Maximum likelihood (ML) analysis was performed using RAxML version 7.0.4 with bootstrapping (1,000 replicates) (Stamatakis et al. 2008) running on the CIPRES web portal (www.phylo.org). The analysis was performed with the model (GAMMA + P-I) and the α parameter was estimated using the same software. Bayesian Inference analysis (BI) was performed using MrBayes v3.1 (Ronquist and Huelsenbeck 2003), the substitution model K2 + G + I was determined as the most suitable using MEGA 5.0 (Tamura et al. 2011) and metropolis-coupled Markov chain Monte Carlo search algorithm was run with 5×10^6 generations. The burn-in and convergence of the chains were determined with TRACER v1.5 (available from http://tree.bio.ed.ac.uk/software/tracer). Twenty-six data partitions were recognised in the RAxML and MrBayes analyses which include introns and exons and splitting of each codon position to a separate partition.

Mating experiments

The genotype of the *MAT* locus was determined using the primers described by Sugui et al. (2010). Opposite mating type strains were paired within and between major clades (Fig. 1) on MEA, PDA and OA plates and incubated at 25, 30 and 37 °C in the dark. The plates were sealed with Parafilm and examined periodically for 3 months under a stereomicroscope for the production of ascomata and ascospores.

Results

Using the above-mentioned isolation techniques, many microscopic fungi were isolated from soil samples, including those that, according to their micromorphological characteristics, belonged to the *Aspergillus* section *Fumigati*. Long-term whitish cottony colonies with varying sporulation types and coloration that were dissimilar from *A. fumigatus* were isolated and characterised using molecular methods, phenotypic methods and mating experiments.

Molecular studies

Eight unique fingerprinting pattern types (and five subtypes in pattern VIII) were observed among 73 isolates (Table 1) when using fingerprinting with *M13-core* primer. Similarly, eight pattern types (and four subtypes in pattern VIII) were observed when using fingerprinting with 834 t primer (Table 1). There were 19 unique combinations resulting from the two fingerprinting methods and for each combination the *benA* and *caM* genes were amplified for at least one isolate.

No major conflicts were observed between single locus trees (ML as well as BI) based on *benA* and *caM*, and the data were combined into one dataset. There were 104 sequences and 1,030 positions in the final dataset of which 520 were variable and 398 were parsimony informative. The single gene alignments were deposited in TreeBASE (submission ID 14615). A pseudogene with stop codons within coding regions which was derived from the calmodulin gene, was amplified for isolate CCF 4477 (see the long branch in Fig. 1). The insertions within exons were eliminated from the alignment. Based on the *benA* gene sequence, this isolate belongs to the *A. udagawae* clade. Two additional protein coding loci (*RPB2* and *act1*) were amplified for the ex-type isolates of the newly described species and were deposited under accession numbers HF937377−HF937384. Similarly, the sequence of ITS region was deposited for the ex-type isolates because of interest in barcoding fungi (HG324081, HF937385, HF937386). However, data for section *Fumigati* are incomplete, and these three loci were not used for the phylogenetic analysis. The sequences of all five loci were able to uniquely determine all species of *A. viridinutans* complex.

Among 73 isolates from the reclamation sites, 68 belonged to the *A. viridinutans* complex. *Aspergillus udagawae* ($n=52$) was the most frequently isolated species, followed by *A. wyomingensis* sp. nov. ($n=12$) and *A. felis* ($n=4$). Two isolates (CCF 4499 and CCF 4482) were conspecific with *A. fumigatiaffinis* and one isolate (CCF 4117) was conspecific with *A. lentulus*. Two isolates (CCF 4149 and CCF 4190) showed unique, fingerprinting patterns, ITS sequences and exhibited 2–3 % dissimilarity on protein coding loci (*benA*, *caM*, *act1* and *RPB2*) each to the other and to the most closely related *N. galapagensis*; these isolates are proposed as the new species *A. brevistipitatus* sp. nov. and *A. conversis* sp. nov.

Mating experiments

MAT1-1 and MAT1-2 idiomorphs were determined for almost all isolates (Tables 1 and 2) based on different lengths of gene products in the assay described by Sugui et al. (2010). The identity of products was verified by DNA sequencing in

several isolates (accession numbers HF937387− HF937392). Systematic mating experiments were performed within and between major clades. Suitable conditions for successful mating differed between species (for details, see Taxonomy section below).

The MAT1-2 gene was amplified in three of four *A. felis* isolates. Five additional isolates from different sources (Table 2) were included in the mating experiments (CCF 4376, IFM 54303 and IFM 60053 which had the MAT1-1 idiomorph, and CBS 135240^{T} and FRR 5680 which had MAT1-2). Fertile cleistothecia were present in almost all possible combinations, and the ascospore morphology was consistent between the crosses. The isolates did not mate with type isolates of *A. wyomingensis*, *N. udagawae* and *A. viridinutans*. The isolates CBS 458.75 (MAT1-1, the ex-type strain of *A. fumigatus* var. *sclerotiorum*; Rai et al. 1971) and IMI 182127 (MAT idiomorph was not identified) which are both closely related to *A. felis* clade, did not produce cleithothecia with any isolate of *A. felis* and as well as with other species. However, both these isolates were held for decades in culture collections, and it is possible that their ability to reproduce sexually was decreased by repeated passaging and degeneration. As no other morphological and physiological differences from *A. felis* were found, these isolates are considered here to represent *A. felis*. The examination of mating bahaviour of genetically similar and fresh isolates would definitely confirm that both these strains represent eighter *A. felis* or separate species. The isolates IMI 280490 and NRRL 6106 previously examined by Varga et al. (2000) are no longer available but based on deposited sequence data also represent *A. felis*. Other two "atypical" isolates IMI 306135 and JV3 mentioned by Varga et al. (2000) are not included in *A. viridinutans* complex and are related to *A. lentulus* and *A. fumisynnematus*.

The ratio of MAT1-1 isolates to MAT1-2 isolates in *A. wyomingensis* ($n=14$) was 8:6. The isolates within the clade produced fertile cleistothecia and did not mate with other species from the *A. viridinutans* complex. The isolates CCF 4416 and CCF 4417^{T} produced particularly high numbers of cleistothecia, and dried cultures with cleistothecia were deposited into the herbarium of the Mycological Department, National Museum in Prague (PRM). *Aspergillus viridinutans* s. str., represented only by the ex-type isolate, had the MAT1-1 idiomorph and was used only to demonstrate cross-sterility with other species.

The isolates designated in this study as *A. udagawae* (Fig. 1) clustered into three subclades (clade 1: all isolates from Wyoming; clade 2: two mating ex-type isolates, IFM 46972^{MT} and IFM 46973^{MT}, from Brazilian soil; clade 3: additional isolates CCF 4479 and CMF ISB 2190 from Illinois and Indiana). The ratio of MAT1-1 isolates to MAT1-2 isolates was 13:42 ($n=55$; not determined in two isolated). Eight MAT1-1 isolates (IFM 46972^{MT}, CMF ISB 2190, CCF 4476, CMF ISB 2687, CMF ISB 2688, CMF ISB 2689, CMF ISB 2690 and CMF ISB 2691) and eight MAT1-2 isolates (IFM 46973^{MT}, CCF 4475, CCF 4478, CCF 4479, CCF 4481, CCF 4491, CCF 4494 and CMF ISB 1972) were chosen for mating experiments in all possible combinations. In the mating assay, only the mating ex-type isolate IFM 46972^{MT} produced fertile cleistothecia with all MAT1-2 strains with exception of CCF 4481. Additional pair producing fertile cleistothecia was CCF 4479×CMF ISB 2689. Cleistothecia without ascospores were produced by pair CCF 4502×CMF ISB 2688. Other crosses did not produce cleistothecia.

Fig. 1 Phylogenetic tree showing relationships of isolates from reclamation sites in Wyoming to other species in section *Fumigati*. The strains isolated in Wyoming are in bold font (accession numbers are listed in Table 1 and 2; for other accession numbers see Hubka et al. (2013b)). Thick lines indicate branches that support Bayesian probabilities greater than 0.95 and a bootstrap value greater than 90 %. Only bootstrap values ≥50 % and Bayesian probabilities ≥0.50 are shown. The ex-type isolates are designated by a superscript T. *Dichotomomyces cejpii* NRRL 5183^{T} was used as outgroup

Future studies are needed to confirm genetic recombination based molecular genetic methods in species from *A. viridinutans* complex, especially in species with high intraspecies genetic diversity such as *A. udagawae* and *A. felis*. However, we believe that the species boundaries as determined here using mating experiment (cross-fertility and cross-sterility) and strongly supported by morphology of ascospore could represent the true species limits.

Phenotypic species differentiation

The *A. viridinutans* complex includes five species. *Aspergillus aureolus* (syn. *A. indohii*) is the only homothallic species within the complex, the remaining species are probably heterothallic, and the teleomorph form (neosartorya-morph) was observed in *A. udagawae*, *A. wyomingensis* and *A. felis* (Fig. 2).

Species differentiation based solely on anamorph phenotype and macromorphology is difficult. However, *A. viridinutans* s. str. could be distinguished from other species within the complex by its slower growth and shorter conidiophores (rarely exceeding 100 μm). Nodding heads occurred in all species within the *A. viridinutans* complex and in some non-related species from section *Fumigati* (see Discussion). In contrast, the morphology of ascospores alone clearly differentiates all heterothallic species within the *A. viridinutans* complex (see sections Taxonomy and Dichotomous key to species from *Aspergillus viridinutans* complex).

No acid production on CREA was observed in *A. felis* or in *A. viridinutans* s. str., *A. brevistipitatus*, or *A. conversis*. In contrast, isolates of *A. fumigatiaffinis* and *A. lentulus* showed strong acid production. Variation in acid production on CREA occurred in isolates of *A. udagawae* and *A. wyomingensis*. In

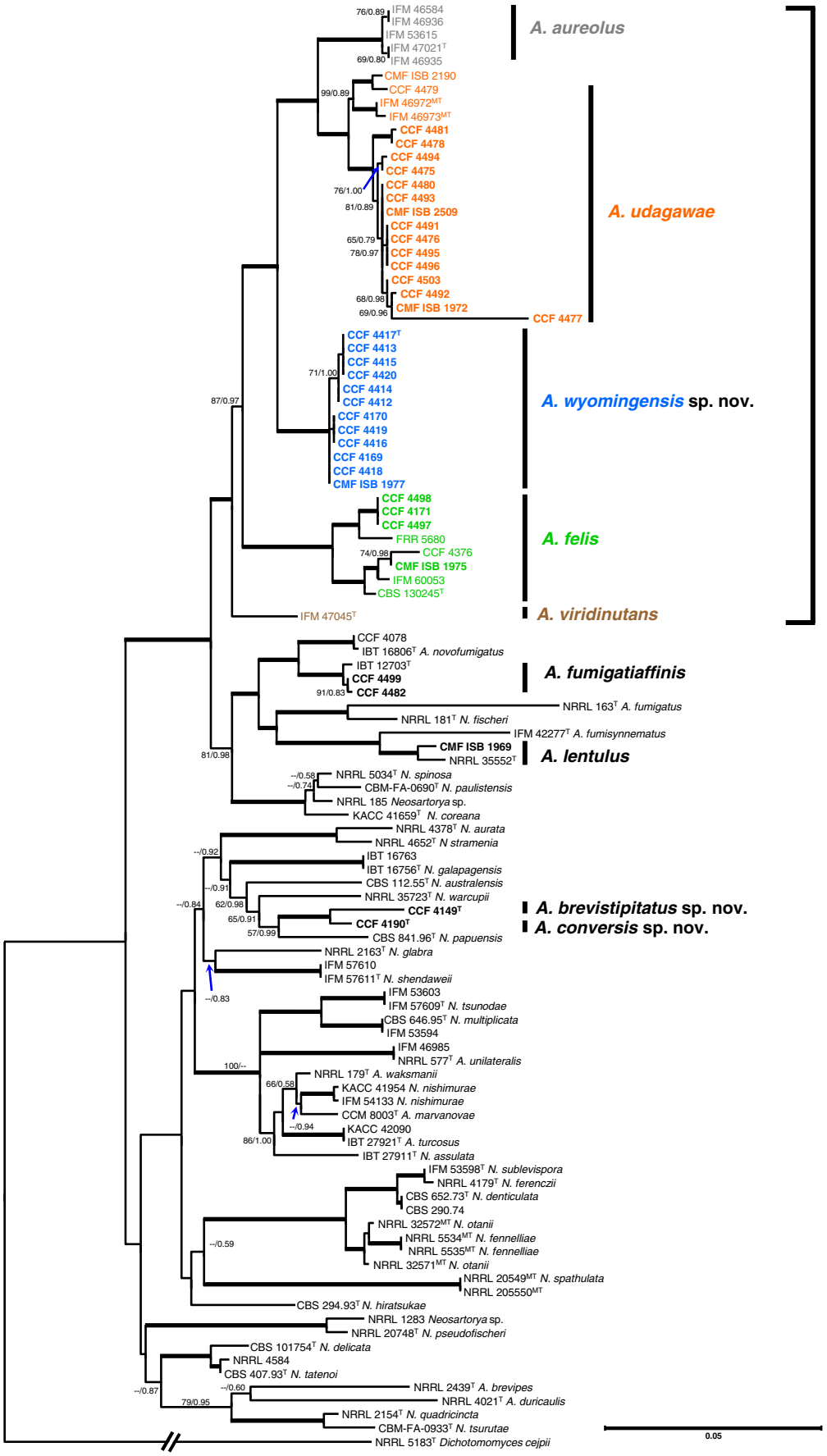

Table 1 Strains from *Aspergillus* section *Fumigati* examined in this study and isolated from reclamation sites in Wyoming (U.S.A.)

Species identity	Culture collection nr.[a]	Locality	Year	Isolation technique[b]	CREA (−/+/++/+++)[c]	MAT locus	M13-core	ISSR 863 t	EMBL accession nrs.	
									benA	*caM*
A. conversis sp. nov.	CCF 4190^T=CMF ISB 2151^T=NRRL 62496^T=IFM 60857^T=CBS 135457^T	Glenrock	2010	DPM	−	MAT1-2	I	I	HF933363	HF933387
A. brevistipitatus sp. nov.	CCF 4149^T=CMF ISB 2152^T=NRRL 62500^T=IFM 60858^T=CBS 135454^T	Glenrock	2010	DPM	−	MAT1-2	II	II	HF933364	HF933388
A. fumigatiaffinis	CCF 4482=CMF ISB 2158	Gillette	2008	DPM	++	MAT1-2	III	III	HE578083	HF933384
	CCF 4499=CMF ISB 2188	Gillette	2011	SWT	+++	MAT1-2	III	III	HF933348	HF933385
A. lentulus	CCF 4117=CMF ISB 1969	Glenrock	2010	DPM	++	MAT1-1	IV	IV	HE578078	HF933386
A. felis	CCF 4148=CMF ISB 1975=IFM 60868	Glenrock	2010	DPM	−	MAT1-1	V	V	HE578084	HF933404
	CCF 4171=CMF ISB 2162=IFM 60852	Glenrock	2010	DPM	−	MAT1-2	VI	VI	HF933350	HF933402
	CCF 4498=IFM 60853	Glenrock	2010	SWT	−	MAT1-2	VI	VI	HF933351	HF933403
	CCF 4497=CMF ISB 1936	Glenrock	2010	TR	−	MAT1-2	VI	VI	HF933349	HF933401
A. wyomingensis sp. nov.	CCF 4413=CMF ISB 2317	Glenrock	2010	TR	++	MAT1-1	VII	VIIa	HF933360	HF933391
	CCF 4170=CMF ISB 2485	Glenrock	2010	DPM	−	MAT1-2	VII	VIIa	HF933356	HF933392
	CCF 4414=CMF ISB 1974=IFM 60856	Glenrock	2010	DPM	+	MAT1-1	VII	VIIa	HF933353	HF933393
	CCF 4169=CMF ISB 2486	Glenrock	2010	SWT	+	MAT1-1	VII	VIIa	HF933354	HF933394
	CCF 4415=CMF ISB 2487	Glenrock	2010	SWT	−	MAT1-1	VII	VIIa	HF933357	HF933395
	CCF 4417^T=CMF ISB 2494^T=CBS 135456^T	Glenrock	2010	SWT	−	MAT1-1	VII	VIIa	HF933359	HF933397
	CCF 4418=CMF ISB 2162=IFM 60855	Glenrock	2010	DPM	+++	MAT1-2	VII	VIIa	HF933355	HF933398
	CCF 4411=CMF ISB 1977=IFM 60854	Glenrock	2010	TR	+	MAT1-2	VII	VIIa	HE578077	HF933389
	CCF 4420=CMF ISB 2491	Glenrock	2010	SWT	−	MAT1-1	VII	VIIa	HF933362	HF933400
	CCF 4412	Glenrock	2010	TR	−	MAT1-1	VII	VIIa	HF933352	HF933390
	CCF 4416=CMF ISB 1976=CBS 135455	Glenrock	2010	DPM	−	MAT1-2	VII	VIIb	HF933358	HF933396
	CCF 4419=CMF ISB 2495	Glenrock	2010	TR	+	MAT1-2	VII	VIIb	HF933361	HF933399
A. udagawae	CCF 4475	Glenrock	2010	TR	−	MAT1-2	VIIIa	VIIIa	HF933366	HF933407
	F7	Glenrock	2010	TR	−	MAT1-2	VIIIa	VIIIa		
	CCF 4503	Glenrock	2010	DPM	−	MAT1-2	VIIIb	VIIIa	HF933365	HF933406
	CMF ISB 2509	Glenrock	2010	DPM	−	MAT1-2	VIIIb	VIIIa	HF933367	HF933408
	CCF 4477	Gillette	2008	DPM	+++	MAT1-1	VIIIb	VIIIa	HF933372	HF933383
	CCF 4491=CMF ISB 1971	Glenrock	2010	TR	+	MAT1-2	VIIIb	VIIIb	HF933370	HF933411
	CMF ISB 1970	Glenrock	2010	TR	+	MAT1-2	VIIIb	VIIIb		
	F61	Gillette	2011	DPM	+++	MAT1-2	VIIIb	VIIIb		
	F62	Gillette	2011	DPM	+	MAT1-2	VIIIb	VIIIb		
	F63	Gillette	2011	DPM	+++	MAT1-2	VIIIb	VIIIb		
	F64	Gillette	2011	DPM	+++	MAT1-2	VIIIb	VIIIb		
	F65	Gillette	2011	DPM	+	MAT1-2	VIIIb	VIIIb		

Table 1 (continued)

Species identity	Culture collection nr.[a]	Locality	Year	Isolation technique[b]	CREA (−/+/++/+++)[c]	MAT locus	M13-core	ISSR 863 t	EMBL accession nrs.	
									benA	*caM*
	F67	Gillette	2011	DPM	++	MAT1-2	VIIIb	VIIIb		
	CMF ISB 2688	Gillette	2011	DPM	++	MAT1-1	VIIIb	VIIIb		
	F69	Gillette	2011	KB	−	MAT1-1	VIIIb	VIIIb		
	CCF 4478=CMF ISB 2193	Gillette	2011	DPM	++	MAT1-2	VIIIb	VIIIb	HF933376	HF933416
	F71	Gillette	2011	TR	+++	MAT1-2	VIIIb	VIIIb		
	CMF ISB 2687	Gillette	2011	DPM	−	MAT1-1	VIIIb	VIIIb		
	F73	Gillette	2011	DPM	−	MAT1-1	VIIIb	VIIIb		
	F74	Gillette	2011	DPM	−	MAT1-2	VIIIb	VIIIb		
	F75	Gillette	2011	SWT	−	MAT1-2	VIIIb	VIIIb		
	F77	Gillette	2011	DPM	+	MAT1-2	VIIIb	VIIIb		
	F78	Gillette	2011	DPM	−	MAT1-2	VIIIb	VIIIb		
	F79	Gillette	2011	DPM	+	MAT1-2	VIIIb	VIIIb		
	F80	Gillette	2011	DPM	+	MAT1-2	VIIIb	VIIIb		
	CCF 4502=CMF ISB 1972	Glenrock	2010	DPM	−	MAT1-2	VIIIb	VIIIc	HE578075	HF933405
	F14	Glenrock	2010	DPM	+	MAT1-2	VIIIb	VIIIc		
	F15	Glenrock	2010	SWT	−	MAT1-2	VIIIb	VIIIc		
	F17	Glenrock	2010	DPM	−	MAT1-2	VIIIb	VIIIc		
	CMF ISB 1978	Glenrock	2010	DPM	+++	MAT1-2	VIIIb	VIIIc		
	F19	Glenrock	2010	DPM	−	MAT1-2	VIIIb	VIIIc		
	F8	Glenrock	2010	TR	−	MAT1-2	VIIIb	VIIIc		
	CCF 4480=CMF ISB 2192	Gillette	2011	CelB	+++	MAT1-2	VIIIb	VIIId	HF933378	HF933418
	CCF 4481=CMF ISB 2191	Gillette	2011	DPM	++	MAT1-2	VIIIc	VIIIb	HF933379	HF933419
	CCF 4492	Glenrock	2010	DPM	−	MAT1-2	VIIId	VIIIc	HF933368	HF933409
	CMF ISB 2691	Glenrock	2010	DPM	+	MAT1-1	VIIId	VIIIc		
	CMF ISB 2690	Glenrock	2010	DPM	−	MAT1-1	VIIId	VIIIc		
	CCF 4494	Glenrock	2010	SWT	−	MAT1-2	VIIIe	VIIIa	HF933373	HF933413
	F58	Glenrock	2010	TR	+	MAT1-2	VIIIe	VIIIa		
	F59	Glenrock	2010	TR	−	MAT1-2	VIIIe	VIIIa		
	CCF 4495	Glenrock	2010	DPM	+	MAT1-2	VIIIe	VIIIb	HF933374	HF933414
	F48	Glenrock	2010	SWT	+	MAT1-2	VIIIe	VIIIb		
	F50	Glenrock	2010	DPM	−	ND[d]	VIIIe	VIIIb		
	CCF 4496	Glenrock	2010	DPM	−	MAT1-2	VIIIe	VIIIb	HF933375	HF933415
	CCF 4493=CMF ISB 1973	Glenrock	2010	SWT	−	MAT1-2	VIIIe	VIIIc	HF933369	HF933410
	CMF ISB 2689	Gillette	2008	DPM	+	MAT1-1	VIIIe	VIIIc		
	F36	Gillette	2008	DPM	+	MAT1-1	VIIIe	VIIIc		
	F37	Gillette	2008	DPM	+++	MAT1-1	VIIIe	VIIIc		

Table 1 (continued)

Species identity	Culture collection nr.[a]	Locality	Year	Isolation technique[b]	CREA (−/+/++/+++)[c]	MAT locus	M13-core	ISSR 863 t	EMBL accession nrs.	
									benA	*caM*
	F38	Glenrock	2010	DPM	+	MAT1-2	VIIIe	VIIIc		
	F40	Gillette	2008	DPM	++	MAT1-2	VIIIe	VIIIc		
	F41	Glenrock	2010	DPM	–	MAT1-2	VIIIe	VIIIc		
	CCF 4476	Glenrock	2010	DPM	++	MAT1-1	VIIIe	VIIId	HF933371	HF933412

[a] Acronyms of culture collections: CMF ISB—Collection of Microscopic Fungi of the Institute of Soil Biology of the Academy of Sciences of the Czech Republic in České Budějovice; CCF—Culture Collection of Fungi in the Department of Botany of Charles University in Prague; IFM—Collection at the Medical Mycology Research Center, Chiba University; NRRL—Agricultural Research Service Culture Collection, Peoria, Illinois, U.S.A.; CBS, Centraalbureau voor Schimmelcultures, Utrecht, Netherlands

[b] *DPM* dilution plate method, *SWT* soil washing technique, *TR* isolation for thermoresistent fungi, *KB* keratin-bait technique, *CelB* cellulose-bait technique, *GSM* gravity settling method

[c] '–' no acid, + weak acid, ++ medium acid, +++ strong acid

[d] *ND* not determined

particular, the colony morphology of *A. udagawae* isolates is highly variable in terms of sporulation rate, reverse colouration and acid production on CREA. Additionally, the inability of many strains to produce fertile cleistothecia with tester strains made *A. udagawae* difficult to identify without molecular methods.

All isolated species had maximum growth temperatures between 42 and 45 °C, all isolates grew at 42 °C, and no isolates grew at 47 °C. *Aspergillus viridinutans* s. str., *A. brevistipitatus* and *A. conversis* had a maximum growth temperature of 42 °C and did not grow at 45 °C. Isolate-dependent growth at 45 °C after 7 d on MEA was observed in *A. wyomingensis* (0–16 mm), *A. udagawae* (0–7 mm) and *A. felis* (0–20 mm).

Aspergillus conversis and *A. brevistipitatus* are phylogenetically distant from all other anamorphic and heterothallic species. Closely related homothallic *Neosartorya* spp. (Fig. 1) are easily differentiated from *A. conversis* and *A. brevistipitatus*. The micromorphology of both these species is reminiscent of *A. brevipes*, *A. unilateralis* and *A. duricaulis* which share with these two species echinulate conidia, short conidiophores and nodding heads (see Diagnoses in the section Taxonomy below).

Taxonomy

Current nomenclature requires the use of only one name for a species (McNeill et al. 2012), whereas previously two or more names could be applied to the same fungus. We continue the trend in naming *Aspergillus* species with pleomorphic life cycle only as *Aspergillus* (Samson et al. 2011; Hubka et al. 2013a, b).

Aspergillus brevistipitatus A. Nováková & Hubka, *sp. nov.* — MB803934; Fig. 3

Etymology. Named after its short conidiophores.

Description. Colonies on CYA 31–37 mm in diam at 25 °C in 7 days, velutinous, wrinkled, sporulation greater in marginal parts, light greenish grey (ISCC-NBS No. 154) to greenish grey (No. 155), darker coloured margin (greyish green – No. 150), no exudate or soluble pigment production, reverse yellowish white (No. 92) to greyish yellow (No. 90). Good growth (40–45 mm in diam) and sporulation at 37 °C, velutinous, wrinkled colonies, olive grey (No. 113) with light orange yellow (No. 70) reverse, exudate yellowish white. Colonies on MEA 36–43 mm, velutinous, wrinkled, light bluish grey (No. 190) to greenish grey (No. 155), no exudate or soluble pigment production, reverse moderate yellow (No. 87) to strong yellow (No. 84). Colonies on YES 40–45 mm in diam, velutinous, wrinkled, yellowish grey (No. 93) to light olive grey (No. 112) with light greenish grey margins (No. 154), no exudate or soluble pigment production, reverse moderate yellow (No. 87). Colonies on CZA 28–30 mm in diam, no sporulation, no exudate or soluble pigment production, reverse colourless. Colonies on CREA 25 mm in diam, no

Table 2 Additional isolates examined in this study

Species identity	Culture collection nr.[a]	Locality	Year	MAT locus	EMBL and GenBank accession nrs.	
					benA	*caM*
A. aureolus	IFM 47021^{T}=CBS 105.55^{T}=NRRL 2244^{T}=IMI 06145^{T}	Soil, Ghana	1950	ND[b]	AF057319	AY689369
	IFM 46584=CCF 4645	Soil, Brazil	1993	ND	HG426057	HG426050
	IFM 46935=CCF 4646	Soil, unknown	–	ND	HG426058	HG426051
	IFM 46936=CCF 4647	Soil, unknown	–	ND	HG426059	HG426052
	IFM 53615=CCF 4571 (ex-type of *A. indohii*)	Soil, Brazil	2001	ND	AB488757	AB488765
A. felis	CBS 130245^{T}	Retrobulbar mass in cat, Australia	<2013	MAT1-2	JX021700	JX021715
	CBS 458.75 (ex-type of *A. fumigatus* var. *sclerotiorum*)	Soil, India	<1971	MAT1-2	AY685178	HG426048
	CCF 4376	Soil of reclamation site, Czech Rep.	2011	MAT1-1	HF933380	HF933422
	FRR 5680	Retrobulbar abscess in cat, Australia	<2005	MAT1-2	HF933381	HF933420
	IFM 54303=CCF 4570	Food, Japan	<2007	MAT1-1	AB248299	AB259973
	IFM 60053=CCF 4559	Abscess near thigh bone (osteomyelitis in man), Japan	2012	MAT1-1	HF933382	HF933421
	IMI 182127	*Pinus caribea*, Sri Lanka	<1974	ND	AF134777	DQ094888
A. udagawae	IFM 46973^{MT}=CBS 114218^{MT}=CCF 4558^{MT}	Soil, Brazil	1993	MAT1-2	AB248303	AY689373
	IFM 46972^{MT}=CBS 114217^{MT}	Soil, Brazil	1993	MAT1-1	AB248302	AY689372
	CCF 4233	Human nail, Czech Rep.	2011	ND		HG426054
	CCF 4479=CMF ISB 2189	Illinois, U.S.A.	2011	MAT1-2	HF933377	HF933417
	CMF ISB 2190	Indiana, U.S.A.	2011	MAT1-1	HG426055	HG426049
A. viridinutans	IFM 47045^{T}=NRRL 4365^{T}=NRRL 576^{T}= CBS 127.56^{T}=IMI 367415^{T}=CCF 4382^{T}	Dung of rabbit, Australia	<1954	MAT1-1	EF669834	EF669904
A. wyomingensis	IFM 59681=CCF 4563	Soil, China	2008	MAT1-2	HG426056	HG426053
	IMI 133982=CCF 4383	Soil, Russia	<1968	MAT1-1	AF134775	DQ094889

[a] Acronyms of culture collections: CMF ISB—Collection of Microscopic Fungi of the Institute of Soil Biology of the Academy of Sciences of the Czech Republic in České Budějovice, CCF—Culture Collection of Fungi in the Department of Botany of Charles University in Prague, IFM—Collection at the Medical Mycology Research Center, Chiba University; NRRL—Agricultural Research Service Culture Collection, Peoria, Illinois, U.S.A.; CBS, Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; FRR—Food Fungal Culture Collection, North Ride, Australia; IMI, CABI's collection of fungi and bacteria, Egham, UK

[b] Not determined

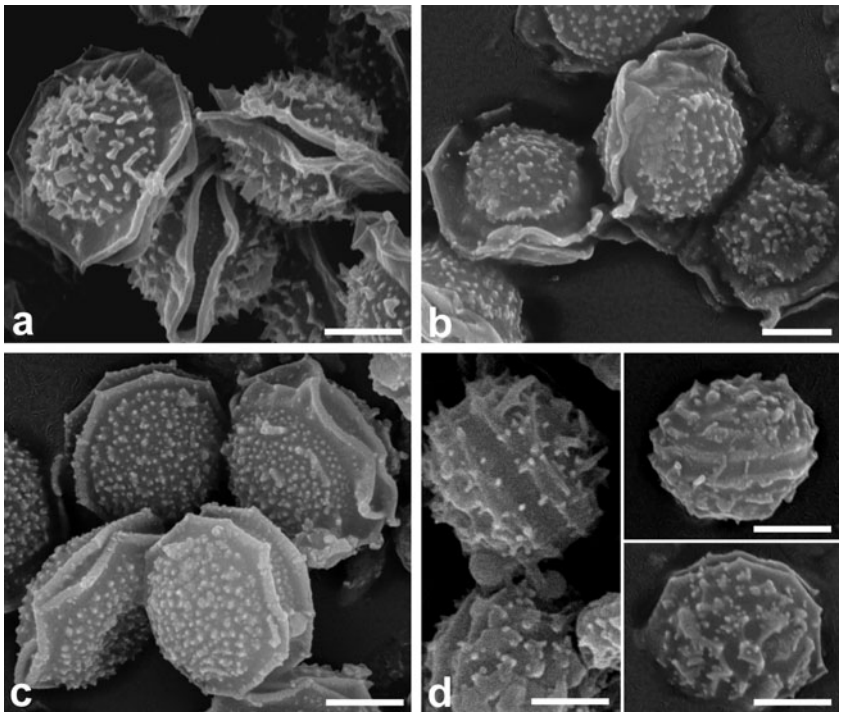

Fig. 2 Comparison of ascospores morphology of homothallic *A. aureolus* and three heterothallic species in *A. viridinutans* complex as observed by SEM. **a** *A. aureolus*; **b** *A. felis*; **c** *A. udagawae*; **d** *A. wyomingensis*. — Scale bars 2 μm

visible sporulation, no acid production. Ehrlich test negative. Maximum growth temperature is 42 °C.

Conidial heads short columnar. Conidiophores arising from aerial hyphae, smooth, most commonly 34–144×2.5−3.2 μm, septate, sometimes extremely short measuring only several μm, nodding heads common. Conidial heads uniseriate, vesicles subglobose to pyriform, 7–13 μm, phialides ampuliform, 3.5−4.5 μm, covering the upper half to two-third of vesicle. Conidia subglobose, rough, verrucose to spinose, 2–2.9 μm.

Diagnosis. The micromorphology is reminiscent of *A. brevipes*, *A. unilateralis* and *A. duricaulis* which also have echinulate conidia and short conidiophores with common nodding appearance. Colonies of *A. brevipes* are purple-red, and *A. unilateralis* has nearly black reverses on CZA. The growth of *A. conversis* and *A. duricaulis* on all media is slower in comparison with *A. brevistipitatus*.

Type. U.S.A., Wyoming, Converse Country, Powder River Basin, Glenrock—Rolling Hills Wind Plant (former Dave Johnson Coal Mine), site recultivated by crested wheatgrass (*Agropyron cristatum*), ex soil, 2010, *A. Nováková*, holotype PRM 860543, a dried culture, isotype PRM 860544, culture ex-holotype CCF 4149^T (= CMF ISB 2152^T=NRRL 62500^T=IFM 60858^T=CBS 135454^T).

Aspergillus conversis Hubka & A. Nováková, *sp. nov.* — MB803935; Fig. 4

Etymology. Relating to Converse County, Wyoming, U.S.A.

Description. Colonies on CYA 25–26 mm in diam at 25 °C in 7 days, velutinous, wrinkled, with white margin, light greenish grey (ISCC-NBS No. 154) to greyish yellow green (No. 122), no exudate or soluble pigment production, reverse yellowish white (No. 92) to pale yellow (No. 89). Colonies at 37 °C 30–40 mm, velutinous, wrinkled, yellowish grey (No. 93), light greenish grey (154) in margin, reverse pale orange yellow (No. 73). Colonies on MEA 30–32 mm, velutinous with floccose centre, light greenish grey (No. 154) with pale green (No. 149) centre, no exudate or soluble pigment production, reverse brilliant yellow (No. 83) to brilliant orange yellow (No. 67). Colonies on YES 32–38 mm in diam, velutinous, wrinkled, light greenish grey (No. 154) to greenish grey (No. 155), exudate yellowish white (No. 92) to pale greenish yellow (No. 104), no soluble pigment, reverse strong yellow (No. 84). Colonies on CZA 28–30 mm in diam, no sporulation, no exudate or soluble pigment production. Colonies on CREA 15–20 mm in diam, no visible sporulation, no acid production, reverse colourless. Ehrlich test negative to very light yellow. Maximum growth temperature is 42 °C.

Conidial heads short columnar. Conidiophores arising from aerial hyphae, smooth, septate, most commonly 47−70(−90)×2.5−4 μm, sometimes extremely short measuring only several μm, nodding heads common. Conidial heads uniseriate, vesicles subglobose to subclavate, 6.5−9(−12) μm, phialides ampuliform, 3.5−5 μm, covering the upper half of vesicle. Conidia ellipsoidal to subglobose, delicately roughened, 2.0−2.8 μm.

Diagnosis. The species is most closely related and similar to *A. brevistipitatus* (see "Diagnosis" above) but the growth of *A. conversis* is slower on all media and at all temperatures. The colonies of *A. conversis* on CZA lack broad areas of submerged growth which are present in *A. duricaulis*.

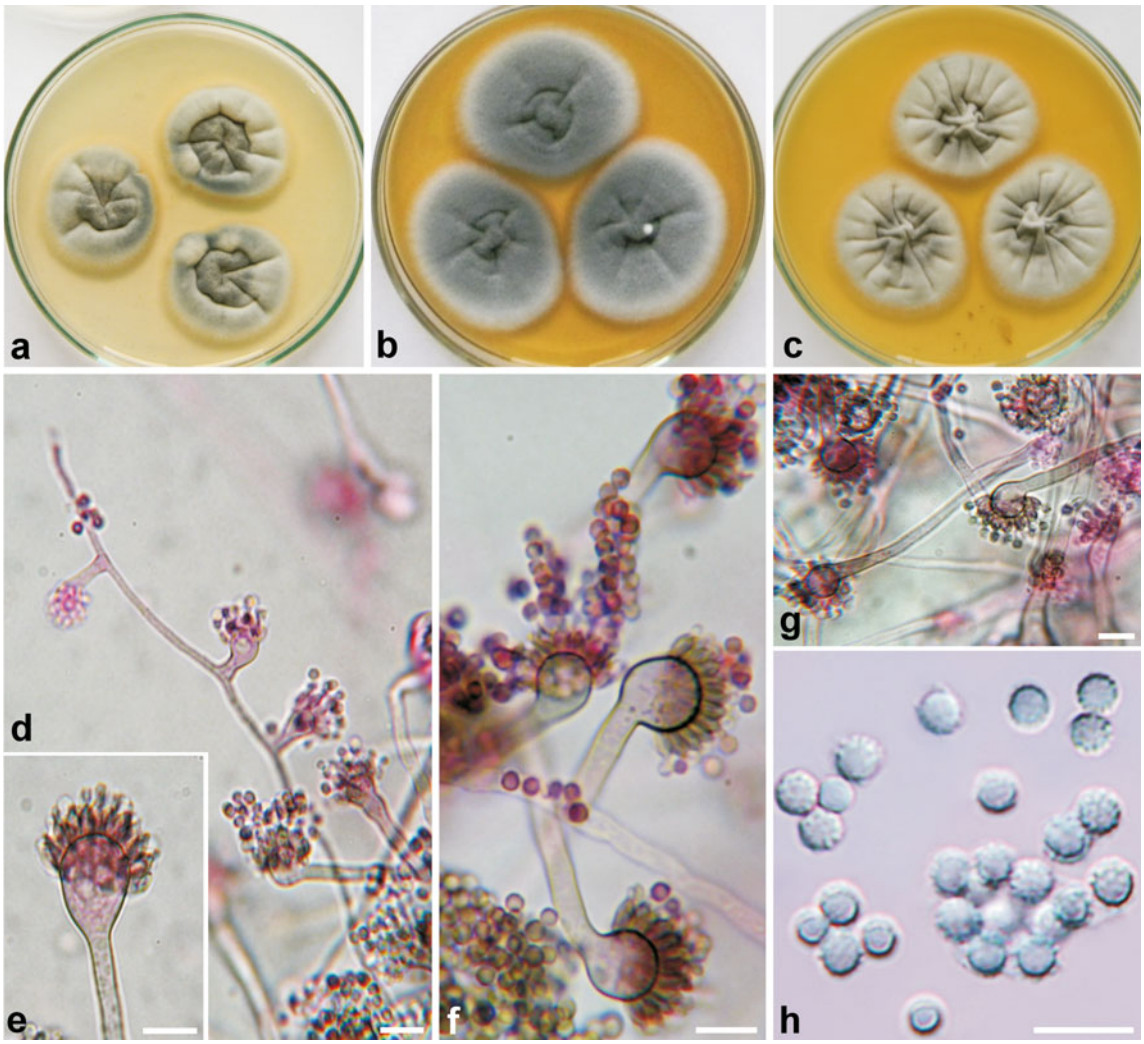

Fig. 3 *Aspergillus brevistipitatus* CCF 4149^{T}. **a**–**c** Colonies incubated 7 days at 25 °C on CYA, MEA and YES, from left to right; **d**–**g** conidiophores; **h** conidia. — Scale bars 10 μm

Type. U.S.A., Wyoming, Converse Country, Powder River Basin, Glenrock—Rolling Hills Wind Plant (former Dave Johnson Coal Mine), site recultivated by crested wheatgrass (*Agropyron cristatum*), ex soil, 2010, *A. Nováková*, holotype PRM 860541, a dried culture, isotype PRM 860542, culture ex-holotype CCF 4190^{T}=CMF ISB 2151^{T}=NRRL 62496^{T}=IFM 60857^{T}=CBS 135457^{T}.

***Aspergillus viridinutans* complex**

Aspergillus aureolus Fennell and Raper 1955, Mycologia 47: 71 — MB292836; Fig. 5

= *Sartorya aureola* (Fennell & Raper) Subram. 1972, Curr. Sci. 41: 760 — MB323108

= *Neosartorya aureola* (Fennell & Raper) Malloch & Cain 1973, Can. J. Bot. 50: 2620 — MB318627

= *Aspergillus aureoluteus* Samson & W. Gams 1985, in Samson & Pitt (eds), Adv. *Penicillium Aspergillus* Syst.: 34 — MB114698

= *Aspergillus indohii* Y. Horie 2003, Mycoscience 44: 398 — MB489533

= *Neosartorya indohii* Y. Horie 2003, Mycoscience 44: 398 — MB489536

Description. Colonies on CYA 54–62 mm in diam at 25 °C in 7 days, floccose, delicatelly furrowed to irregularly wrinkled, pale yellow (ISCC-NBS No. 89), light yellow (No. 86) to strong yellow (No. 84) with yellowish white margins, no exudate, no diffusible pigment, reverse deep yellowish brown (No. 75) to medium olive brown (No. 95) with 3–5 mm medium yellow (No. 87) margins. Colonies at 37 °C 65–70 mm in diam, plane to irregularly furrowed, floccose to lanose with umbonate centre, visible ascomata in some strains, yellowish white (No. 92) to pale yellow (No. 89), light olive grey (No. 112) sporulation in some strains, no exudate, no diffusible colour, reverse reverse yellowish white, pale orange yellow (No. 73) to strong yellow (No. 84). Colonies on MEA 38–70 mm in diam, velutinous to floccose with umbonate centre, delicately furrowed to irregularly wrinkled, yellowish

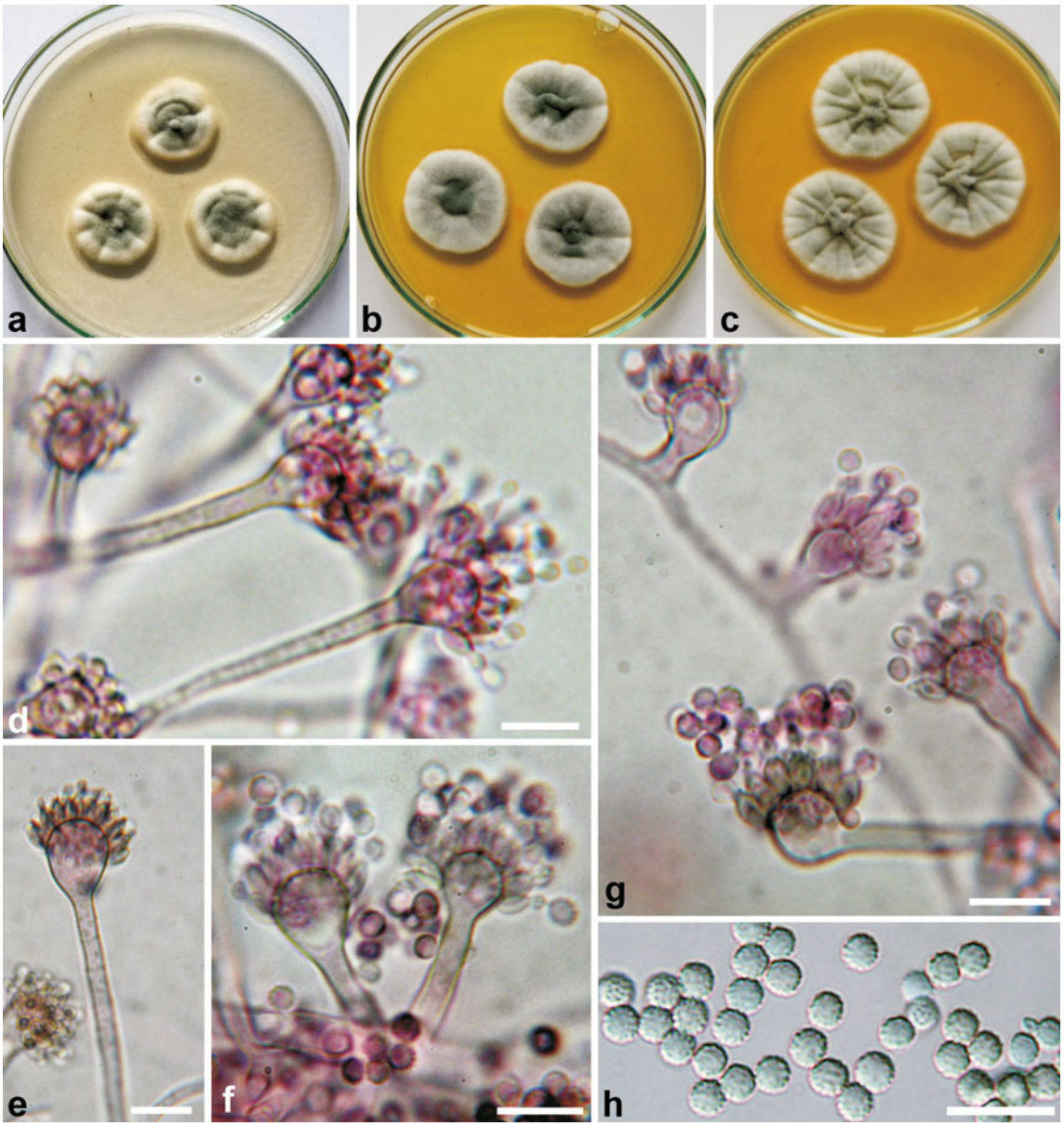

Fig. 4 *Aspergillus conversis* CCF 4190^{T}. **a**–**c** Colonies incubated 7 days at 25 °C on CYA, MEA and YES, from left to right; **d**–**g** conidiophores; **h** conidia. — Scale bars 10 μm

white (No. 92), pale yellow (No. 89) to brilliant orange yellow (No. 67), no exudate, no diffusible colour, reverse strong yellow (No. 84), deep orange (No. 51) to strong brown (No. 55) with light yellow (No. 86) to strong yellow margins. Colonies on YES 43–58 mm in diam, floccose to lanose, plane, yellowish white (No. 92) with pale yellow tint in the colony centre, brilliant orange yellow (No. 67) to pale orange yellow with paler margins, no exudate, no diffusible colour, reverse brilliant yellow (No. 83) with vivid orange yellow or strong to vivid orange (No. 68 and 66) colonies centre or strong orange (No. 50) to vivid orange (No. 48). Colonies on CZA 48–53 mm in diam, floccose to lanose, plane, yellowish white (No. 92) to pale yellow (No. 89), colony centre pale yellow (No. 98) to greyish yellow (No. 90) or with pale orange yellow (No. 73) coloured central parts, no exudate, no diffusible pigment, reverse yellowish white with strong yellow (No. 84) colony centre with several dark greyish yellow (No. 91), light olive brown (No. 94) or dark olive brown (No. 96) spots or yellowish white with pale yellow tint in colonies centre. Colonies on CREA 28–43 mm in diam, sparse colourless to yellow coloured mycelium with visible ascomata or dense mycelial ring in some strains, weak acid production under colonies. Ehrlich test negative.

Conidial heads columnar. Conidiophores arising from aerial hyphae, smooth, septate, up to 450.0×2.5−3.5(−5) μm, nodding heads rarely present. Conidial heads uniseriate,

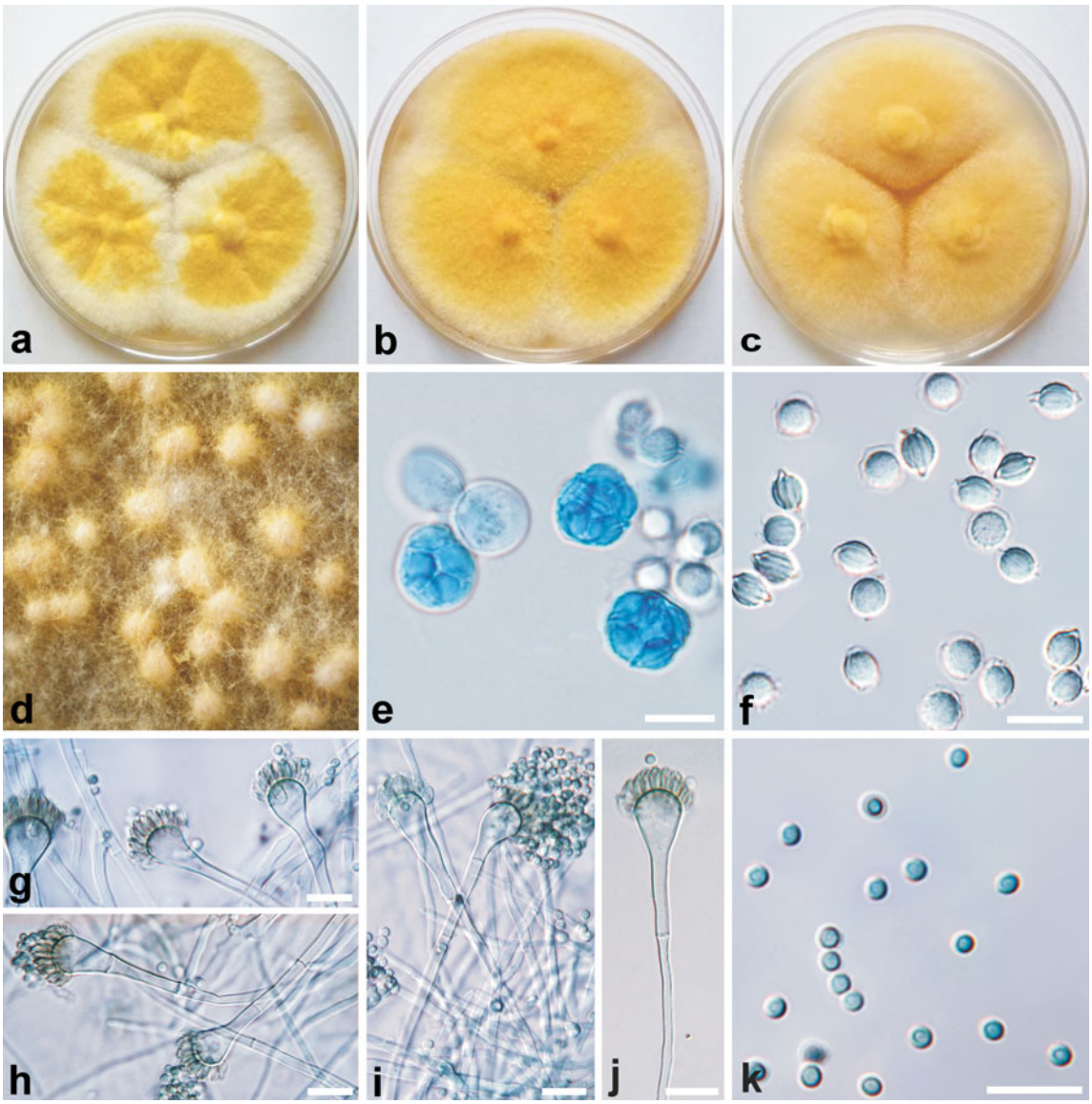

Fig. 5 *Aspergillus aureolus* IFM 46935. **a**–**c** Colonies incubated 7 days at 25 °C on CYA, MEA and YES, from left to right; **d** cleistothecia; **e** asci; f. ascospores; **g**–**j** conidiophores; **k** conidia. — Scale bars 10 μm

vesicle clavate, 8–13.5 μm, phialides ampuliform covering the upper half of vesicle. Conidia broadly subglobose, delicately roughened, 2.1−2.8 μm.

Homothallic species; the ascomata visible after 1 week of incubation on CYA, CYA37, MEA, YES and CREA. Cleistothecia yellowish white to yellow, globose or subglobose, 200–650 μm in diameter, covered by a felt of yellowish hyphae; asci eight-spored, globose to subglobose, 10.5−13.5×10–12 μm; ascospores lenticular, spore bodies (4−)4.5−5.5 μm in longer axis, with two well-separated, 1–1.5 μm wide, irregular equatorial crests, convex surface echinulate and tuberculate. The species is able to grow at 42 °C and does not grow at 45 °C.

Diagnosis. The species is homothallic in contrast to other members of *A. viridinutans* complex. The most of isolates have typical yellow to golden yellow colonies, most conspicuous on MEA and CYA. The ascospores have echinulate and tuberculate convex surface, broad equatorial crests and are very similar to those of heterothallic *A. felis*. Colonies in shades of yellow and orange can be found in several other species (*A. auratus*, *A. laciniosus*, *A. multiplicatus* and *A. stramenius*) and all of them can be distinguished by different ascospores morphology and spectrum of produced secondary metabolites (Samson et al. 2007a). *Aspergillus laciniosus* and *A. auratus* have ascospores with convex surface smooth or slightly roughened by light microscopy, in addition *A. auratus* has slower growth parameters on all media and temperatures and has smaller cleistothecia. *Aspergillus laciniosus* is able to grow at 47 °C in contrast to *A. aureolus*. Ascospores of *A. multiplicatus* lack equatorial crests and have ribbed to

reticulate ornamentation. The cleistothecia of *A. stramenius* are smaller and its ascospores are less conspicuously roughened.

Ecology. Soil of tropical countries (Brazil, Ghana, Liberia, Suriname), passion-fruit juice (Fiji). References: Fennell and Raper (1955); Peterson (1992); Horie et al. (1995); Horie et al. (2003); Samson et al. (2007a).

Specimens examined. Five isolates, see Table 2.

Nomenclatural notes. The new botanical code (McNeill et al. 2012) enables the use of some well known holomorphic names that were treated as invalid in the dual nomenclature system and have priority over names proposed later separately for teleomorph and anamorph. This is true for *A. aureolus*. However, inappropriate type (living culture) was designated by the Fennell and Raper (1955), and the species is neotypified here. Incorrect type designation before 1st January 1958 do not affect validity of published name [Art. 40.1; McNeill et al. 2012].

Type. Ghana, ex soil, 1950, *C. F. Charter*, a dried culture Herb. CBS 105.55 derived from the living culture CBS 105.55 is here designated as neotype. Ex-neotype culture CBS 105.55^{T}=NRRL 2244^{T}=IFM 47020^{T}=IMI 06145^{T}.

Aspergillus felis Barrs, van Doorn, Varga & Samson 2013, Plos One 8: e64871 — MB560382; Fig. 6

=? *Aspergillus fumigatus* var. *sclerotiorum* J.N. Rai, S.C. Agarwal & J.P. Tewari 1971, J. Ind. Bot. Soc. 50: 66 — MB347792

Description. Colonies on CYA 52–65 mm in diam at 25 °C in 7 days, floccose, plane to slightly wrinkled in central part of

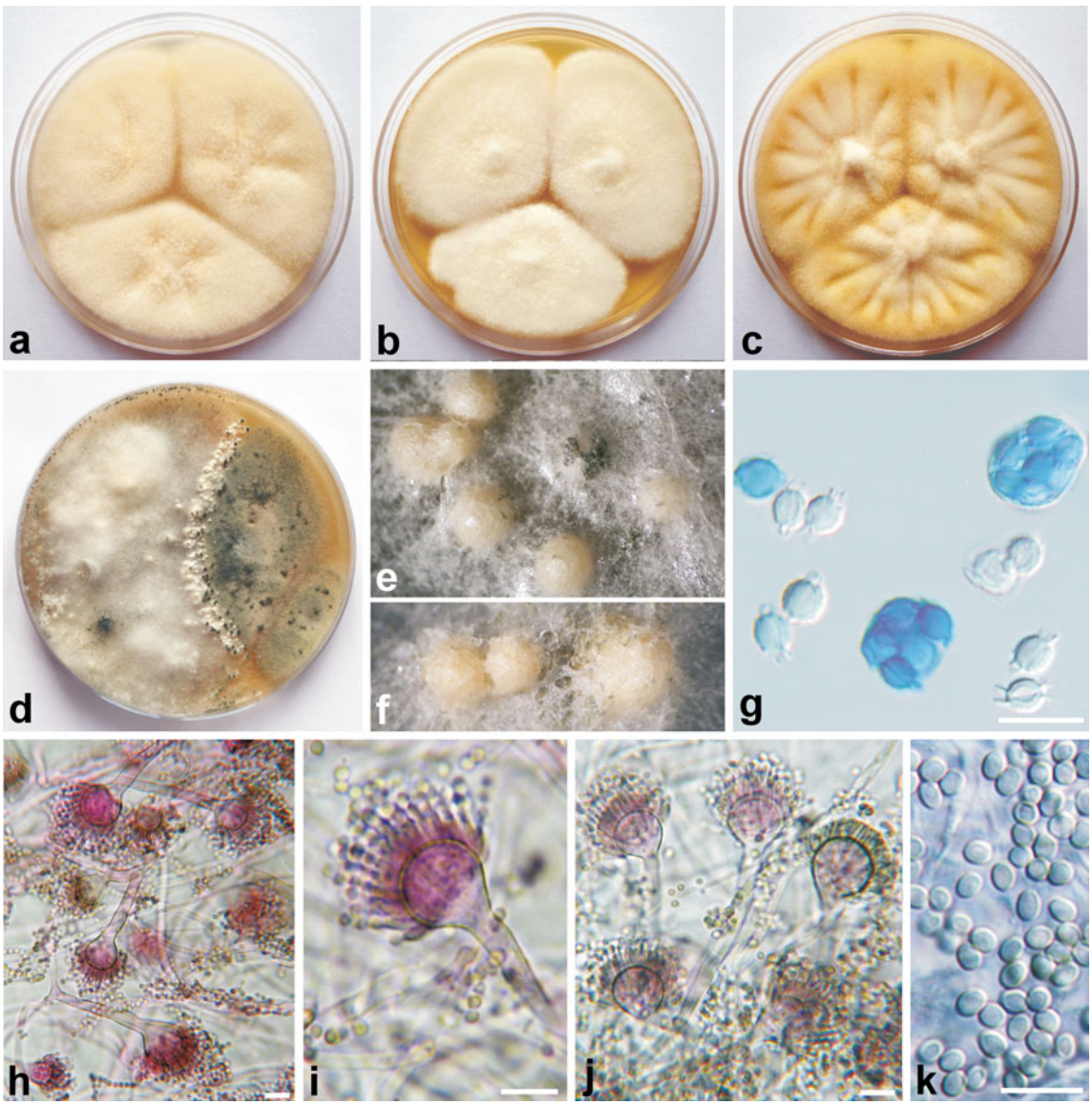

Fig. 6 *Aspergillus felis* CCF 4497. **a**–**c** Colonies incubated 7 days at 25 °C on CYA, MEA and YES, from left to right; **d** fertile cleistothecia as a result of crossing of isolates IFM 60053 and FRR 5679 on MEA after 6 weeks. **e**–**f** cleistothecia. **g** asci and ascospores. **h**–**j** conidiophores; k. conidia. — Scale bars 10 μm

colonies, yellowish white (ISCC-NBS No. 92) to very pale green (No. 148), pale green (No. 149) in marginal parts, greyish yellow green sporulation (No. 122) after 14 days, no exudate or soluble pigment production, reverse yellowish white (No. 92) to pale yellow (No. 89). Colonies at 37 °C 65–70 mm, lanose, irregularly wrinkled, yellowish white (No. 92), reverse pale yellow (No. 89). Colonies on MEA 54–68 mm, floccose, white to yellowish white (No. 92) to very pale green (No. 148), after 14 days greenish grey (No. 155) to greyish green (No. 150) sporulation, no exudate or soluble pigment production, reverse brilliant yellow (No. 83) to strong yellow (No. 84). Colonies on YES 65–70 mm in diam, floccose, regularly wrinkled, white, light yellow (No. 89) to very pale green (No. 148), no exudate or soluble pigment production, reverse moderate yellow (No. 87). Colonies on CZA 45–48 mm in diam, plane, whitish yellow. Colonies on CREA 40–55 mm in diam, poor mycelial growth, no acid production, reverse colourless. Ehrlich test negative to very light violet. Only some isolates were able to grow restrictedly (up to 20 mm) at 45 °C.

Conidial heads short columnar. Conidiophores arising from aerial hyphae, smooth, up to 252×3–5.5(−6.5) μm, nodding heads occasionally present. Conidial heads uniseriate, vesicles subglobose to subclavate, (7−)9−18(−20) μm, phialides ampuliform covering the upper half of vesicle. Conidia broadly subglobose, delicately roughened, 2.3−2.8 μm.

Heterothallic species; the ascomata visible after 3 weeks of incubation on MEA, OA and PDA at 25, 30 and 37 °C, mature ascospores present after 4–6 weeks. Cleistothecia yellowish white, globose or subglobose 300−1,000(−1,250) μm in diameter, glabrous or covered by a loose felt of white hyphae; asci eight-spored, globose to subglobose 10–12×9.5–11 μm; ascospores lenticular, spore bodies 3.2–5 μm in longer axis, with two well-separated, 1–1.5(−2) μm wide, irregular equatorial crests, convex surface echinulate and tuberculate.

Diagnosis. The morphology of ascospores with markedly echinulate and tuberculate convex and broad equatorial crests (> 1 μm wide) differentiates *A. felis* from other heterothallic species in *A. viridinutans* complex. Some other heterothallic species have somewhat similar ascospores but all of them can be differentiated using morphology and physiology. The convex surface of ascospores of *A. spathulatus* is smooth, *A. nishimurae* has broadly ellipsoidal conidia and is able to grow at 47 °C in contrast to *A. felis*, the convex surface of ascospores of *A. fennelliae* and *A. otanii* is only delicately roughened, and their colonies on MEA have greyish colour.

Ecology. Soil (Czech Republic, India, U.S.A., Zambia), *Pinus caribea* (Sri Lanka), indoor air (Germany); human pathogen (or clinical material contaminant in some cases)—lungs, upper respiratory tract, thigh bone, cornea, nail (Japan, Portugal, Spain, U.S.A.), animal pathogen—thoracic mass and retrobulbar abscess in cats (Australia, Japan, UK), vitreous humor in dog (Australia). References: Rai et al. (1971); Varga et al. (2000); Katz et al. (2005); Yaguchi et al. (2007); Alcazar-Fuoli et al. (2008); Vinh et al. (2009a); Coelho et al. (2011); Barrs et al. (2012); Shigeyasu et al. (2012); Barrs et al. (2013); Kano et al. (2013); Peláez et al. (2013)

Specimens examined. Eleven isolates, see Tables 1 and 2. A dried colony from a paired culture of isolates CCF 4171= CMF ISB 2162=IFM 60852×IFM 60053 was deposited as PRM 860735 and PRM 860736.

Type. Australia, retrobulbar mass in domestic short-haired cat, holotype CBS H-21125, culture ex-holotype CBS 130245^{T}.

Aspergillus udagawae Horie, Miyaji and Nishim. 1995, Mycoscience 36: 199 — MB412533; Fig. 7

= *Neosartorya udagawae* Y. Horie, Miyaji & Nishim. 1995, Mycoscience 36: 199 — MB413573

Description. Colonies on CYA 40–60 mm in diam at 25 °C in 7 days, velutinous to effuse, wrinkled, yellowish white (ISCC-NBS No. 92) to light greenish grey (No. 154) but strongly coloured in some strains with moderate greenish blue (No. 173), dark bluish green (No. 165) to dark greenish blue (No. 174) colour with sporulation in marginal area or on the whole colony surface, no exudate or soluble pigment production, reverse yellowish white (No. 92). Colonies on CYA at 37 °C 65–70 mm, lanose, yellowish white (No. 92) to greenish grey (No. 155) to dark greenish grey (No. 156) in parts with visible sporulation, reverse pale yellow (No. 89). Colonies on MEA 42–65 mm in diam, floccose, plane to very fine wrinkled, white to yellowish white (No. 92), in some strains with pale yellow in the centre, sporulation in very light bluish green (No. 162) to moderate bluish green (No. 164), no exudate, no diffusible pigment in some strains or yellow pigment in other ones, reverse yellowish white (No. 92) to vivid yellow (No. 82) in the case of diffussible pigment production. Colonies on YES 62–75 mm in diam, floccose, plane to wrinkled, yellowish white (No. 92) to light greenish grey (No. 154), no exudate or soluble pigment production, reverse yellowish white (No. 92). Colonies on CZA 37–47 mm in diam at 25 ºC, velutinous, plane to sporadicaly wrinkled, with 0.3–0.5 mm submerse margins, yellowish white (No. 92), no exudate, no soluble pigment, reverse yellowish white. Colonies on CREA 46–50 mm in diam, floccose, pure sporulated, without acid production in some strains to slight or very strong acid production in other ones. Ehrlich test negative. Only some isolates were able to grow restrictedly (up to 7 mm) at 45 °C, all grew at 42 °C.

Conidial heads columnar. Conidiophores arising from the aerial hyphae up to 500×(2.5–)3–6.5 μm with stipes smooth, vesicles hemispherical to subglobose, 11–18(−19.5) μm, nodding heads occasionally present. Conidia globose, subglobose to ellipsoidal, smooth, 2.4–3.2 μm.

Heterothallic species; the ascomata visible after 3 weeks of incubation on MEA at 25 °C, mature ascospores present after 5–6 weeks. Cleistothecia yellowish white, globose or subglobose 300–600(−1,000) μm in diameter, glabrous or

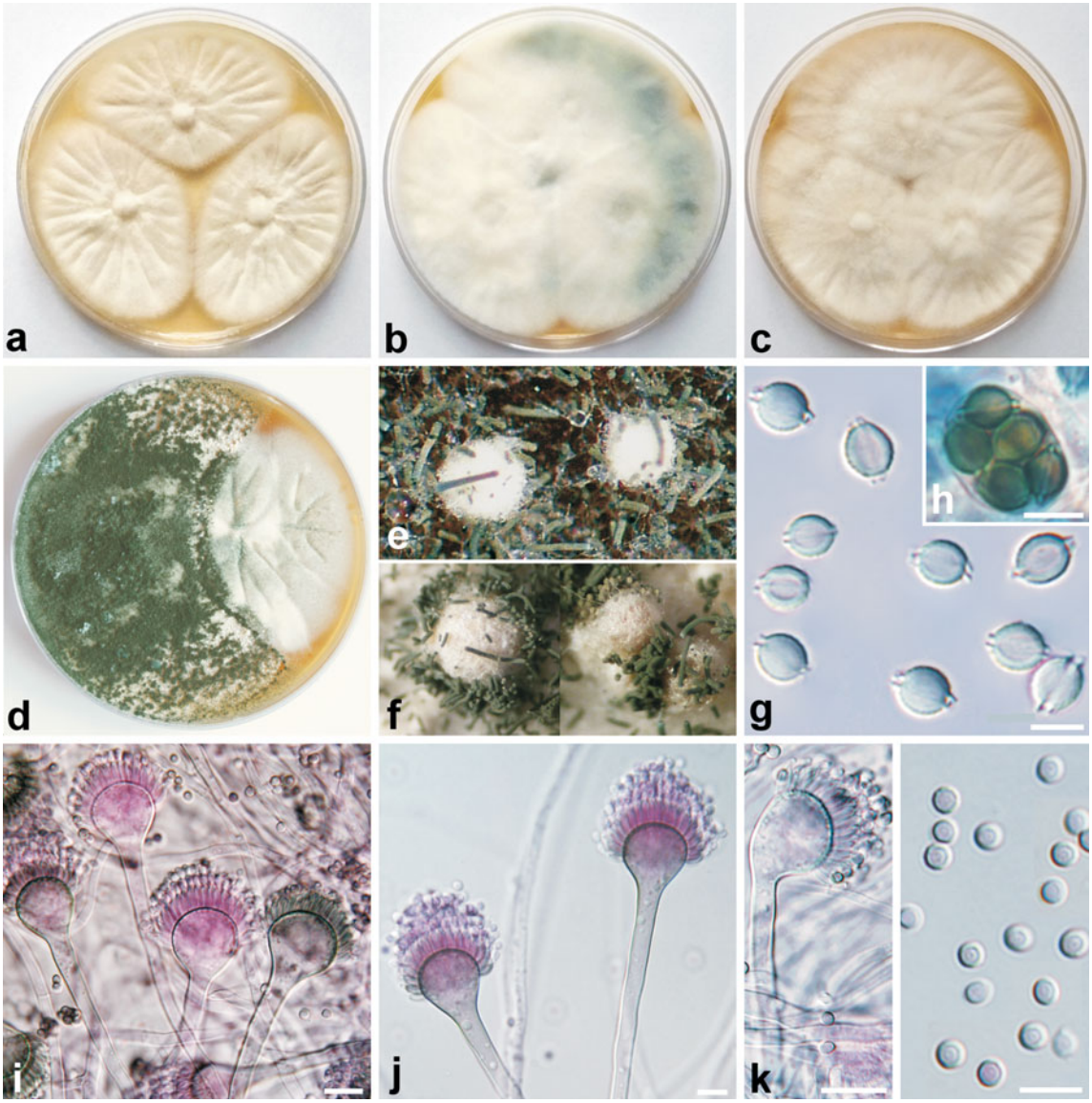

Fig. 7 *Aspergillus udagawae* CCF 4493. **a**–**c** Colonies incubated 7 days at 25 °C on CYA, MEA and YES, from left to right; **d** fertile cleistothecia as a result of crossing of mating ex-type isolates IFM 46972^{MT} and IFM 46973^{MT} on MEA after 6 weeks. **e**–**f** cleistothecia. **g** ascospores. **h** ascus. **i**–**k** conidiophores; **l** conidia. — Scale bars: g–h=5 μm; i–l=10 μm

covered by a loose felt of white hyphae; asci eight-spored, globose to subglobose 9.5–11.5×9–11 μm; ascospores lenticular, spore bodies 4.1–5.2 μm in longer axis, with two well-separated, 0.5–0.8 μm wide equatorial crests, convex surface tuberculate.

Diagnosis. The species can be differentiated from other heterothallic species by the ascospores with tuberculate convex surface and two well visible equatorial crests that do not exceed 1 μm. The ascospores of recently described teleomophic stage of *A. lentulus* (Swilaiman et al. 2013) have similar morphology with tuberculate convex surface and equatorial crests <1 μm (Céline M. O'Gorman, personal communication). However, reticulate convex surface texture may be more pronounced in *A. lentulus* ascospores. The isolates of *A. lentulus* always grow at 45 °C and produce different spectrum of secondary metabolites (Samson et al. 2007a).

Ecology. Soil (Brazil, Korea, U.S.A.), food (Japan), human pathogen (or clinical material contaminant in some cases)—lungs, upper respiratory tract, brain, cornea, nail (Czech Republic, Italy, Japan, U.S.A.), animal pathogen—respiratory tract and retrobulbar mass in cats (Australia, Japan). References: Horie et al. (1995); Katz et al. (2005); Balajee et al. (2006); Vinh et al. (2009b); Yaguchi et al. (2007); Kano et al. (2008); Balajee et al. (2009); Hong et al. (2010a, b); Posteraro et al. (2011); Gyotoku et al. (2012); Kano et al. (2013)

Specimens examined. Fifty-seven isolates, see Tables 1 and 2.

Type. Brazil, São Paulo, Botucatú, Lagoa Seka Avea, ex soil in plantation, 1993, *Y. Horie*, holotype CBM-FA-0711, a dried

colony from a paired culture of isolates MAT1-1 IFM 46972^{MT}= CBS 114218^{MT}×MAT1-2 IFM 46973^{MT}=CBS 114217^{MT}.

Aspergillus viridinutans Ducker and Thrower 1954, Aust. J. Bot. 2: 355 — MB292864; Fig. 8

Description. Colonies on CYA in 28–40 mm in diam at 25 °C in 7 days, velutinous to floccose, plane to slightly wrinkled and umbonate in central part of colonies, yellowish white (ISCC-NBS No. 92) to pale yellow (89), no exudate or soluble pigment production, reverse pale orange yellow (No. 73) to light orange yellow (No. 70). Colonies at 37 °C 40–50 mm, velutinous, irregularly wrinkled with umbonate central part, greyish white (No. 153) to light greenish grey (No. 154), reverse light orange yellow (No. 70) to pale orange yellow (No. 73) in marginal part of the colony or greyish yellowish brown (No. 80) to moderate olive brown (No. 95) to greyish green (No. 150). Colonies on MEA 31–33 mm, velutinous to floccose, plane to lightly wrinkled, umbonate in the centre, white margin 1–3 mm, sporulation pale green (No. 149), greyish green (No. 150) to light greyish grey (No. 154) in the central umbonate part of colony, reverse light yellow brown (No. 76) with yellowish white margin or yellowish white to pale orange yellow (No. 73) to dark orange yellow (No. 72) in the centre. Colonies on YES 32–37 mm, floccose, irregularly lightly wrinkled, yellowish white, yellowish white to pale yellow (No. 89), pale orange yellow exudate, droplets to 1 mm, some of them to 2–3 mm in diam, reverse light yellow brown (No. 76) to moderate orange yellow (No. 71). Colonies on CZA 19–25 mm in diam at 25 °C, velutinous, plane with umbonate centre, yellowish white (No. 92) with

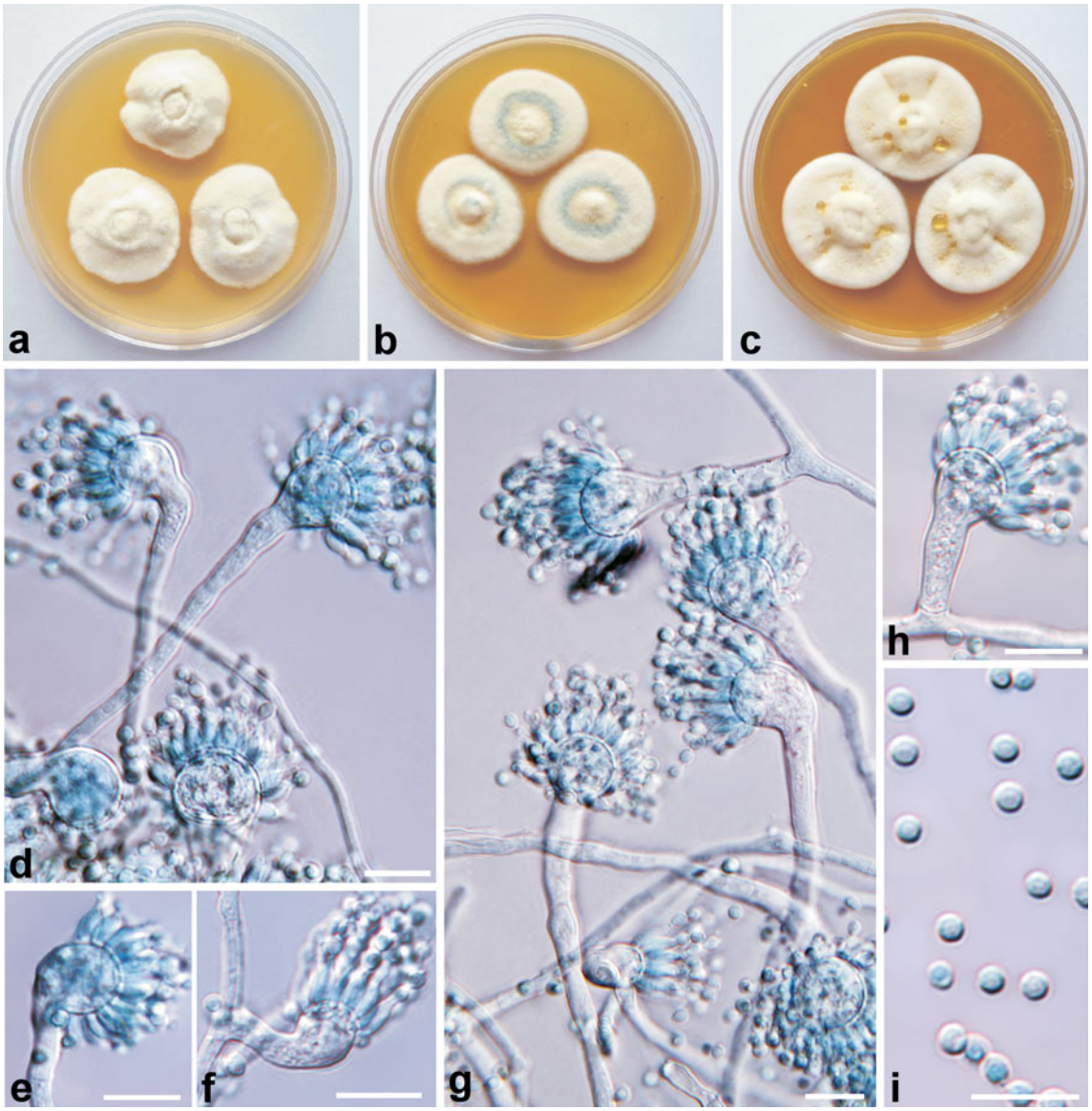

Fig. 8 *Aspergillus viridinutans* IFM 47045^{T}. **a**–**c** Colonies incubated 7 days at 25 °C on CYA, MEA and YES, from left to right; **d**–**h** conidiophores; **i** conidia. — Scale bars 10 μm

light greenish grey (No. 154) to pale green (No. 149) sporulation, no soluble pigment, reverse yellowish white. Colonies on CREA 18–21 mm, effuse, yellowish white, no visible sporulation, no acid production. Ehrlich test negative.

Conidial heads columnar. Conidiophores arising from aerial hyphae, smooth, most commonly 20–70(−112)×3–4(−5) μm, sometimes extremely short measuring only several μm, nodding heads common. Conidial heads uniseriate, vesicles globose, 7.5–12(−14) μm, phialides ampuliform covering the upper half of vesicle. Conidia globose to subglobose, smooth to delicately roughened, 2–2.8 μm.

Diagnosis. The species can be differentiated from other anamorphic or heterothallic species by overall relatively slow growth parameters, inability to grow at 45 °C, colourless reverse on CZA, presence of nodding heads, short conidiophores, globose and almost smooth conidia.

Ecology. Dung of *Oryctolagus cuniculus* and soil (both at Frankston, Australia). References: McLennan et al. (1954).

Specimens examined. The species is only represented by the ex-type isolate.

Nomenclatural notes. Original material represented by illustration is extant for *A. viridinutans*, however the species was lecto- and later neotypified by a specimen dried from the original living culture (Samson and Gams 1985; Pitt and Samson 2000). In this study, we designated a lectotype (iconotype) to supersede neotype designated by Pitt and Samson (2000) and this "neotype" is here designated as epitype.

Type. Australia, Frankston, dung of *Oryctolagus cuniculus*, lectotype designated here—Fig. 2 in McLennan et al. 1954, Aust J Bot 2: 359; epitype designated here—Herb. IMI 62875, a dried culture, culture ex-epitype CBS 127.56^{T}=NRRL 4365^{T}=NRRL 576^{T}=IFM 47045^{T}=IMI 367415^{T}=CCF 4382^{T}.

Aspergillus wyomingensis A. Nováková, Dudová and Hubka, *sp. nov.* —MB803936; Fig. 9

Etymology. Named after the state of Wyoming (U.S.A.).

Description. Colonies on CYA in 52–58 mm in diam at 25 °C in 7 days, velutinous, wrinkled, yellowish white (ISCC-NBS No. 92) with poor sporulation on the colony margin (pale yellow green—No. 121) after 14 days, no exudate or soluble pigment production, reverse pale yellow (No. 89). Colonies at 37 °C 65–70 mm, floccose to lanose, wrinkled, yellowish white (No. 92), reverse pale yellow (No. 89). Colonies on MEA 43–44 mm, floccose, plane, yellowish white (No. 92), no exudate, soluble pigment present after 14 days—brilliant greenish yellow (No. 98) to vivid greenish yellow (No. 97), reverse light yellow (No. 86) with moderate yellow (No. 87) parts, brilliant greenish yellow (No. 98) to vivid greenish yellow (No. 97). Colonies on YES (60−)68−70 mm in diam, velutine to floccose, wrinkled, yellowish white (No. 92), no exudate, no soluble pigment, reverse strong yellow (No. 84) to deep yellow (No. 85). Colonies on CZA 38–42 mm in diam, plane, whitish yellow. Colonies on CREA 46–50 mm in diam, poor mycelial growth, acid production strong or only under the colony. Ehrlich test negative. Only some isolates were able to grow restrictedly (up to 16 mm) at 45 °C, all grew at 42 °C.

Conidial heads columnar. Conidiophores arising from aerial hyphae, smooth, up to 275.0×(3−)4−6.5(−7) μm, nodding heads occasionally present. Conidial heads uniseriate, vesicles subglobose to globose, pigmented, 11−19(−24) μm, two-thirds covered by ampuliform phialides. Conidia subglobose, delicately rough, 1.7−2.8(−3.3) μm, light green in mass.

Heterothallic species; the ascomata visible after 3 weeks of incubation on OA at 25, 30 and less abundant at 37 °C, mature ascospores present after 4–5 weeks. Cleistothecia white, globose or subglobose 180−500(−600) μm in diameter, covered by a dense felt of white hyphae; asci eight-spored, globose to subglobose 10–12×10–11 μm; ascospores lenticular, spore bodies (3.2−)3.6−5 μm in longer axis, equatorial crests absent or are very low and difficultly distinguishable by light microscopy, shallow equatorial furrow is present, short ribs, rough tubercles and echines are present on the convex surface and clearly visible using light microscopy, a part of ascospores lack ornamentation as well as equatorial crests and furrow.

Diagnosis. The morphology of ascospores with convex surface covered by ribs and echines and very low to absent equatorial creast differentiating *A. wyomingensis* from all species in *A. viridinutans* complex as well as from all other heterothallic species in section *Fumigati*.

Ecology. Soil (USA, Russia, China). References: Varga et al. (2000).

Specimens examined. Fourteen isolates (see Tables 1 and 2). A paired culture of isolates CCF 4416 (= CMF ISB 1976= CBS 135455)×CCF 4417^{T} (= CMF ISB 2494^{T}=CBS 135456^{T}) was deposited as PRM 860737–8.

Type. U.S.A., Wyoming, Converse Country, Powder River Basin, Glenrock—Rolling Hills Wind Plant (former Dave Johnson Coal Mine), site recultivated by crested wheatgrass (*Agropyron cristatum*), ex soil, 2010, *A. Nováková*, holotype PRM 861504, a dried culture, isotype PRM 861505, culture ex-holotype CCF 4417^{T}=CMF ISB 2494^{T}.

Dichotomous key to species from *Aspergillus viridinutans* complex

1a) homothallic ……***A. aureolus***
1b) heterothallic or anamorphic ……2
2a) colonies on MEA after 7 d at 25 °C<35 mm; conidiophores on MEA <120 μm ……***A. viridinutans***
2b) colonies on MEA after 7 d at 25 °C>35 mm; at least some conidiophores on MEA >120 μm ……3
3a) ascospores without equatorial crests or with very low crests (< 0.2 μm) ……***A. wyomingensis***
3b) ascospores with two clearly distinguishable equatorial crests (> 0.5 μm wide) ……4

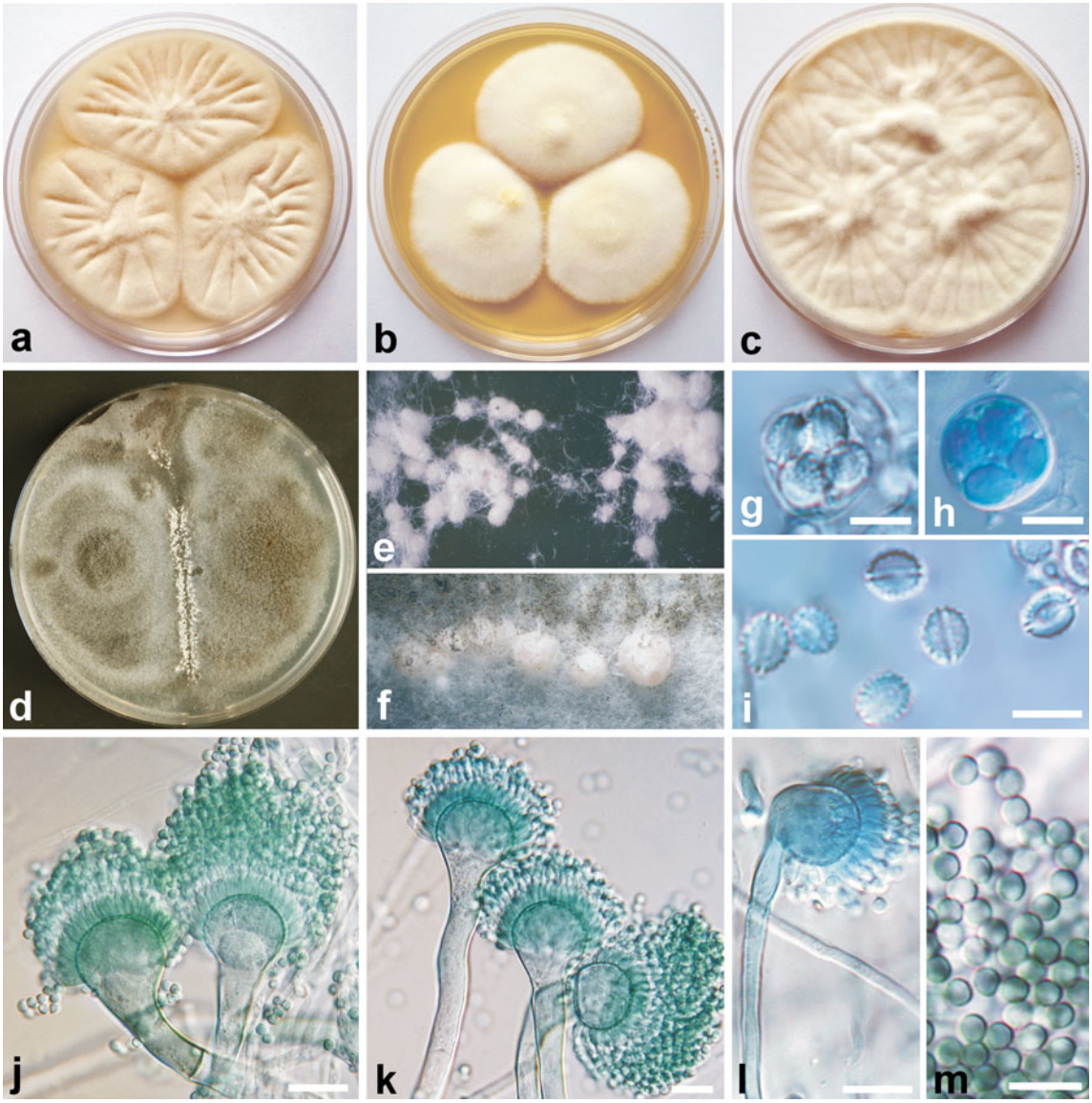

Fig. 9 *Aspergillus wyomingensis* CCF 4417^{T}. **a**–**c** Colonies incubated 7 days at 25 °C on CYA, MEA and YES, from left to right; **d** fertile cleistothecia as a result of crossing CCF 4416 and CCF 4417^{T} on OA after 6 weeks. **e**–**f** cleistothecia. **g**–**h** asci. **i** ascospores. **j**–**l** conidiophores; **m** conidia. — Scale bars: g–i=5 μm; j–m=10 μm

4a) equatorial crests narrower than 1 μm ….....….***A. udagawae***
4b) equatorial crests wider than 1 μm ……..……***A. felis***

Discussion

The isolates traditionally named *A. viridinutans* show considerable phenotypic variability but typically share nodding heads (some vesicles borne at an angle to the stipe) and relatively poorly sporulating colonies with abundant aerial mycelium (in comparison with *A. fumigatus* s. str.) as important morphological characteristics. Previous studies based on molecular data confirmed that *A. viridinutans* includes several lineages but the species boundaries were not clearly determined, and *A. viridinutans* was retained as a species complex (Varga et al. 2000; Katz et al. 2005; Hong et al. 2010b). *Aspergillus viridinutans* was originally described from rabbit dung (Australia) (McLennan et al. 1954) and seems to be very rare species according to the species concept presented here. As far we know, no other isolate identical to the ex-type strain based on molecular methods has been published. As discussed by Varga et al. (2000), the use of nodding heads as a morphological characteristic is misleading because it is present in not only the *A. viridinutans* complex but also many non-related species from the section *Fumigati,* including *A. brevipes* (Smith 1952), *A. duricaulis* (Raper and Fennell 1965), *A. marvanovae* (Hubka et al. 2013b), *A. unilateralis* (McLennan et al. 1954), *N. pseudofischeri* (Paden 1968;

Peterson 1992) and two newly described species, *A. brevistipitatus* and *A. conversis*.

Species of the section *Fumigati,* including those of the *A. viridinutans* complex, are most commonly isolated from soil and clinical material (Katz et al. 2005; Yaguchi et al. 2007; Hong et al. 2010a, b). In this study, we examined many isolates belonging to *A. viridinutans* complex which were dominant inhabitants of reclamation site soils. Seven species, including three undescribed species, were identified using an approach combining morphological, physiological, molecular and mating data. The boundaries between species from the *A. viridinutans* complex were established with an emphasis on our and previous mating experiment results. Three teleomorphs of heterothallic *A. felis*, *A. udagawae* and *A. wyomingensis* sp. nov. showed different ascospores morphology which is treated here as the most important phenotypic feature for these species (Fig. 2). None of these three species produced cleistothecia when paired with the ex-type isolate of *A. viridinutans*. The MAT1-1 idiomorph was partially characterised in the ex-type isolate of *A. viridinutans* IFM 47045^{T} using the primers *alpha1* and *alpha2* (Sugui et al. 2010). No product was observed on electrophoretograms when using the primers HMG1 and HMG2 designed for the MAT1-2 locus. These results suggest that *A. viridinutans* is also heterothallic, although no opposite mating type isolate is currently available for mating experiments.

Aspergillus felis and *A. udagawae* both include several strongly supported lineages which could be considered to represent separate species (Fig. 1) when only molecular data are taken into account. However, mating experiments clearly defined species boundaries in *A. felis*, as the opposite mating type isolates readily produced fertile cleistothecia and did not mate with species of the other clades. Similar results were obtained for *A. wyomingensis*. The interpretation of the mating experiment results for *A. udagawae* was problematic. The ascospores were observed in only limited number of crosses, most commonly with the mating ex-type isolate IFM 46972^{MT}. However, these crosses were distributed across all three major subclades (Fig. 1), supporting *A. udagawae* as relatively large species. Substructuring in *A. udagawae* isolates based on sequence data was also observed by Yaguchi et al. (2007) and Sugui et al. (2010). Previous mating experiments were either unsuccessful or successful in a very limited number of clinical isolates of *A. udagawae* (Balajee et al. 2006; Yaguchi et al. 2007; Vinh et al. 2009b; Sugui et al. 2010). Some isolates examined by Sugui et al. (2010) and Matsuzawa et al. (unpubl. data) were similar or identical based on sequence to a majority of *A. udagawae* isolates from Wyoming and were mated successfully with the ex-type strain. These data suggest that *A. udagawae* is one genetically diverse species with decreased ability to produce fertile ascomata rather than multiple cryptic species. A decline in the level of sexual reproduction was also reported for the human pathogen *A. fumigatus* (O'Gorman et al. 2009).

In contrast to Sugui et al. (2010) who observed a MA1-1:MAT1-2 ratio of 6:4 among clinical isolates of *A. udagawae*, we observed considerable bias toward MAT1-2 over MAT1-1 isolates (42:13). A comparable number of opposite mating type isolates were identified among *A. felis* (MAT1-1:MAT1-2, 4:6) and *A. wyomingensis* (8:6) isolates. Unequal ratios between MAT1-1 and MAT1-2 isolates in *A. udagawae* populations could indicate decreased sexuality levels, as observed in some other genera (Brygoo et al. 1998; Yun et al. 2000). Further studies from geographically distant regions are needed to support our observation and to exclude the link between virulence and mating type idiomorph which was, for example, described in *Cryptococcus neoformans* (Nielsen et al. 2003).

Based on sequence data previously deposited in GenBank, all clinically important cases of human and animal infections due to species from the *A. viridinutans* complex belong to the *A. udagawae* (Katz et al. 2005; Balajee et al. 2006; Vinh et al. 2009b; Yaguchi et al. 2007; Kano et al. 2008; Balajee et al. 2009; Posteraro et al. 2011; Gyotoku et al. 2012; Kano et al. 2013) and *A. felis* clades (Katz et al. 2005; Yaguchi et al. 2007; Alcazar-Fuoli et al. 2008; Vinh et al. 2009a; Coelho et al. 2011; Barrs et al. 2012, 2013; Shigeyasu et al. 2012; Kano et al. 2013; Peláez et al. 2013). No clinically relevant cases have been reported for *A. viridinutans* s. str. or *A. wyomingensis* sp. nov. The majority of species in *A. viridinutans* complex (*A. felis*, *A. udagawae* and *A. wyomingensis*) seems to be worldwide distributed in soil, *A. aureolus* is probably restricted to soil of tropical countries (see "Ecology" in section Taxonomy).

An unexpected species spectrum, with the notable absence of *A. fumigatus*, was obtained using a combination of several isolation techniques from reclamation site soils. Our results and data previously published by Hong et al. (2010a) for arable soil indicate that the spectrum of species from the section *Fumigati* in soil is relatively wide and is not restricted to *A. fumigatus* which was usually referred to as predominant. On the other hand, reclamation sites and arable soil share unstable environmental conditions affected by human-mediated external interventions, and non-*fumigatus* species could play a specific role in these sites. Data on the occurrence of section *Fumigati* members in climax soils that have been verified by molecular methods are not currently available, and the species spectrum could be diametrically different. *Aspergillus udagawae*, an opportunistic human and animal pathogen with increasing incidence in the U.S.A. and worldwide, has never been reported as a dominant species in soil. Reclamation sites and dump soils could be an important source of this pathogen, together with other non-*fumigatus* opportunistic pathogens such as *A. felis* and *A. lentulus*.

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after brown coal mining in the U.S.A. and Europe: a bioindication potential and a role in pedogenesis. This research was also partially supported by the Ministry of Education, Youth and Sports (CZ.1.07/2.3.00/20.0055 and CZ.1.07/2.3.00/30.0003). Molecular genetics analyses were supported by the project GAUK 607812.

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